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One-pot enzymatic resolution/separation of enantiomers using green solvents
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Vogal(ais): Prof. Susana Barreiros

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Antes de mais, gostaria de dizer que esta tese é o produto de inúmeras contribuições, sem as quais não teria chegado tão longe e que vão muito para além dos nomes na primeira página.

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Abstract:

In the context of "green" chemistry and sustainable processes, the main goal of this work is to develop a process that would circumvent the current complications of racemic sec-alcohol separation, using alternative solvents and selective enzymatic resolution.

In this work the ability of enzymes to perform the resolution of sec-alcohols to obtain high added-value enantiomers is advantageously exploited in the production of pure chiral compounds. Candida rugosa lipase is capable of selectively converting one of the enantiomers of menthol into a different chemical compound with substantial different properties. Following this enzymatic catalysis, a separation method is used recurring to alternative solvents properties to separate the enantiomer that does not react obtaining a pure chiral compound.

The main goals of this research are to finding both a vinyl ester and an acid anhydride capable of reacting selectively with the racemic menthol through catalyzed reaction using Candida rugosa lipase and test independently the acylating agents at various parameters that influence the conversion and enantioselectivity of the process such as temperature, enzyme concentration, parallel chemical reaction and solvent effect.

Through this work we were successful in testing these two different chemical compounds obtaining high values for conversion and enantioselectivity. In the case of propionic anhydride we obtained 51% of conversion, 89% and 74% of enantiomeric excess of substrate and product, respectively, at 310.15 K in [Omim][PF_6]. In the case of vinyl decanoate, we obtained 44.4% of conversion, 90.7% of enantiomeric excess of substrate, at 310.15 K in [Hmim][PF_6].

Keywords: Candida rugosa lipase, enantioselectivity, racemic menthol, ionic liquids, supercritical carbon dioxide, green solvents.

Resumo:

No contexto da química "verde" e do desenvolvimento de processos sustentáveis, o principal objectivo deste trabalho é desenvolver um processo que contorne as atuais complicações com a separação de álcoois secundários racémicos usando solventes alternativos e resolução enzimática selectiva.

Neste trabalho, a capacidade das enzimas para efectuar a resolução de álcoois secundários para se obter enantiómeros de elevado valor acrescentado é vantajosamente explorada na produção de compostos quirais puros a partir do mentol racémico. A lipase de *Candida rugosa*, uma enzima muito selectiva, é capaz de converter selectivamente um dos enantiómeros do mentol num composto químico diferente e com propriedades substancialmente diferentes. Após esta catálise enzimática, um método de separação é usado para recorrendo às propriedades dos solventes alternativos para separar o enantiómero que não reage e obter um composto quiral puro.

Os objectivos principais desta pesquisa são encontrar tanto um éster de vinilo como um anidrido ácido capaz de reagir selectivamente com a mistura racémica de mentol através de uma reacção catalisada pela lipase de *Candida rugosa* testando independentemente os diferentes agentes acilantes e outros parâmetros que podem influenciar a conversão e enantioselectividade do processo, tais como temperatura, concentração de enzima, reacção química paralela e efeitos do solvente.

Através deste trabalho, fomos bem-sucedidos em testar estes dois compostos químicos diferentes, obtendo elevados valores de conversão e de enantioselectividade. No caso do anidrido propiónico obteve-se 51% de conversão, 89% e 74% de excesso enantiomérico do substrato e do produto, respectivamente, a 310,15 K em [Omim] [PF_6]. No caso de decanoato de vinilo, obteve-se 44,4% de conversão, 90,7% de excesso enantiomérico do substrato, em 310,15 K em [Hmim] [PF_6].

Palavras-chave: Lipase de Candida rugosa, enantioselectividade, mentol racémico, líquidos iónicos, dióxido de carbono supercrítico, solventes verdes.

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Acronyms

CRL – *Candida rugosa* lipase

DCC - N,N'-Dicyclohexylcarbodiimide

DMAP - 4-Dimethylaminopyridine

E – enantioselectivity

ee – enantiomeric excess

ee_P – enantiomeric excess of the product

ee_S – enantiomeric excess of the substrate

GC – gas chromatography

IL – Ionic liquid

$scCO_2$ – supercritical carbon dioxide

SCF – supercritical fluids

VOC- Volatile organic compounds

[Bmim][BF_4] - 1-Butyl-3-methylimidazolium tetrafluoroborate

[Bmim][PF_6] - 1-Butyl-3-methylimidazolium hexafluorophosphate

[Bmim][Tf_2N] - 1-Butyl-3-Methylimidazolium bis(trifluoromethanesulfonyl)imide

[Hmim][PF_6] - 1-Hexyl-3-methylimidazolium hexafluorophosphate

[Omim][PF_6] - 1-Methyl-3-octylimidazolium hexafluorophosphate

Chapter I

Introduction

In this chapter an overview of the proposed goals for the present work is presented, followed by a brief introduction to green chemistry contemplating ionic liquids and supercritical carbon dioxide, with a focus on the proposed work. In this chapter we also explain menthol, its uses and currently processes used in purification. The chapter finishes with a detailed explanation of the desired reactions, and the utilized enzyme.

I. Introduction

a. Purpose of the work

The main goal of the present work was the development of one-pot enzymatic system to separate two enantiomers of menthol, using for that "green" solvents such as ionic liquids and supercritical carbon dioxide. To make the separation feasible, a chemical change has to occur on the desired menthol molecule, to change its properties. The chemical reaction suitable for this was an acylation recurring to a vinyl ester such as vinyl decanoate or to an acid anhydride, such as propionic anhydride. Such reaction was required to achieve a high yield and purity of the product. For the separation we would use the properties for supercritical CO_2, to selectively separate one molecule from the other. This reaction would be performed in a batch reactor using supercritical CO_2 as carrier for that target menthol molecule, and the transformed menthol molecule would then be returned to its previous state.

On the next image we show a simple mechanism for this system.

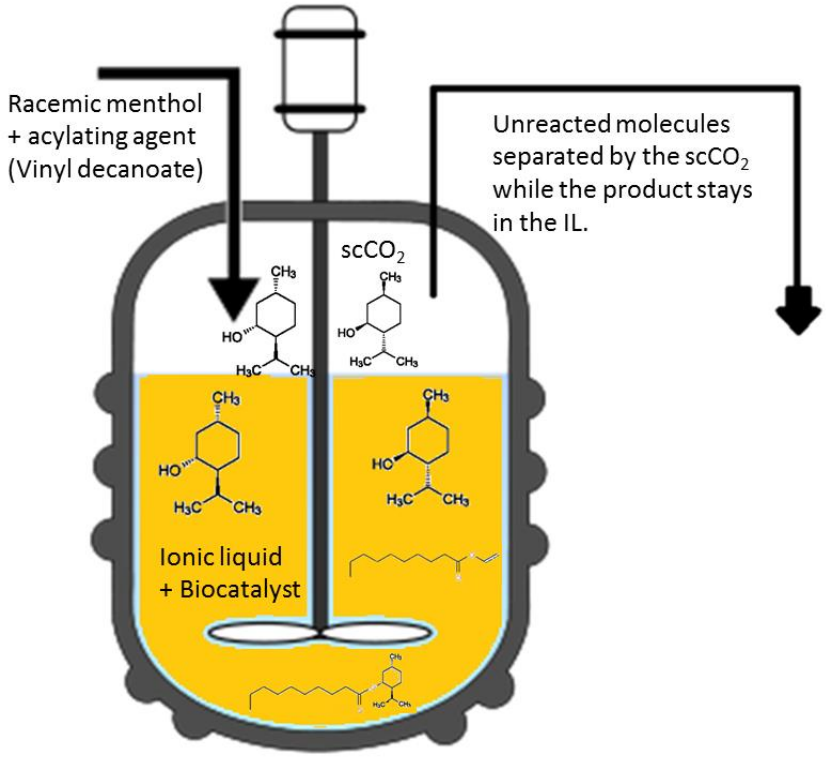

Figure I.1 - Figure illustrating the intended mechanism.

b. Green chemistry

Over the past two decades, the chemistry community has been mobilized to develop new processes that are less hazardous to the human health and the environment. This new approach has received increasingly more and more attention over the years. There are many designations for this pursuit, but they all lead to a movement towards cleaner chemistry with the knowledge that the consequences of chemistry do not stop with the properties of the target molecule or the efficacy of a particular reaction[1].

This leads to Green Chemistry, which is the design, development, and implementation of chemical products and processes to reduce or eliminate the use and generation of substances hazardous to human health and the environment[2].

The green chemistry revolution is providing an enormous number of challenges to those who practice chemistry in industry, education and research. With these challenges however, there are an equal number of opportunities to discover and apply new chemistry, to improve the economics of chemical manufacturing and to enhance the much-tarnished image of chemistry[1].

For those of us who have been given the capacity to study chemistry and practice it as our livelihood, it is and should be expected that we will use this capacity wisely. With this knowledge comes the burden of responsibility, because, in a world moving towards evolution, in which everyone takes a part, we can no longer pretend that our actions have no effect in this world molded by our actions. The benefits of science cannot be seen as an excuse to act ignorantly and the time has come to weigh each of our actions, acting pro-actively creating benign processes to whose consequences do not lead to irreparable damaging to the environment.

Another lesson learned is that nothing is benign and all substances and all activity have some impact just by their being, and in this case, the dosage makes the poison. What is being discussed when the term benign or environmentally benign chemistry is used is simply an ideal. Almost all processes must create waste, but we must as well aim for that impossible perfection that is a waste free process[3]. Chemists working toward this goal have made dramatic advances in technologies that not only address issues of environmental and health impacts but do so in a manner that satisfies the efficacy, efficiency and economic criteria that are crucial to having these technologies incorporated into widespread use. It is exactly because many of these new approaches are economically beneficial that they become market catalyzed[3].

By designing for sustainability at this fundamental level, it challenges innovators to design and utilize matter and energy in a way that increases performance and value while protecting human health and the environment. The principles of Green Chemistry today need to become the core for tomorrow's chemistry, integrating sustainability into science and its innovations[4].

Overpowering the environmental unacceptability and poor atom economic of typical processes are the goals of green chemistry. The emphasis will be on batch type processes involving liquid phase reactions as practiced by fine, specialty chemical and chemical intermediate manufacturers around the world.

The green chemistry goal is to remove all elements from the accounts other than those involved in the organic chemistry and push the organic chemistry towards 100% selectivity to the desired product.[4]

The ambition towards clean technology in the chemical industry and the emergence of green chemistry related issues in chemical research and education are not short term 'fashions', as same thought years ago. Now and in the future, the synthetic chemist will need to be as concerned about atom efficiency as the synthetic route and the process chemist will need to be as concerned about the waste produced as the product to be made[1].

Chemists working pursuit of this objective have made dramatic advances in technologies that not only address the environmental and health impacts cause by the industry but do so in a way that satisfies the efficacy, efficiency and economic criteria that are crucial to have these technologies accepted and incorporated in that same industry. The green chemistry revolution is also providing an enormous number of challenges to industry, education and research and with those came equal number of opportunities to discover and apply new chemistry, to improve the economics of chemical manufacturing and to enhance the image of chemistry.[5]

The chemical industry is consistently regarded less favorably than all the others, the petroleum, gas, electricity, lumber and paper industries have a more satisfying opinion by the consumers. The negative public opinions, in other hand, contrast with the tremendous economic success of the industry. It's one of the most successful and diverse sectors of manufacturing industry in most regions of the world, with an enormous range of products that make an invaluable contribution to the quality of our lives. That same industry may vary in size, having capacities ranging from a few tons per year in the fine chemicals area to 500,000 tons per year in the petrochemicals area. The main reasons given for unfavorable opinions of the chemical industry are concerns over adverse environmental impact, safety and waste[6].

Why is green chemistry so important? Up to the year of 1993, the U.S. Environmental Protection Agency reported 30 billion pounds of chemical released to air, land and water in their Toxic Release Report. This data may cover releases from a variety of industrial sectors, including only 365 of the approximately 70,000 chemicals available in commerce by the day. Of the industrial sectors that are covered by the toxic release inventory, the chemical manufacturing sector is understandably the largest releaser of chemicals to the environment, releasing more than 4 times as many pounds to the environment as the next highest sector[1].

Many of these laws require companies either explicitly through methodology-based regulations or implicitly through performance-based regulations to have a variety of waste

handling, treatment, control and disposal processes in place to meet environmental mandates. All of their wastes must be controlled, accounted and treated, requiring for those processes the acquisition of equipment with high capital costs and it's far easier for the companies to ignore those laws and pay the respective fines.

From this, we acquired that from an economic standpoint, it is clear that we not only want to have sustainable technology but we want it to be cost neutral at minimum and profitable in the overall process when it's possible.

The ideal angle for green chemistry to develop is one that demonstrates new techniques and methodologies which allow industry to continue their tradition of innovation while shifting financial resources that are now expended on environmental costs to further research and development.

Knowing what is green chemistry and its principles, we can sum up the principal areas of research, development and commercialization:

- Nature of the Feedstocks or Starting Materials
- Nature of the Reagents or Transformations
- Nature of the Reaction Conditions
- Nature of the Final Product or Target Molecule[4]

We can see that these 4 elements are closely related, and in some cases one implies the other, however, we must address them separately, so we can identify the areas where incremental improvements can be made and design more environmentally benign syntheses.

All this four elements are related and incorporated in "The Twelve Principles of Green Chemistry"[4] those are a philosophy of chemical research and engineering that encourages the design of products and processes that minimize the use and generation of hazardous substances. The principles are as followed:

I. Prevention

It is better to prevent waste than to treat or clean up waste after it has been created.

II. Atom Economy

Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.

III. Less Hazardous Chemical Syntheses

Wherever practicable, synthetic methods should be designed to use and generate substances that possess little or no toxicity to human health and the environment.

IV. Designing Safer Chemicals

Chemical products should be designed to affect their desired function while minimizing their toxicity.

V. Safer Solvents and Auxiliaries

The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and innocuous when used.

VI. Design for Energy Efficiency

Energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.

VII. Use of Renewable Feedstocks

A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable.

VIII. Reduce Derivatives

Unnecessary derivatization (use of blocking groups, protection/ deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.

IX. Catalysis

Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.

X. Design for Degradation

Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.

XI. Real-time analysis for Pollution Prevention

Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.

XII. Inherently Safer Chemistry for Accident Prevention

Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

During the this work, we aimed at many of this principles, but mostly at I, II, III, IV, V, VII and IX, making this process a very green one.

In the development of this work, we focus on two of the principal areas of research in green chemistry. First, on alternative synthetic transformations and alternative reagents by reducing risk to human health and the environment through the elimination or reduction of toxic substances, using routes that utilize more benign chemicals as reagents necessary to carry out particular transformations or change the actual transformations themselves.

In second, and most important, we seek alternative reaction conditions used for the synthesis of the aimed chemicals. These alternatives have a significant effect on the pathway's overall environmental impact. It’s easy to evaluate the amount of energy used by one process versus another is quite easily evaluated in economic terms but is not currently as easily evaluated in environmental terms. Even though there are some programs like life cycle assessment (LCA)[7], that give the overall impact of the production/activity of a given industrial activity they are,

essentially, statistical approximations. It appears that because of this difficulty in measurement, and not necessarily as a judgment on relative importance, that the majority of Green Chemistry research on reaction conditions has been centered on the substances utilized as part of those conditions.

Most of the environmental concern in the manufacture of chemicals came, not only from merely the chemicals that are made or the chemicals from which they are made, but with all the substances associated with their manufacture, processing, formulation and use. Those substances can increase substantially the environmental burden of a chemical process. Most of these substances have a great impact because they are solvents to the process, used in reaction media, separations and formulations, and some of them are highly volatile and lost in the process, like organic solvents. Because of that, they have come under increased scrutiny and regulatory restriction based on concerns for their toxicity and their contributions to air and water pollution. Long since, the enterprises became more and more interest in using alternative solvents with low impact in the environment, low volatility and economically appealing.

One way to measure the potential environmental acceptability of a chemical processes is the E factor[8]. The E factor is defined as the mass ratio of waste to desired product. For this calculation we account with everything but the desired product as waste and this can give as the actual amount of waste produced in the process[8].

This will account in, not only with the chemical yield, but also includes reagents, solvents losses, all process aids and, even fuel, with the exception of water.

By considering typical E factors in various segments of the chemical industry, we can evaluate the magnitude of the produced waste and the environmental problems that may rise from that process.

Fine chemicals and pharmaceuticals manufacture are changing their processes to catalytically reactions instead of antiquated 'stoichiometric' reactions. These, had a tremendous amount of waste generated in the manufacture of organic compounds and most of that waste consisted primarily of inorganic salts used in the stoichiometric reaction. This adds to the estimated 85% of the total mass of chemicals involved in pharmaceutical manufacture comprises solvents and their recovery efficiencies are usually around 50–80%. Most of that loss came from the process itself and from removal of residual solvent from products, most of the times, achieved by evaporation or distillation[9,10]. We may also account with spillage and evaporation which inevitably leads to atmospheric pollution and worker exposure to volatile organic compounds (VOCs) leading to serious health issues [2].

One of the most active areas of investigation in alternative solvents and in Green Chemistry in general has focused on the use of supercritical fluids (SCFs) and ionic liquids (IL's) as solvents.

These solvent systems are being investigated systematically for their usefulness in a wide range of reaction types for their low cost, low volatility and for being innocuous solvents (depending on the choice of an ion and cation) that can supply "tunable" properties. But for being a reasonable option, is crucial to demonstrate technical efficacy, superior performance and ability to undertake biocatalysis in order to evaluate the true advantages offered by the environmental and risk reduction benefits.

c. Enzymes

i. Biocatalysis

Enzymes are biocatalysts that can be found anywhere in nature, they increase the reaction velocity by decreasing activation energy necessary to undergo a reaction; this will contribute to the stabilization of the substrate. These catalysts are highly efficient from the standpoint of providing increased rate of reaction in relation to the uncatalyzed reaction.

From the energy point of view, the enzymes are also very efficient since operating temperatures and moderate pressures, as well as moderate range of pH values [11].

However, enzymes do differ from most other catalysts in that they are highly specific for their substrates, but at the same time, there are thousands of them, capable of catalyzing about 4,000 biochemical reactions. Biocatalyst, like enzymes have several advantages over their chemical counterparts, in particular regioselectivity (preference for one of several identical functional groups in the substrate molecule), the enantioselectivity (preference for one enantiomer of a racemic mixture) and chemoselectivity (favoring one functional group of the substrate instead of the others) [12]. Usually, enzymes are much larger than the substrates they work on, and only a small portion of the enzyme is directly involved in catalysis, the active site. This region, that contains these catalytic residues, binds the substrate and then carries out the reaction. But enzymes also contain sites that are used to bind other molecules, like cofactors, which are needed for catalysis. They also have sites for feedback regulation, where small molecules can bind to influence the enzyme’s activity. These molecules can be direct or indirect products of the reaction or substrates.

Long ago, the industry understood the benefits of recurring to enzymes to catalyze their reactions. The increasing demand for enzymes for industrial applications is precisely related to the selectivity for the substrate: the ability to discriminate distinct but structurally similar

substrates. That knowledge increased the demand from the industry for enzymes for industrial applications that are both selective and have a very high yield for the desired reactions [13,14].

ii. Effects on enzyme activity

An enzymatic reaction is the conversion of one molecule into another; a chemical reaction catalyzed at the reactive sites on the enzyme. Enzymes are very complex molecules, it is reasonable to expect that many parameters will affect the rate of catalytic activity[15]. Enzyme activity can be affected by other molecules: inhibitors are molecules that decrease enzyme activity; activators are molecules that increase activity. Many drugs and poisons act like enzyme inhibitors in essential physiological processes. Even so, other factors may act upon enzyme stability, reaction rate and selectivity as: water content, temperature, pressure. Differences in temperature may deactivate or denaturate enzymes and change their reaction rate[16]. The temperature may affect catalytic activity of the enzyme, due to increases in the kinetic energy. As the temperature of a system is increased it is possible that more molecules per unit time will reach the activation energy. Thus the rate of the reaction may increase. The number of collisions per unit time will also increase. As the temperature of the system is increased, the internal energy of the molecules in the system will increase. The internal energy of the molecules may include the translational energy, vibrational energy and rotational energy of the molecules, the energy involved in chemical bonding of the molecules as well as the energy involved in nonbonding interactions [11]. Other factor that affects enzymes and is important to the work presented in this thesis is pressure. Pressure may act directly and indirectly on enzyme stability and activity. Pressure directly affects protein structure by changing its tertiary and quaternary structure, this may lead to changes in the active site and or cofactors site of ligation. This may change the enzyme specify to its substrate, enzyme stability and even reactivity. These changes are unlikely to happen when enzymes are used in $scCO_2$ at pressures up to 300 bar. The biggest influence of the pressure in enzyme activity happens indirectly on the reactants itself, by changing their solubility and the reaction rate. Even so, enzymes are usually immobilized to prevent pressurization and depressurization effects on their properties [16].

iii. Classes of enzymes

Enzymes are classified according to the report of a Nomenclature Committee appointed by the International Union of Biochemistry (1984). This enzyme commission assigned each enzyme a recommended name and a 4-part distinguishing number. The enzyme commission (EC) numbers divides enzymes into six main groups according to the type of reaction catalyzed:

Table I.1 - Enzyme classification accordingly IUPAC-IUBMB[17].

Classes	
EC1	Oxidoreductases
EC2	Transferases
EC3	Hydrolases
EC4	Lyases
EC5	Isomerases
EC6	Ligases

Form this table we are interested in lipases, EC3, which will be spoken further in this introduction.

d. Ionic liquids

Until 20 years ago, most chemical reactions have been carried out in molecular solvents. Indeed, barely a chemical process exists in which a solvent is not personally involved in both synthesis and separation stages. In most organic reactions, choice of solvents is of crucial importance; often the solvent is the major component. The search for new solvents having wider-ranging properties continues to be a significant part of organic research. For two millennia, most of our understanding of chemistry has been based upon the behavior of molecules in the solution phase in molecular solvents. However, a new class of solvent has emerged, the ionic liquids (IL). IL are a group of new organic salts that exist as liquids at a low temperature (<100 ◦C). An important feature of ILs is their immeasurably low vapor pressure besides chemical and thermal stability, nonflammability, high ionic conductivity, a wide electrochemical potential window and tolerance to strong acids[18,19]. For this reason, they are called ‘green’ solvents, in contrast to traditional volatile organic compounds (VOCs). These solvents consist entirely of ionic species

and are often fluid at room temperature or close by. They have many fascinating properties which make them of fundamental interest to all chemists: as they are made up of at least two components which can be varied, they can be designed for a specific task, having the desired properties. The reactions carried out in ionic liquids are both the thermodynamically and kinetically different from those in conventional molecular solvents, making chemistry different.

Therefore, they have been extensively investigated as solvents or co-catalysts in a wide range of reactions including organic catalysis, [18–20] inorganic synthesis, biocatalysis[21,22], and polymerization[23,24].

At first, it seemed difficult and complex to experience classic reactions on ionic liquids, but with time it turned out to be exceptionally easy and many results came from those experiments.

In general, ionic liquids consist of one or two ions that are both the ions are large, this brings the cation to a low degree of symmetry. These factors tend to reduce the lattice energy of the crystalline form of the salt, and hence lower the melting point[25].

Ionic liquids may come in two main categories:

-simple salts (made of a single anion and cation) and

-binary ionic liquids (salts where an equilibrium is involved).

As been said before, ionic liquids are designer solvents, and this means that their properties can be adjusted to suit the requirements of a particular process. We may vary properties such as melting point, viscosity, density, and hydrophobicity so it suits the reaction we want carried out. These changes are made by varying the structure of the ions. Another quite important property is the miscibility with water in these ionic liquids.

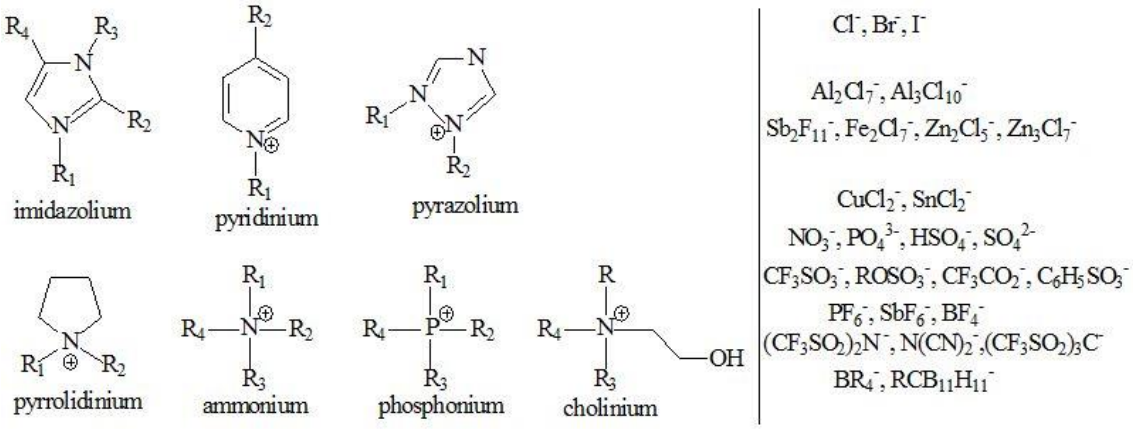

Figure I.2 - Most common ions and cations featuring in ionic liquids[132].

As ionic liquid raised attention, chemistry developed more and more of them as a way increase the robustness of processes, water-stable ionic liquids have become increasingly important. In that process, a number of ionic liquids have been found to be hydrophobic, but yet

readily dissolve many organic molecules, with the exception of alkanes and alkylated aromatic compounds (such as toluene). As an example we present [Bmim][PF_6][26], which forms triphasic mixtures with alkanes and water, being unable to dissolve them.

Due to the unique properties of ionic liquids, some volatile products can be separated from their catalyst by distillation, as the ionic liquid has effectively no vapor pressure and therefore cannot be lost. There also exists the possibility of extraction with supercritical solvents developed in this thesis. A recent example is the use of supercritical carbon dioxide to extract naphthalene from [Bmim][PF_6][27].

IL can either be neutral or acidic and may be classified accordingly to the reactions during their synthesis and for the anions source. We can obtain Lewis acidic or neutral ionic liquids. IL are, for example, composed of bulky 1,3-dialkylimidazolium, alkylammonium, alkylphosphonium or alkylpyridinium organic cations and inorganic anions such as most frequently $AlCl_4^-$, BF_4^- or PF_6 but also NO_3, ClO_4, CF_3CO_2, CF_3SO_3 or CH_3CO_2 and other anions. The most commonly used neutral ionic liquids include 1-butyl-3-methylimidazolium hexafluorophospate or tetrafluoroborate abbreviated as [Bmim][PF_6] and [Bmim][BF_4] correspondingly[28].

In neutral ionic liquids, special conditions are not usually required to carry out reactions. There is no need to exclude water, or to carry out the reaction under an inert atmosphere, plus with the ability to produce ionic liquids with the desired properties it allows us an easy separation of the product, making reactions in ionic liquids extremely straightforward to carry out.

The main benefit of this is that the ionic liquid and catalyst can be recycled and reused after solvent extraction or direct distillation of the product from the ionic liquid making the process greener.

Another useful use of ionic liquids is in bio-transformations, they may be used in room-temperature situations as a direct replacement for conventional organic solvents in multiphase bioprocess operations. One of these situations includes the liquid-liquid extraction of the antibiotic erythromycin and two-phase biotransformation processes[29].

Due to the tunable properties and behavior of the ionic liquid they may be adjusted to suit an individual reaction type, to increase product yields to very high levels and to reduce the amount of waste produced in a given reaction. Often the ionic liquid can be recycled, and this leads to a reduction of the costs of the process, with improve benefiting to the environment. It must also be emphasized that reactions in ionic liquids are not difficult to perform and usually require no special apparatus or methodologies, giving in most cases quicker and easier reactions to carry out than in conventional organic solvents.

Even through their incredible perspectives as green solvents, the LCA for an IL is not actual green in some cases, not only because there are exceptions to the general no volatility property, mostly due to decomposition of the ionic species when they undergo highly conditions

of temperature and pressure[30]. Other negative point of IL is due to their synthesis during which, in most cases, some conventional volatile organic solvents are used to synthesize and recover ILs[31]. To some extent, air pollution is transferred to synthesis and recovery of ILs. Even if applications of IL indeed helps to reduce air pollution, it is possible to release them into environment by accidental spills into effluents, which might cause water or soil pollution, and especially cause aquatic contamination[32–34].

The lack of environment-friendly in ILs preparation is due to the series of reactions and purification steps employing various volatile organic compounds as described for [Bmim][BF_4], it is often that synthesis of ILs has a more severe environmental impact than preparation of conventional molecular solvents[35].

Recent research on their eco-toxicity and degradability has shown that some ILs are not as eco-friendly as we expected. We should, then hold a balanced view on ILs and their applications. In order to improve their greenness, more effort should be made in green synthesis of ILs, reducing their toxicity and increasing their degradability, developing novel applications and seeking their suitable recovery and purification approaches.

Many reactions are carried out in one media to later be extracted from there, this process is an energy-efficient technology used by engineers for many years recurring to two immiscible phases (conventionally an organic phase and an aqueous phase). However, many of the used organic solvents are toxic and flammable VOCs. With safety and environmental friendliness improvement in mind, this conventional separation technique can be update with the use of ILs as substitutes because of their stability, nonvolatility and adjustable miscibility and polarity. ILs can be hydrophilic and hydrophobic depending on the structures of cations and anions.[3637] But the anion seems more important in determining the water miscibility of ILs[37]. ILs based on $[PF_6]^-$ and $[Tf_2N]^-$ are normally water immiscible, therefore, they are the solvents of choice for forming biphasic systems in most IL extraction applications.

i. Biocatalysis in Ionic Liquids

Biocatalysis in ionic liquids have presented enhanced activity, stability, and selectivity, as compared to those observed in conventional organic solvents. Advantages of using ionic liquids over the use of normal organic solvents as reaction medium for biocatalysis also include their high ability of dissolving a wide variety of substrates, especially when using highly polar ionic liquids.

For more than three decades, the use of nonaqueous environments instead of in aqueous media has been widely discussed and their importance and applicability have been well recognized [12,38]. The emerging of ionic liquids as nonaqueous reaction medium presented as an

attractive solution for biocatalysis, promising to solve a great amount of problems and gained increasing attention, sometimes through remarkable results [22,39,40].

High polarity is one of the most special properties for ionic liquids and it may be determined based on the shift of the charge-transfer absorption band of a solvatochromic probe, such as Reichardt's dye, in the presence of the solvent[41]. A correlation between the decrease in both the chain length of the alkyl substituents on the imidazolium ring of the cation and the anion size is accompanied with an increase in polarity[25]. This polarity values are, sometimes, sensitive to temperature and presence of water[42]. With this high polarity, ionic liquids present an ideal ability to dissolve a wide range of different substances including polar and nonpolar organic, inorganic, and polymeric compounds and despite of their high polarity, most of them are hydrophobic and cannot dissolve more than 1% of water, which may affect the physical properties of the ionic liquids[43]. However, the solubility of water in ionic liquids may vary[39], with [Bmim][PF_6] and [Bmim][BF_4] similar on Reichardt's polarity scale, but only the last one is completely water-miscible[40]. Ionic liquids are generally immiscible with many organic solvents, polar and nonpolar, like *n*-hexane (nonpolar), dichloromethane(polar) and tetrahydrofuran (polar)[40]. This immiscibility either with water or organic solvents has made them feasible to be used to form two-phase systems and able to perform extractions.

When comparing to typical organic solvents, ionic liquids are much more viscous (35–500 cP viscosity against 0.6 cP for toluene and 0.9 cP for water at 25 ◦C)[40,44]. Viscosity of the reaction medium may control the enzyme activity by affecting the mass-transfer limitations. Therefore, a lower reaction rate would be expected in an ionic liquid with a higher viscosity.

The viscosity of an ionic liquid represents its tendency to form hydrogen bonding and the strength of its van der Waals interactions, these interactions are lowered by increasing the temperature and thereafter the entropy of the system or by adding some organic co-solvents. As seen in fatty acids, the longer alkyl chains the stronger the van der Waals interactions, in this case that happens with the increase of the alkyl chain in the cation, the same presents with larger anion sizes. This brings one obvious advantage of using ionic liquids over the use of normal organic solvents is that the physical and chemical properties of the ionic liquids, including their polarity, hydrophobicity, viscosity and solvent miscibility, can be finely tuned by altering the cation, anion and attached substituents. So, by manipulating the solvent properties, one is allowed to design an ionic liquid for specific reaction conditions, such as to increase the substrate solubility, to modify the enzyme selectivity, or to tailor the reaction rate.

i. Effect of solvent properties of ionic liquids towards enzymes and enzymes activity

It has been reported that active enzyme in ionic liquids, normally do not dissolve but remain suspended as a powder or immobilized by a solid support, maintaining their structure and functionality like when in organic solvents. It has also been reported that, in ionic liquids, a variety of enzymes are capable of performing catalytic activities, with equal or higher activity than those observed in conventional organic solvents[45,46].

For example, the lipase-catalyzed resolution of amino acid ester in ionic liquids can be improved by adjusting the solvent parameters of the ionic liquids such as their nature, polarity, and concentration[47]. Obviously, it is necessary to work out the controlling factors that affect the enzyme activity in ionic liquids so as to be able to optimize and to take advantage of biocatalysis in such a new reaction medium.

ii. Hydrophobicity

Hydrophobicity affects biocatalysis by the affecting the water surrounding the protein. An IL capable of coordinate water with their anion will denaturate enzymes, this is cause by the removal of water surrounding the enzyme, providing her with the necessary flexibility to catalyze the reaction. The logarithm of the partition coefficient of a solvent in an octanol/water mixture, logP, has been, over the years the best way to determine enzyme activity, and is has been suggested that more hydrophobic solvents, like heptane (logP= 4,4), are more favorable for enzymatic reactions than those such as methanol, with a low logP (logP=-0.764)[48]. But there are exceptions to this rule; ionic liquid cannot be evaluated by the logP concept. During the determinations of logP for ionic liquids, extremely low values (−2.90 to−2.39) were obtained due to the fact that the imidazolium ring presented in the dialkylimidazolium-based ionic liquids absorbs strongly at approximately 211 nm[49]. This range of values, suggested that ionic liquids are highly hydrophilic and would likely denaturante enzymes, but this is not true[49], rendering this measurement inappropriate for determining enzyme activity in ionic liquids.

iii. Polarity

Against what is expected, higher reactions rates are observed in more polar ionic liquids, which was to the opposite of the trend shown when the reaction was conducted in normal organic solvents. Lozano et al., who studied transesterification reaction catalyzed by-chymotrypsin in different ionic liquids reached the same conclusion[50], even when they changed the lipase to free Candida Antarctica lipase B in the presence of 2% (v/v) of water the activity of the enzyme increased with the increase in the polarity of the ionic liquid[51]. There are, however, studies of other reactions showing no relationship between the reaction rate and the polarity of the solvent[45,49,52].

This studies proved that exists a relationship between the polarity and the reaction rate, but there might be others conditioning factors. The reaction rate may be more reliable upon viscosity than in polarity. To support this argument, the polarity of ionic liquids only varies in a relatively narrow range (0.6–0.7)[39,52] when compared to the viscosity of those ionic liquids which change over a much broader range (35–500 cP)[44]. In other hand, there seems to be a correlation between polarity and viscosity of ionic liquids. The balance between the length of the alkyl chain and the anion size may not be a very simple one and bigger alkyl chain may contribute more to the viscosity than the size of the anion to the polarity. This brings us to the conclusion that the viscosity effect commands the reaction to higher rates with more polar ionic liquids[50,52]. And finally, normal comparisons and methodologies cannot be applied when using ionic liquids, the comparison of reactions rates in different ionic liquids when the same amount of water was present in the reaction system is erroneous, in this case, the solvent with a higher polarity would have less water associated with the enzyme and more water remaining in the solvent, resulting in a lower reaction rate[53]. More water associated with the enzyme leads to an increase in enzyme activity due to an improvement in the mobility of the protein molecule[54], solubility of substrates and/or products[52] and the ionic interactions. To all of the observations made above, we must add a more important one, compared to other organic solvents in the same range of polarity, like methanol or ethyleneglycol, ionic liquids normally do not inactivate enzymes being suitable for reaction.

iv. The effect of water

It is well-known, that the key determinant of the enzyme properties (e.g. activity, stability, and specificity) is related with the amount of water surrounding the enzyme giving her mobility and making possible to achieve the free energy necessary for her to denaturate or to

stabilize[55]. The hydration level of the enzyme is best correlated with the thermodynamic water activity (a_w)[56].

It's expected, ionic liquids to act on enzymes in similar way organic solvents do, but it's not certain. In organic solvents, enzyme activity and performance is affected by different interactions: the organic solvent will stripping off the essential water that is associated with the enzyme; change protein conformation and/or active center by interaction leading to changes in protein dynamics; and interactions with substrates and products directing reactions with them or by altering their partitioning between the organic and aqueous phases [57].

It is well known that water is required to any reaction affecting the conversion rate and enantioselectivity, but the same amount of water may correspond to different availability to the enzyme, this is due to a longer hydrophobic alkyl chain in the ionic liquid cation, for example and thus holding less tendency to strip off the essential water and presenting a considerably higher activity in [Omim][PF_6] than in [Bmim][PF_6]. In some cases, even when we have comparable water uptake in low water activities, the enzyme is more active in ionic liquids than in organic solvents[55].

v. Enzyme inactivation in ionic liquids

Ionic liquids have other effect also with excipients, pH changes and impurities, but we will turn our attention to their capacity to inactivate enzymes. Usually, enzymes do not dissolve in ionic liquids, if they do, they inactivate. To prevent that to happen, we must guarantee that the amount of enzyme added is very much higher than the ionic liquid solubility limit. Only the enzyme in excess will remain functional[58].

This denaturation of the protein structure is presumably related to the ionic nature of the ionic liquid: its cation or anion may interact with the charged groups of the enzyme, either in the active site or at its periphery, causing changes in the enzyme's structure.

Some studies showed the role of anions is more crucial to denaturate than of cations, enzymes are usually active in ionic liquids containing BF_4, PF_6, and Tf_2N anions[50], but inactive in those containing anions such as NO_3, CH_3CO_2, CF_3CO_2, and CF_3SO_3[49]. This can be explained in two ways: first, the enzyme-compatible anions exhibit lower hydrogen bond basicity, which minimizes interference with the internal hydrogen bonds of an enzyme[59]. Take PF_6^- anion as an example, it spreads its negative charge over six fluorine atoms. On other hand, the enzyme-compatible anions exhibit lower nucleophilicity and thus would show lower tendency to change

the enzyme's conformation by interacting with the positively charged sites in the enzyme structure[60].

vi. Enzyme stability in ionic liquids

How really stable is an enzyme in ionic liquids? It's well-known that enzyme stability is far greater in organic media, especially at a low water activity, than in aqueous solution. This is given by hydrophobic reaction medium which allows the preservation of the essential water molecules surrounding the protein structure and the maintenance of its structure by reducing the protein–ion direct contact. This behavior is already described in literature [61].

Other experiments, using five ionic liquids based on quaternary ammonium cations were performed and proven that this cation acted as a stabilizing agent to the enzyme much alike hexane, increasing in the free energy of deactivation and extending the half-life of the same enzyme 2000x-fold. These observations are in line with the increased hydrophobicity of the cation alkyl side chain.

Protein spectroscopy of an enzyme in ionic liquids, showed that the significant thermodynamic stabilization effect offered by the ionic liquid may result from alteration in the protein hydration level and the structural compaction, it was also suggested that some other factors such as free volume contributions, ionic interactions and confinement effects may also contribute to the protein stabilization by ionic liquids. This proves that enzymes are not very accepting of the ionic liquid as a media, but they work because they don't have the necessary free energy to denature.

vii. Enzyme selectivity in ionic liquids

Every time you exchange the media, new properties arise, with the change to ionic liquid new selectivities appeared when compared to organic and aqueous solvents. In these properties we may count: enhanced enantioselectivity and regioselectivity. In ionic liquids, some enzymes are even more enantioselective and regioselective than in their natural conditions. Some of them continue to do it, even when the temperature is elevated, for example, Frater et al.[62] have reported, *Candida rugosa* lipase in ionic liquids kept its activity and enantioselectivity much better than in traditional organic solvents at higher temperatures.

Various research groups have reported that lipases showed higher enantioselectivity when used for kinetic resolution of chiral alcohols in ionic liquids when compared to organic solvents [45,46,63,64]. This results show that enantioselectivity is extraordinarily dependent on the nature of the ions present in the reaction medium. In the lipase-mediated acetylation of racemic p-chiral hydroxymethanephosphinates and hydroxymethylphosphine oxides [64]: enantioselectivity in [Bmim][[PF_6] was up to six times higher than in common organic solvents, but was negligible in [Bmim][BF_4][63]. The enzyme-mediated kinetic resolution in ionic liquids has shown its advantages when conducted at elevated temperatures due to the high enzyme thermostability in ionic liquids.

In sum, clear signs showed the great potential of the use of ionic liquids as alternative solvents for biocatalysis. They presented enhanced activity, stability, and selectivity besides suppression of side reactions. Their nature and high polarity permits ionic liquids to be used as reaction medium for biotransformations of a wide range of substrates, especially those highly polar, which gives many problems in organic solvents. Ionic liquids with their low vapor pressure makes then perfect to became the future media of a wide range of reactions not because they are harmful for the environment, but because they have little losses and can by almost completely recycled. Even more, it's not very often we have the opportunity to work with a media that can be finely tuned by selecting appropriate combinations of cations and anions, thus allowing the ionic liquids to be specifically designed for every different reaction.

There are still many steps to be given, there is too many we don't understand, to be able to take full advantage of these new solvents we need to understand biocatalysis at a molecular level: how does the ionic liquid behaves around the enzyme and how does it affects enzyme functioning such as activity, stability, and selectivity. Ionic liquids particular ionic nature allows them to strongly charge-charge interact with the enzyme leading to being either activated or inactivated. Taking all in consideration, we cannot account only with one particular property of ionic liquids, but with a complex multiparameter approach may be required which should have more ability to reflect the possible enzyme–solvent interactions.

e. Supercritical Carbon Dioxide

i. *Carbon dioxide*

Carbon dioxide (CO_2) is known for being a greenhouse gas being the second biggest contributor to the effect on Earth along with methane and ozone and behind water vapor. The rising values of CO_2 is a rising problem with tremendous effect in climate change[65]. The energy supply and the industry are the biggest contributors to this phenomenon as shown in fig I.4:

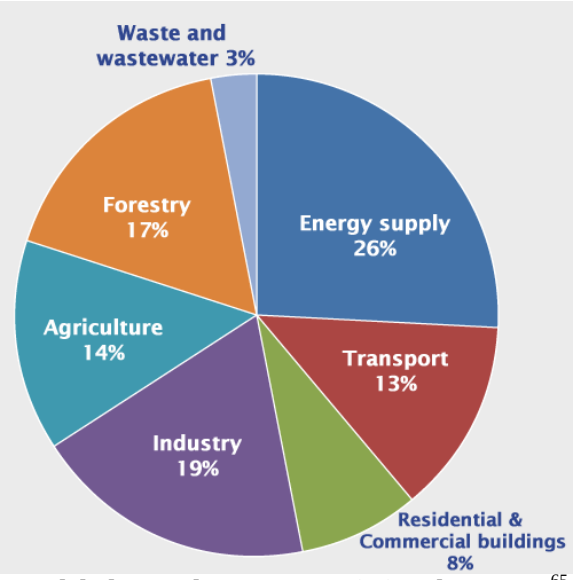

Figure I.3 - Global Greenhouse Gas Emissions by Source[65].

New technologies are being developed to stop and diminish this problem and use the emissions in a way that benefits humans and do not harm the nature and the environment. Along these ideas, we have: ways of capture the Carbon dioxide from power plants by removing it from the atmosphere and retaining it (stored) within plants and soil supporting the plants or alternatively, capturing it (either before or after fossil fuel is burned) and then it can be stored (sequestered) within the earth [66]. So Carbon dioxide is readily available, inexpensive, nontoxic, nonflammable and chemically inert under many conditions, environmentally acceptable, liquefiable at reasonable pressures and most of all, a waste. So carbon dioxide would be the perfect solvent.

Supercritical fluids represent a highly viable alternative to conventional solvents. A supercritical fluid is any fluid above its critical point (Figure I.5). The critical point of a pure substance is the highest temperature and pressure at which liquid and vapor can coexist in

equilibrium, beyond that point we obtain a single phase where both the liquid and vapor phase became identical and impossible to distinct them, this phase is called them supercritical phase[67].

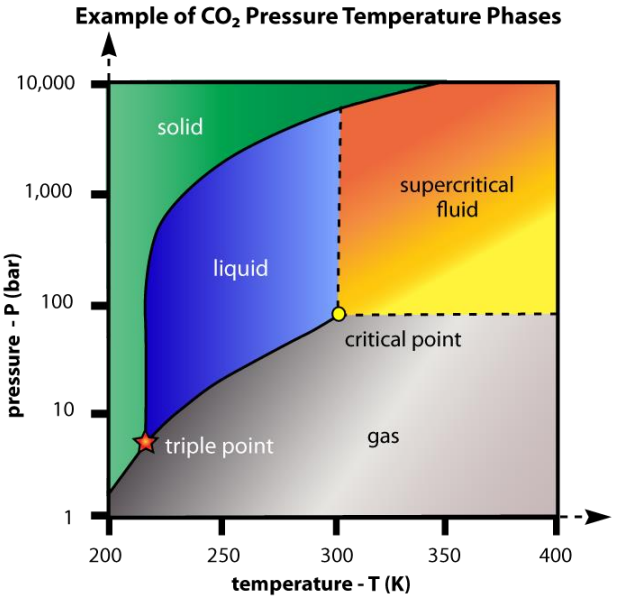

Figure I.4 - Carbon dioxide pressure-temperature phase diagram[68].

A supercritical fluid cannot be liquefied by however large pressure is applied mainly due to the intense molecular motion which prevents a positive balance in favor of intermolecular attractions necessary for the formation of a liquid phase. Supercritical fluids or compressed gases have properties between liquid and gas, being able to solvate a wide range of organic compounds, products and reagents having at the same time the viscosity of a gas increasing immensely mass transfer.

The most popular supercritical fluid is the carbon dioxide because what we said before: low toxicity, high availability and existence in the nature many times seen as a pollutant, but not only, between all supercritical fluids, supercritical carbon dioxide (sc-CO_2) has a low critical point compared to the other (as can be seen in table I.2) , this critical point has a critical temperature so low it's suitable to be used as a solvent in biocatalisys [69].

Table I.2 - Critical properties of various solvents commonly used as supercritical fluids[69].

Solvent	Molecular weight	Critical temperature	Critical pressure	Critical density
	g/mol	K	Mpa	g/cm^3
Carbon dioxide (CO2)	44.01	304.1	7.38	0.469
Water (H_2O)	18.015	647.096	22.064	0.322
Methane (CH4)	16.04	190.4	4.60	0.162
Ethane (C2H6)	30.07	305.3	4.87	0.203
Propane (C3H8)	44.09	369.8	4.25	0.217
Ethylene (C2H4)	28.05	282.4	5.04	0.215
Propylene (C3H6)	42.08	364.9	4.60	0.232
Methanol (CH3OH)	32.04	512.6	8.09	0.272
Ethanol (C2H5OH)	46.07	513.9	6.14	0.276
Acetone (C3H6O)	58.08	508.1	4.70	0.278

The uses of supercritical fluids have several advantages, although their lower solubilization capacity of reactants, products and of hydrophobic molecules in comparison to the organic solvents commonly used. The solubilization of hydrophobic compounds is a common problem for aqueous systems leading to low productivities and in a supercritical system we may elevate productivity performing these reactions. We can also obtain a better mixing of the compounds due to the properties of sc-CO_2 leading to better heat and mass transfer and a faster reaction in comparison to organic solvents. In supercritical fluids, natural catalysts can be used with natural solvents in a green process.

Ease of manipulation of the physical properties of the solvent by simply changing the pressure or temperature is a unique property of supercritical systems (tuning properties). Albeit in supercritical fluids, the process requires a great amount of energy to reach supercritical conditions and when it reaches it, structures like the reactor may suffer from corrosion and salt deposition (in the case of supercritical water)[70]. Supercritical conditions are, at the best, very harsh conditions to everything in direct contact and to add, the prices of the equipment are extremely high and require continue maintenance and supervision. For sc-CO_2 to become a wide alternative, very important problems must be solved, such as reducing the energy requirement associated with compressing CO_2, improve sequential reactions without depressurization between them and perform reactions in gas-expanded liquids which greatly enhances solubility of polar reactants and catalysts compared to pure $scCO_2$ [71].

CO_2 is the most suitable compound for supercritical conditions with its gas like diffusivity and liquid alike density along with elevated mass transfer to increase reaction rates turning $scCO_2$ into an economically sustainable solvent. The solubility of a certain compound

may be adjusted by changing the temperature and pressure of the sc-CO_2. This happens because the solubility of any compound in sc-CO_2 is linked to its density which in turn, is controlled by the pressure and temperature applied. The manipulation of both of these parameters will result in an increase or decrease of the solubility of that same compound in the solvent; this can be used as a cracking/recovery method for products, reactants and/or even side products that are unwanted in the reaction medium by various reasons. This precipitation of compounds can also be used not only to segregate solvent-free products and unreacted reactants but, as well, to reutilize the carbon dioxide fashioning a cost-efficient process[72]. Using sc-CO_2 and retrieving products and reactants recurring to a depressurizations step, reduces energy demand when compared to processes requiring further steps for purification and removal of, many times, toxic solvents.

Most of the time, and to a well understood process, it is only required to adjust the pressure and temperature, altering sc-CO_2 solubility of a certain compound to a value known to precipitate the target molecule. This way, we can save money, resources and infrastructures in a given process.

Recently, one of the most successful application of scCO_2, the decaffeination of coffee beans become one of the most popular decaffeination processes[73]. This broadened the already vast uses of scCO_2 in potential to be implemented in the food industry and it happens probably because of the attraction for a non-toxic solvent and the relatively high value of the products. A key factor in the success of the decaffeination process is that the caffeine can be recovered from the high pressure CO_2 by extraction with water rather than by releasing the pressure. This has a positive impact on the energy costs of the process[73].

There are also two major successful industrial-scale processes for hydrogenation in scCO_2. The first is part of the synthesis of vitamins by Hoffman la Roche [74,75] and the other was a multi-purpose plant built by Thomas Swan & Co Ltd for hydrogenation of isophorone.

ii. Enzyme catalyzed reaction on sc-CO2 and the separation process

In recent years, lipases were very often used instead of conventional catalysts as biocatalysts turning processes more efficient and productive with higher selectivity and fewer environmental problems. Lipases were discovered at nearly 100 years ago when microbiologist C. Eijkmann reported that several bacteria could produce and secrete lipases. Then, it became proved that lipases remain enzymatically active in organic solvents[76]. What is it that makes lipases so attractive? Firstly, they usually display exquisite chemoselectivity, regioselectivity and

stereoselectivity. Secondly, they are readily available in large quantities because many of them can be produced in high yields from microbial organisms. Thirdly, the crystal structures of many lipases have been solved, facilitating considerably the design of rational engineering strategies. Finally, they do not usually require cofactors nor do they catalyze side reactions. And if the enzyme is immobilized, it can be used several times reducing production costs.

To the process described in this work, the most important reaction is the transesterification, which follow the general mechanism shown below:

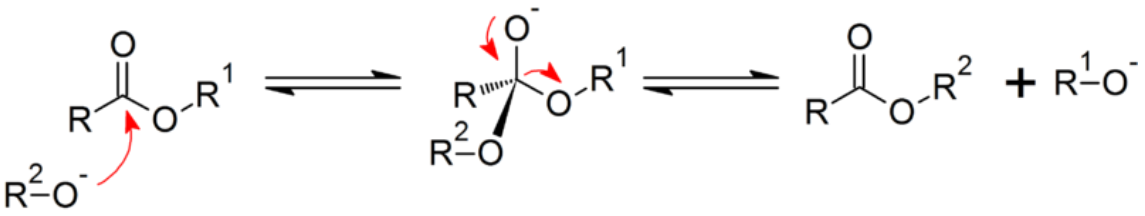

Figure I.5 -General mechanism of a transesterification reaction[77].

This reaction mechanism will be taken into notice later in this introduction when we discuss the target molecule for this project, the (-) Menthol.

The use of enzymes in supercritical media have several advantages over conventional solvents, among them possibly enhanced reaction rate due to the higher mass transfer of reactants compared to organic solvents since the low viscosity of the media promote collisions between the reactants accelerating the reaction rate and given the solubility capacity of sc-CO_2, the reaction medium will be a homogenous phase which accelerates the reaction since there is no interphase mass transfer to limit the reaction rate and slow the reaction [78].

After the reaction takes place, and as said before, sc-CO_2 can be adjusted to retrieve a target product and then be recycled. During the separation, the most important parameters are the solubility and phase equilibrium of the system, because at constant temperatures, higher solubilities can be achieved by increasing pressure. Still, with a fixed pressure, an increase in temperature will decrease fluid density and lead to different solubility of a given compound.

Another important factor in separation using a supercritical fluid is vapor pressure since an increase in temperature will increase vapor pressure of that compound and its solubility. By manipulating this as a system, we may choose which compounds retrieve from the media adjusting their solubilities and at the same time separating them.

f. Menthol: what is, where does it comes from and its properties.

Menthol is the world's best-selling aroma ingredient with a global production capacity about 20,000 metric tons a year and a market value of 1,6 billion Dollars per year. There is high demand on extremely pure product of superior quality. Its market divides in: food industry like chewing gums and confectionery mints, in the cosmetic and pharmaceutical industry. In pharmaceutical industry serves both as taste-masking excipient and as intermediate in pharmaceutical synthesis.

Menthol oil can be extracted from plants of the *Lamiaceae* family like *Mentha*, *Peppermint* and *Mentha arvensis*, which gives plants of the *Mentha* species the typical minty smell and flavour. Peppermint oil and cornmint oil from *Mentha arvensis*, prepared by steam distillation from the fresh flowering tops of the plant, contains 50% and 70% of (-)-menthol, respectively [79]. That same volatile oil has a mixture of menthol enantiomers, the composition of that mixture depends of its source and different mixtures have different effects depending on the quantity and abundance of the enantiomers present.

Molecular menthol ($C_{10}H_{20}O$, Mw 156.27) is a secondary alcohol with a waxy, and crystalline appearance, clear or white in color, which is solid at room temperature and melts slightly above, approximately at 36ºC – 45ºC, depending on the enantiomer present or the mixture of two or more enantiomers and the purity of the mixture. The main form of menthol occurring in nature is (−)-menthol, which is assigned the (1R,2S,5R) configuration, but there are eight stereoisomers in total, and they can all be present in different quantities, depending on the source. Menthol has three asymmetric carbon atoms in its cyclohexane ring, and therefore occurs as four pairs of optical isomers; (-)- and (+)-menthol, (-)- and (+)- neomenthol, (-)- and (+)- isomenthol and (-)- and (+)- neoisomenthol. The eight possible stereoisomers structures are shown in the next page:

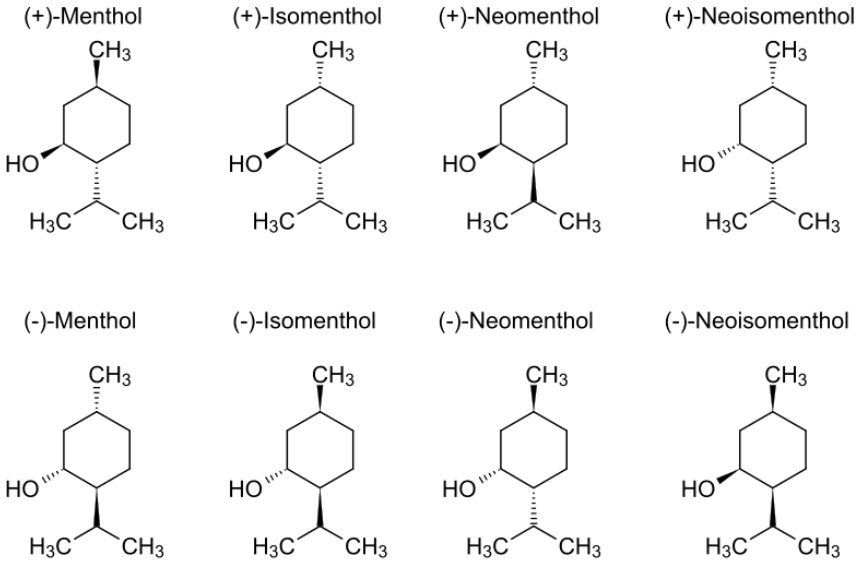

Figure I.6 - Illustration of the eight possible stereoisomers of menthol present in the nature[133].

Menthol has been used for medicinal purposes for over one hundred years in the West, but our knowledge of this fascinating compound is still very limited despite its very widespread use. Menthol has local anesthetic and counterirritant qualities, and it is widely used to relieve minor throat irritation. Menthol also acts as a weak kappa opioid receptor agonist. Pure (-)-menthol can be obtained from *cornmint* oil by recrystallization from low-boiling point solvents. Peppermint oil made from *Mentha piperita* contains up to 50% menthol, but due to its high price, peppermint oil is not used for the production of menthol. Peppermint oil is mostly produced in the USA and is used mainly as a flavoring for toothpastes, other oral hygiene products and chewing-gum [79].

(-)-Menthol is the isomer that occurs most widely in nature and is the one assumed by the name menthol. It has the characteristic peppermint odor and exerts a cooling sensation when applied to skin and mucosal surface. The other isomers of menthol have a similar, but not identical, odor and do not have the same cooling action as (-)-menthol[79].

In the next image, the different characteristic of menthol stereoisomers are showed on 4 different charts one for each pair of the four natural optical isomers.

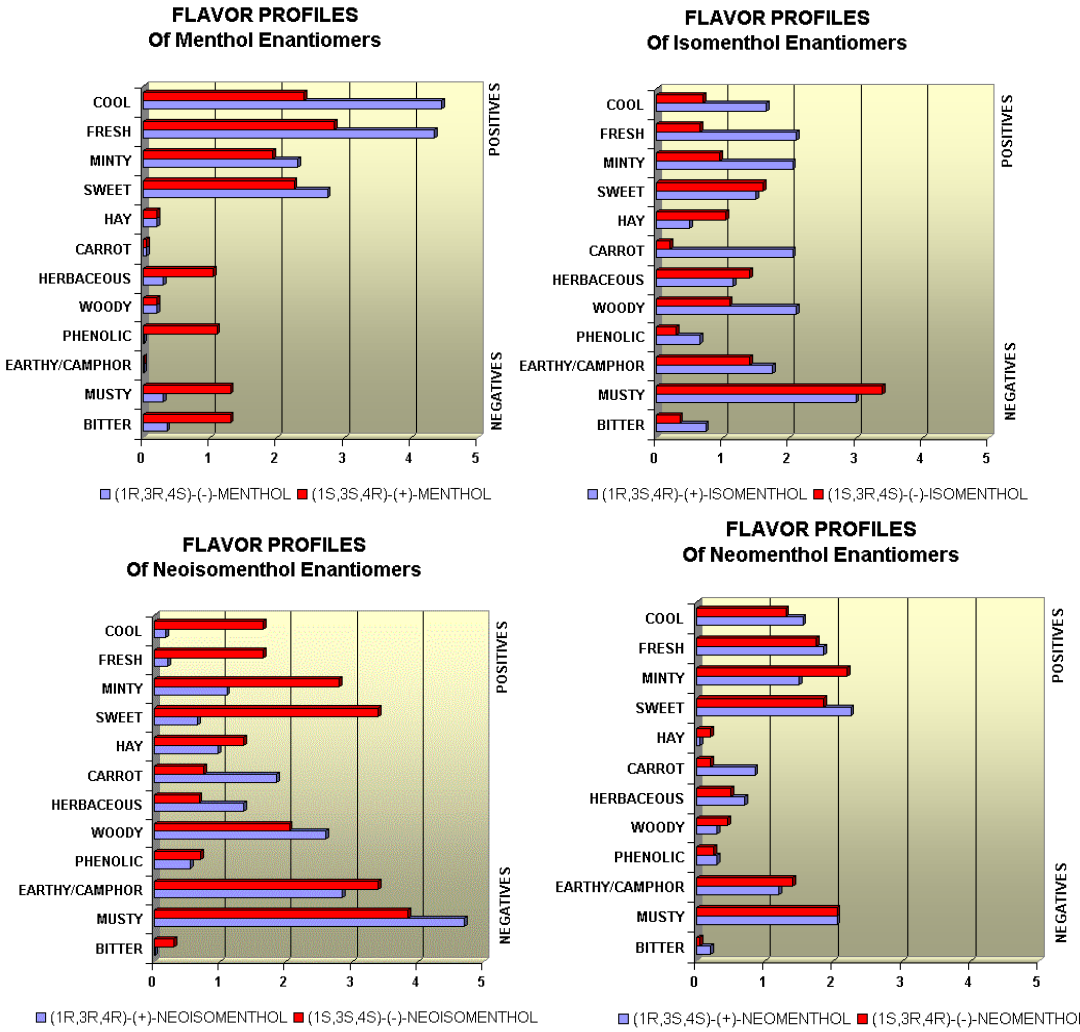

Figure I.7 - Different characteristics of the eight menthol stereoisomers according to consumers and differentiation of characteristics between the appreciated ones and those undesirable to be presented in products[80].

From the presented graphics we may acknowledge the (-) - Menthol as the enantiomer with the most desirable characteristics and with lesser negative ones of the other seven stereoisomers. Both the negative and the positive enantiomers have similar effects, but only the (-) - Menthol has any anesthetic affect, being able to induce analgesia through the activation of the central k opioid system, making it extremely valuable to the industry [81].

The majority of (-) - menthol is still obtained by freezing the oil of *Mentha arvensis* to crystalize the menthol present. Menthol can also be extracted or synthesized from other essential oils such as citronella oil, eucalyptus oil and Indian turpentine oil. There have been many efforts

to produce menthol from other readily available materials, but only two processes are sustainable both industrially and commercially: Haarmann and Reimer (H&R) and the Takasago processes.

The first process starts from inexpensive m-cresol and propylene to produce thymol (step 7), them this compound is hydrogenated to give the mixture of the eight isomers of menthol.

From there, a fractional distillation gives racemic (+/-) - menthol (step 8) which is converted into racemic benzoate (step 9). This mixture is resolved recurring to fractioned cristilization. By saponification (-) - menthol is obtained whereas the mother liquour gives (+)-menthol. The other seven undesired isomers are recycled in a separate racemization step and reused.

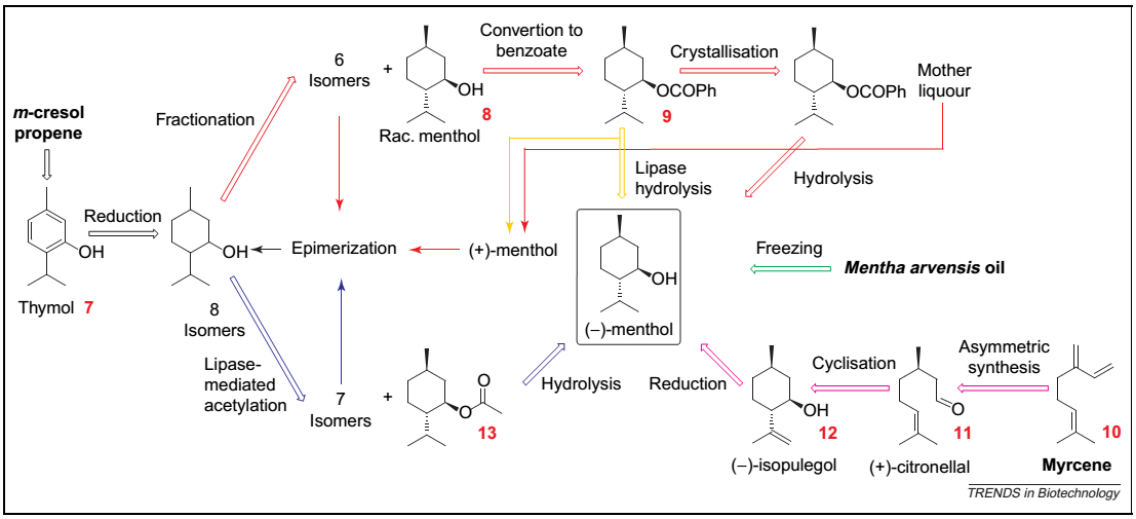

Figure I.8 - Industrial production of (-) – menthol. In red we have Haarmann and Reimer process, in green the extractive process and in violet we have Takasago process. Also on the image we have the new biocatalytic process[82].

The other process, the Takasago process, uses asymmetric synthesis as the main step of is process, which starts when myrcene (step 10) is converted into diethygeranylamine. From there, diethygeranylamine is isomerized to (C)-citronellal by the action of a chiral rhodium phosphine catalyst (RhI-(S)-BINAP) [82]. After this step, (C)-citronellal (step 11) is transformed into (K)-menthol, this transformation is performed through cyclisation to (K)-isopulegol (step 12) followed by hydrogenation. Meanwhile, two other alternatives to the first process have been described; the first one are based on lipase resolution of racemic menthol benzoate (step 9) by lipase mediated using highly enantioselective enzymes like Candida rugosa lipase giving (-)-menthol with a high yield and purity [83]. The other process is based on an enantio- and diastereoselective acylation from a pool of the eight isomers of menthol producing a enantiomeric excess over 96%. Then, the ester is separated from the unreacted isomers by distillation and then hydrolysed to give pure (-)-menthol [84]. This process is currently patented to AECI Ltd [85].

The bigger problem with freezing plant extracts oils like Mentha arvensis is that we cannot separate all the enantiomers present there, some of them have the same physical characteristics, like melting point but are undesirable, that's the case of (+) – menthol. In the next table we present the melting points of the most relevant menthol stereoisomers:

Table I.3 - Melting points of the most relevant menthol Stereoisomers[86].

Compound	Melting point (°C)
(-) – menthol	43 - 45
(+) – menthol	43 - 45
(+) – isomenthol	77- 80
(+) – neomenthol	-22

As seen above, (-) and (+) ,menthol have the same physical properties, but different characteristics. One way to separate this to enantiomers is to transform one into a different molecule, giving it different properties so we can apply simple methodologies to separate them. With this, we arrive at the main purpose of this thesis, how to separate efficiently (-) and (+) menthol using green chemistry and achieving a high value product with extremely purity and low production costs.

g. Transesterification and acylation:

Transesterification is the process of exchanging the organic group R″ of an ester with the organic group R′ of an alcohol. These reactions are often catalyzed by the addition of an acid or base catalyst. The reaction can also be accomplished with the help of enzymes (biocatalysts) particularly lipases (E.C.3.1.1.3), as in this case.

The scheme of reaction is a very simple one and it's presented in figure I.6 (pag.25).

When an alcohol and an ester are combined with a catalyst, which can be acid, base, or enzymatic in nature, it starts a chemical reaction. This change causes the organic group R″ of the alcohol to switch places with the organic group R′ of the ester. The result is a new ester and alcohol. This reaction is used for many different purposes. The esters can be created for their use in perfumes, herbicides and other industrial strength chemicals. But they can, as well, be used in the much known reaction for production of biodiesel,[87] which has attracted much attention today

due to the crisis in fossil fuels and their escalating prices and pollution. Bio diesel is a chemical compound made by the transesterification of vegetable oil. The triglycerides, or fat, in the oil are converted into useable fuel through the process.

So, in this work, we intend to convert only one of menthol enantiomers in a ester, for this purpose we use a vinyl ester (example showed in fig. I.12) and an acid anhydride (Fig I.11). We will find and use different molecules of each functional group and analyze which one is better in terms of selectivity and conversion.

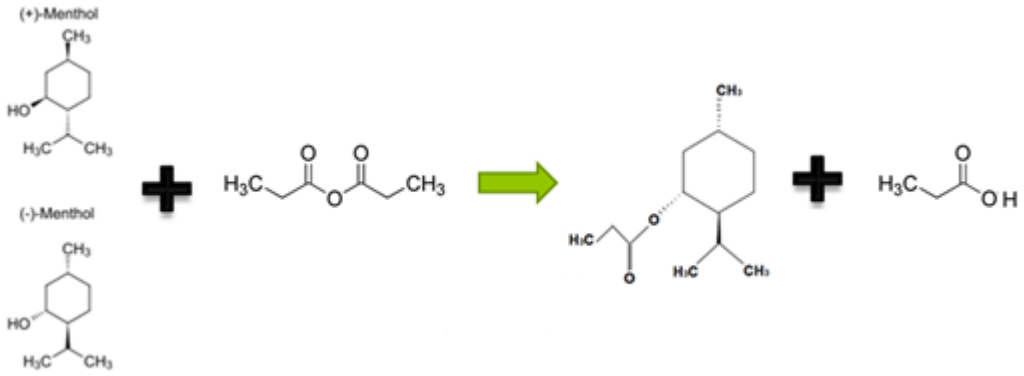

Figure I.9 - Selective transesterifcation of (-) – menthol using propionic anhydride. This reaction also produces propionic acid (not depicted) besides the target propionic menthyl.

In this reaction of the sec-alchool with the anhydride, we produce a product with low selectivity because anhydrides are very reactive and will indiscriminately react with both enantiomers via a parallel chemical reaction.

Reaction using a vinyl decanoate:

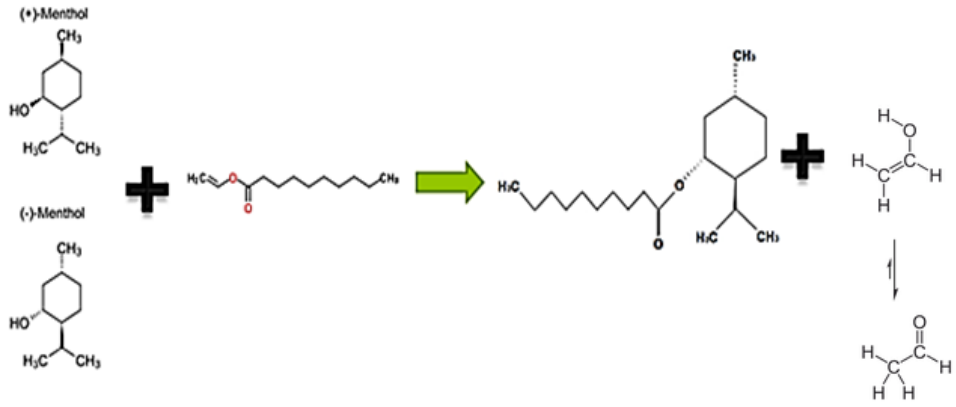

Figure I.10 - Reaction of rac-menthol with a vinyl ester, in this case, vinyl decanoate producing menthyl decanoate and other enol molecule which will tautomerize.

The reaction, in this case, of vinyl decanoate with rac-menthol, will produce menthyl decanoate and other enol molecule which, in turn, will tautomerize and transform into a ketone making the reaction irreversible. The later reaction is more appealing, because it can only occur with the use of a catalyst, like an enzyme. If we use a very specific enzyme, we can obtain a very pure product with high enantiomeric excess. Furthermore, because we recur to a long chain ester, the product will be very different from the starting molecule, giving it different properties and the ability to be separate from the other molecule using simple and inexpensive processes.

h. Candida rugosa lipase

Extracellular lipases (EC 3.1.1.3) produced by microorganisms are often investigated because of their many different uses in biotechnological processes. There has been an increasing worldwide initiative taken up in the screening of lipase-producing organisms and their possible utilizations in the growing biotechnology for human well-being[88].

Lipases are a growing interest from investigators and industry for decades, they are very versatile being capable of utilize a great amount of different substrates, and capable of withstand a wide range of reactional conditions without the need of a cofactor. These enzymes are easy to produce and with a great yield from microorganisms, such as fungi and bacteria. They have structures easily resolved and very known which facilitates engineering new lipases for the use in industry with desired properties[89]. lipases can be used in various reactions, such as esterification, transesterification and hydrolysis of esthers[89].

One of the most attractive lipases is from *Candida rugosa* due to its high activity and availability both in hydrolysis and esterification. *Candida rugosa* lipase (CRL) gathers more applications than any other biocatalyst. It has a day-by-day stronger role in the food and flavor industry, the production of ice cream and single cell protein, biocatalytic resolution of life-saving pharmaceuticals (like Ibuprofen), carbohydrate esters (waxs) and amino acid derivatives unobtainable by conventional chemical synthesis, potent biocide making, biosensor modulations, eco-friendly approach and bioremediation, biosurfactants in detergent making, and even in cosmetics and perfumery. These reactions involve, mainly, hydrolysis and synthetic reactions for the resolution of racemic mixtures, drug design, acylglycerols, and flavor and fragrances[88].

Candida rugosa lipase was successfully used in a wide range of catalytic reactions in both aqueous and water-restricted environments which include: biocatalyst for esterification,

transesterification, and polyesterification. Especially in transesterification, *Candida rugosa* lipase gives good results in comparison to a number of other commercially available lipases, such as those from *Rhizomucor miehei*, *Chromobacterium viscosum* and *Pseudomonas fluorescens*[90]. This enzyme has been used over the past 20 years in Japan and the United States for the hydrolysis of milk fat to produce flavor compounds, being proved that is toxicologically safe for food applications.

The yeast *Candida cylindracea* (reclassified as *Candida rugosa*) is a non-sporogenic, pseudofilamentous, unicellular, and non-pathogenic who synthesizes and secretes a mixture of lipase isoenzymes that have been studied by several authors aimed at their purification and characterization, in order to promote the growing lipase industry [91–93].

Each of the isoforms proteins is encoded by a separate gene. All the genes encode 534 amino acids and their respective protein products have an apparent MW of 60 kDa [92,94,95].

Candida rugosa lipase is a single domain protein with an α/β hydrolase fold. The flap, located between the last strand, β_3, of the small β- sheet and strand β_2 of the large β-sheet, is tightly fixed at its base by the Cys 60-Cys 97 disulfide bridge and the Glu 95- Arg 37 salt bridge. The loop has an elongated shape and lies flat on the protein surface above the active site [96].

The flap/lid that canopies the active site has 31 amino acids, mainly hydrophobic on the side directed towards the active site, and hydrophilic on its external face [97]. Both the amphipathic nature of the lid and the specific amino acid sequence may be important for lipase specificity and activity [96].

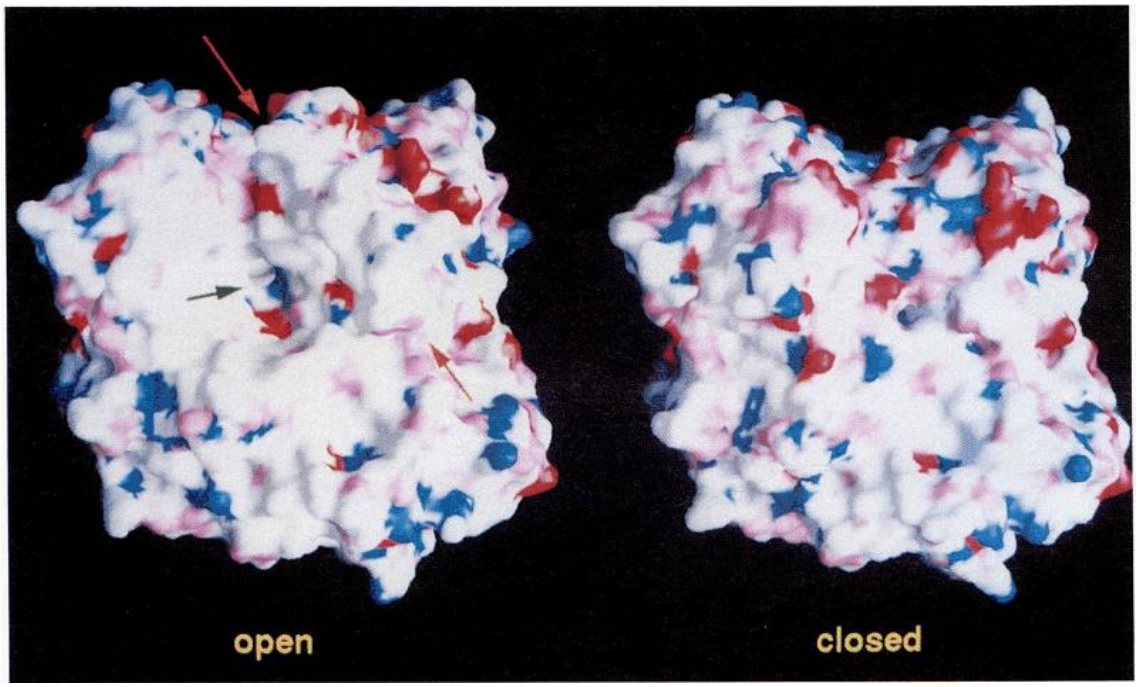

Figure I.11 - Image of CRL with and without the lid open and where is positioned[96].

The catalytic center of lipases consists of a serine protease-like triad, Ser-His-Asp/Glu, and their hydrolysis of ester bonds of triacylglycerols is believed to comprise an enzymatic mechanism similar to that of the serine proteases [96].

Commercially available CRL, obtained from various sources, show remarkable variations in catalytic efficiency, substrate specificity, and enantioselectivity. Previous work on the purification of a *Candida rugosa* lipase preparation has shown that the enzyme has two distinct forms (A and B). The two forms have a similar amino acid composition, N-terminal sequence and molar mass, but differ in their neutral sugar content and hydrophobicity [90].

Native or wild-type CRL obtained by conventional fermentation techniques is a mixture of various isoforms (isozymes) due to the differential response of the different genes, in both the wild-type and mutant strains, to the varied fermentation conditions employed by different enzyme suppliers[95,98]. This factor limits the application of crude CRL, because of the mixed isoforms, in the production of specific compounds with high purity and reproducibility. This also brings lack of reproducibility of reactions which can be attributed to the variable proportion of isoforms, amount of water in the crude lyophilized lipase, and the amount of lipolytic protein present in the powdered enzyme.

In commercial crude CRL preparations, only three (LIP1, LIP2, and LIP3) have been successfully identified. This happens because the yeast *Candida rugosa* does not utilize the

universal codon CTG for leucine. These suggest that the specificity and stability of lipase preparations can be changed by engineering the culture conditions, which will in turn result in change of the isoform compositions [92,99–101].

The sequence homology of the isoforms is 85–90% amino acids, and even through all lipases share a common structural motif there are differences between them. All lipases have essentially two binding sites, one for the substrate or acyl group and the other for alcohol binding.

Many studies revealed that CRL has a preference for short-chain fatty acids[102–104]. Measurements made in toluene (based on Vm/Km) showed a high preference for C4, C8, C10 and C12 fatty acids. For efficient catalysis to occur the presence of a water–lipid interface is required[105]. One great advantage of lipases is that their reactions have no cofactor requirement.

The enantioselectivity of CRL is correlated by a rule based on the sizes of the substituents at the stereocenter. This rule foretells which enantiomer of a racemic secondary alcohol reacts faster for 51 of 55 cyclic substrates of lipase from *Candida rugosa* (CRL) and also implies that the most efficiently resolved substrates are those having substituents which differ significantly in size. Thus, the rule is not useful for acyclic substrates of CRL [106].

This lipase is a soluble enzyme and the direct use of pure lipases which perform catalysis for a long time has been very limited due to their low molecular stability and high cost, so and in these cases they are preferentially used in entrapment technology because of their proven advantages:

(a) enzymes can be reused;

(b) processes can be operated continuously and can be readily controlled;

(c) products are easily separated;

(d) effluent problems and materials handling are minimized;

(e) enzyme properties (like activity and thermostability) can be altered favorably;

(f) the process is more cost effective [107,108].

This entrapment is based on the coupling of enzymes to the lattices of a polymer matrix or enclosing them in semi-permeable membranes, tight enough to prevent the leaching of protein, however allowing the diffusion (mass transfer) of substrates and products to and fro[109]. Both natural and synthetic polymers are used as matrices in many immobilization techniques.

Chapter II

Materials and methods

In this chapter the experimental procedures are concisely presented, with the detail considered necessary to allow the reproduction of the results. Identification of equipment suppliers is presented where necessary. Unless otherwise stated reactants were from Sigma-Aldrich and were of the highest purity available and all weighing was performed using a Precisa 205A Superbal-Series Balance.

II. Materials and methods

a. Catalysts:

Novozyme 435, Lipozym RM-IM and Lipozym TL-IM from Novozymes and Lipase from *Candida rugosa* (CRL), Type VII L1754.

b. Reactants:

- (+/-) – Menthol Aldrich ≥ 98% GC, 100g
- Vinyl Decanoate, 95%, 10g
- Propionic anhydride, ≥ 99%
- Tridecane, ≥ 99%
- Butyric acid, Merck, ≥ 99.5%
- Propionic Acid, ≥ 99.5%
- Acetic anhydride, Fluka, ≥ 98%
- Vinyl Laurate, Fluka, ≥ 99.0%
- Vinyl Stearate, ≥ 99%
- Lauric acid, 99%
- Decanoate acid, ≥ 99%
- Dichloromethane, ≥ 99%
- 4-Dimethylaminopyridine (DMAP), ≥ 99%
- Dicyclohexylcarbodiimide (DCC), ≥ 99%
- Silica gel 60M, Macherey-Nagel
- Ethyl acetate, Carlo Erba, ≥ 99.8%

c. Materials:

- Karl fischer 831 KF coulometer Metrohn
- Haake d1 immersion circulator
- Reliance electric master XL speed reducer compressor
- Labnet accuplate hotspot stirrer
- Vacuunbrand GMBH MZ 2C NT

- Termoquest Trace GC 2000 series
- Asus barebone PC
- Auto sampler Thermo Finnigan AS2000

d. Procedures:

Organic synthesis of (-) Menthyl Laurate and (+/-) Menthyl Laurate :

To a solution of Lauric acid (220.5 mg, 1.1 mmol, 1.25 eq) in dry CH_2Cl_2 (3 mL), DCC (230 mg, 1.1 mmol, 1.25 eq) was added. After 1 h, (-) Menthol (138.1 mg, 0.875 mmol, 1 eq) and DMAP (12.5 mg, 0.10 mmol, 0.125 eq) were added. The reaction was completed after 2 h. The mixture was filtered, and the filtrate was washed with H_2O. The organic layer was dried, filtered, and concentrated under reduced pressure. Then the mixture was purified by flash chromatography using a mixture of n-Hexane and Dichloromethane (9:1) as washing solvent. The filtrate was later concentrated by removal of the solvent using a Rotary evaporator (BÜCHI Rotavapor R205) to evaporate it. For the synthesis of (+/-) Menthyl Laurate, we proceeded as previously, just changing the (-) Menthol used to the racemic one. The products were later evaluated by gas chromatography, NMR H_1 and mass spectrometry, and the purity determined by Professor Marco Ritcher and Professor Paula Branco.

Organic synthesis of (-) Menthyl Decanoate and (+/-) Menthyl Decanoate :

To a solution of Decanoic acid (172.26 mg, 1.1 mmol, 1.25 eq) in dry CH_2Cl_2 (3 mL), DCC (230 mg, 1.1 mmol, 1.25 eq) was added. After 1 h, (-) Menthol (138.1 mg, 0.875 mmol, 1 eq) and DMAP (12.5 mg, 0.10 mmol, 0.125 eq) were added. The reaction was completed after 2 h. The mixture was filtered, and the filtrate was washed with H_2O. The organic layer was dried, filtered, and concentrated under reduced pressure. Then the mixture was purified by flash chromatography using a mixture of n-Hexane and Dichloromethane (9:1) as washing solvent. The filtrate was later concentrated by removal of the solvent using a Rotary evaporator (BÜCHI Rotavapor R205) to evaporate it. For the synthesis of (+/-) Vinyl Decanoate, we proceeded as previously, just changing the (-) Menthol used to the racemic one. The products were later evaluated by gas chromatography, NMR H_1 and mass spectrometry, and the purity determined by Professor Marco Ritcher and Professor Paula Branco.

Calibration curves for menthol, propionic anhydride and propionic acid:

To a 2 ml volumetric flask, we add the menthol mass to make concentrations of 25; 50; 75; 100; 150; 200 mM. All exact weights were noted and taken into account as the real mass. The tridecane was added in a concentration of 20 mM, approx. 35 mg, but the real weight of the

added was noted, as well. We proceeded in the same way for the propionic anhydride and propionic acid. Then the curves were made as presented in appendix 1 for the calculation of each concentration.

Reactions on n-Hexane with propionic anhydride:

In a 10ml flask with a 5ml solution of n-Hexane we added 260mg of menthol (333,33mM), 213,5µl of propionic anhydride (333,33mM) and 200mg of CRL after pre-heating a bath over a hotplate from labnet (model: accuplate). We add tridecane as internal standard to the reaction media 24,4 µl (20mM). 100 µl samples were taken at 0h, 6h, 24h, 48h, 72, 96 added 500 µl of n-Hexane and filtered using 0.20 µl pore syringe filters. After that, all samples were analyzed by gas chromatography in a suitable program.

Reactions on n-Hexane with vinyl decanoate:

In a 10ml flask with a 5ml solution of n-Hexane we added 260mg of menthol (333,33mM), 372.67µl of vinyl decanoate (333,33mM) and 200mg of CRL or less, depending of the experiment, after pre-heating a bath over a hotplate from labnet (model: accuplate). We add tridecane as internal standard to the reaction media 24,4 µl (20mM). 100 µl samples were taken at 0h, 6h, 24h, 48h, 72h, 96h added 500 µl of n-Hexane and filtered using 0.20 µl pore syringe filters. At all time, a reaction media without enzyme was present to serve as blank and samples were taken and analyzed with the reaction ones by gas chromatography in a suitable program.

Reactions on n-Hexane with vinyl laurate:

In a 10ml flask with a 5ml solution of n-Hexane we added 260mg of menthol (333,33mM), 432.7µl of vinyl laurate (333,33mM) and 200mg of CRL after pre-heating a bath over a hotplate from labnet (model: accuplate). We add tridecane as internal standard to the reaction media 24,4 µl (20mM). 100 µl samples were taken at 0h, 6h, 24h, 48h, 72h, 96h added 500 µl of n-Hexane and filtered using 0.20 µl pore syringe filters. At all time, a reaction media without enzyme was present to serve as blank and samples were taken and analyzed with the reaction ones by gas chromatography in a suitable program.

Reactions on n-Hexane with vinyl stearate:

In a 10ml flask with a 5ml solution of n-Hexane we added 260mg of menthol (333,33mM), 515mg of vinyl stearate (333,33mM) and 200mg of CRL after pre-heating a bath over a hotplate from labnet (model: accuplate). We add tridecane as internal standard to the reaction media 24,4 µl (20mM). 100 µl samples were taken at 0h, 6h, 24h, 48h, 72h, 96h added 500 µl of n-Hexane and filtered using 0.20 µl pore syringe filters. At all time, a reaction media

without enzyme was present to serve as blank and samples were taken and analyzed with the reaction ones by gas chromatography in a suitable program.

Reactions on n-Hexane with butyric anhydride and acetic anhydride:

In a 10ml flask with a 5ml solution of n-Hexane we added 260mg of menthol (333,33mM), 100 mg of CRL enzyme, 122,08 µl of tridecane (120mM) and a concentration of 333.33 mM of the respective anhydride, 157.25 µl in the case of acetic anhydride and 269.9 µl in the case of butyric anhydride. Each reaction was taken separately so we could measure the reactivity and selectivity of each anhydride. 100 µl samples were taken at 0h, 6h, 24h, 48h, 72h, 96h added 500 µl of n-Hexane and filtered using 0.20 µl pore syringe filters. At all time, a reaction media without enzyme was present to serve as control and samples were taken and analyzed with the reaction ones by gas chromatography in a suitable program.

Reaction in scCO_2:

The experiments were made in a steel cell with variable volume. We added 333.33 mM of menthol, tridecane 20 mM and 333.33 mM of the acylating agent (propionic anhydride or vinyl decanoate), depending on the experiment in course. Then we put the cell inside a thermostatic bath and we set the volume to 10 mL using the piston with the help of a water pressure generator. Then the cell was filled with CO_2 which was compressed until 150 bar. The stirring initiated and samples were taken at 5 min, 6h, 24h, 48h and 72h. Samples were taken from a 150 µl loop and left bubbling in 500 µl of n-Hexane. The loop system was cleaned with 1 ml of n-Hexane which was added to the sample and taken for GC analysis. A photograph of the installation is depicted next:

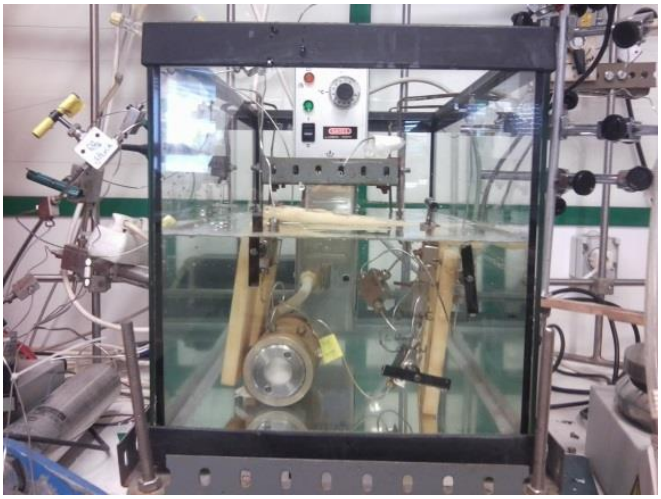

Figure II.1 - Experimental set-up for the batch reaction.

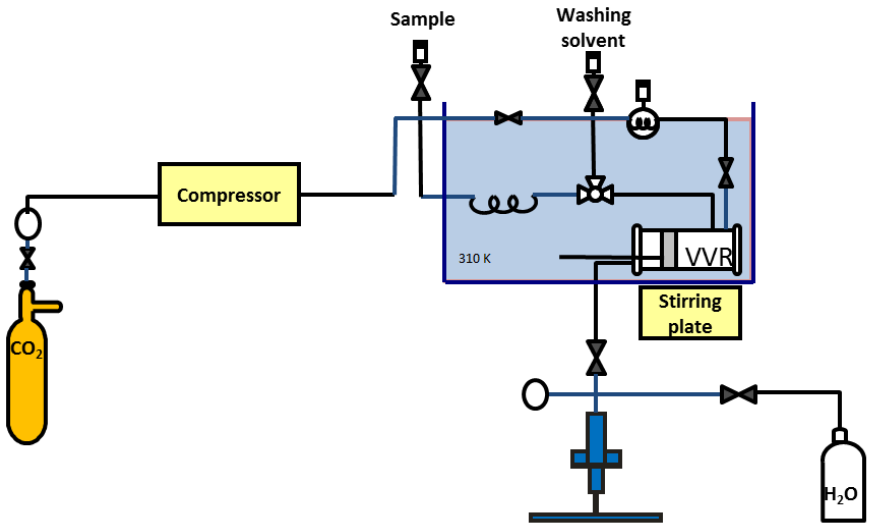

Figure II.2 - Experimental apparatus used for the batch reaction.

Reactions on ionic liquids:

In a 10ml flask, we weighted, accordingly to its density, approximately 2ml of each ionic liquid in study. Those ionic liquids were pre-treated: we dried them using a vacuum pump (Vacuubrand GMBH+ MZ 2C NT) at a low pressure for 48h. After that, we weighted them once more, and added the reaction: 104 mg of menthol (333.33 mM), 200 mg of CRL enzyme and the aciling agent: vinyl decanoate or propionic anhydride in a concentration of 333.33 mM, 149 µl and 427 µl, respectively. Approximately 100 µl (weighted) samples were taken at 0h, 6h, 24h, 48h and 72h. Samples were treated by dissolving them in 8 ml of ethyl acetate after which we passed them in a flash chromatography using a Pasteur pipette filled with silica gel (M60 from Macherey-Nagel) and cotton to prevent the escape of the silica from the pipette. The chromatography was executed to extract the ionic liquid from the sample and prevent its entry in the GC column. To the filtered solution we added 1ml of a solution of tridecane in ethyl acetate with the concentration necessary to make 2,5 mM in the final solution. 1ml of the final 9ml solution was taken and set in a GC vial to analyze in a suitable program. A photograph of the reaction undergoing is depicted in the next page:

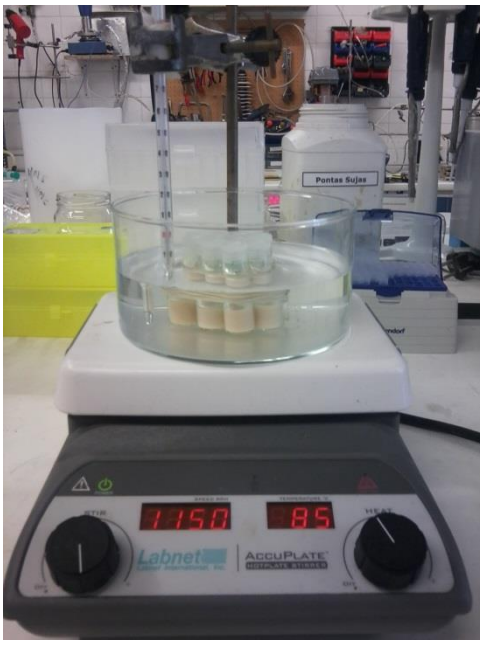

Figure II.3 - Experimental set-up for the ionic liquid experiments.

e. Sample Preparation and Injection:

The sample analysis is made by gas chromatography (GC) which is a commonly used method of separating components of a complex mixture. The GC system consists of gas supplies for the mobile phase and the detector, flow controls for the gases, an injector, an oven for heating the column, a detector, and a data recording device. Common carrier gases are N_2, H_2, or He.

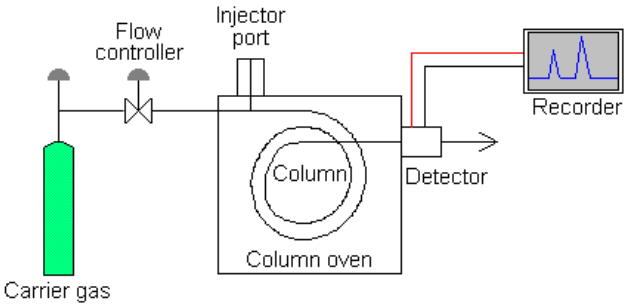

Figure II.4 - Gas chromatography apparatus scheme.

Hydrogen often gives the best results, but it is flammable and therefore somewhat hazardous. Helium gives almost as good of results as H_2 and is inert, but is two times the Hydrogen's price.

Liquid Injection

This was the chosen method for introducing a sample into a GC and it's also the most common. It's preformed with a microliter syringe, hence this process has become known as injection. Generally the sample is introduced through a gas-tight rubber septum. The injector is generally heated to volatize the sample. There must be enough volume in the detector to allow for the expansion of the solvent upon vaporization.

Samples analyzed with capillary columns are necessarily small which makes sample introduction more difficult than one would think. As with any analytical procedure, the measurement of the sample must be accurate and reproducible, but there are other important aspects to injecting a sample into a GC. Because of the wide range in volatiles often present in a sample, it can be difficult to introduce a representative sample because these differences in volatility often results in a phenomenon called discrimination. Discrimination results when the syringe needle is inserted into the injector and some of the high boiling compounds cling to the syringe as the solvent evaporates. If the needle is pulled out too quickly, some of the high boiling compounds will remain in the needle and will be removed with the syringe. There are a number of techniques to reduce needle discrimination. The one used in this case was the recurring of rapid Injection, with autosampler. The sample is propelled out of the needle before the solvent evaporates. This requires the use of a glass wool plug or other injector modification. It is possible that small droplets of solvent will enter the column rather than just vapors. The plug of glass wool can stop these aerosols from entering the column.

Split/Splitless Injectors

Split/splitless injectors are probably the most common types. These injectors can be operated in split or splitless modes. Splitless injection (where the split vent is closed) attempts to transfer the entire sample to the column and is used for trace analysis detecting compounds in the ppm range. The transfer of the sample to the column is slow so solvent focusing is important for obtaining narrow peaks. Because of the slow transfer time, much of the sample remains in the hot injector for a long period of time which may allow for sample degradation.

In split mode, only a small portion (maybe 1-10% of the sample) moves into the column, and the rest is sent to waste, in your case only 2% enters the column. This is used when the analytes are in high concentration and would overload the column. This is especially important in very small diameter columns (0.25 mm or less) because they have very small capacity. Split injection produces narrow peak widths and high reproducibility and is good for dirty samples. However, there is a problem with backflash and molecular weight discrimination. Higher molecular weight compounds may not completely vaporize and reach the split point as an aerosol. Aerosols are swept out the split vent without proper mixing and therefore, higher molecular weight components are underrepresented in the portion of the sample entering the column.

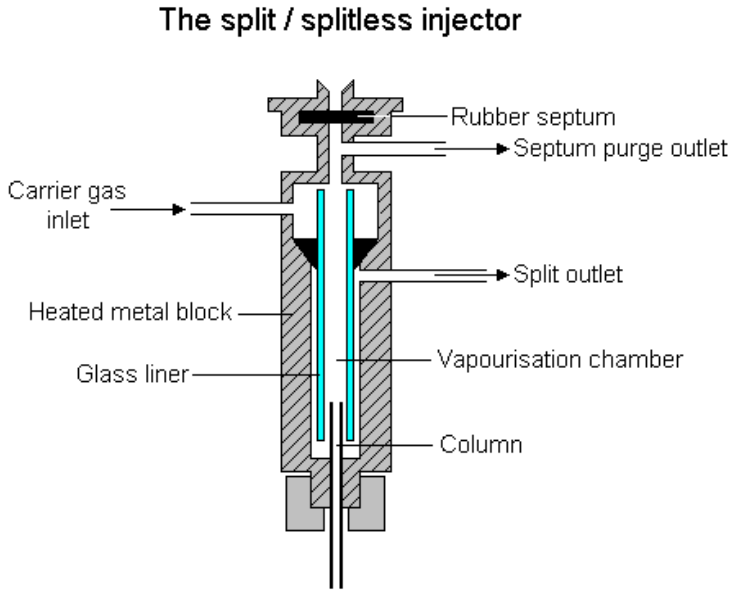

Figure II.5 - Figure describing a GC injector and were the main components are located.

When a liquid solvent is transferred into the hot injector, it immediately vaporizes and expands. The injector must be designed to contain this expansion. The injector usually contains a glass liner and must be large enough to allow gas to expand, if it's not, a problem called backflash can occur. What happens is that the sample expands so much that it expands to the septum purge area and part of the sample can be lost through the septum purge.

There are simple ways to minimize backflash, for example: Optimize sleeve, pack it with glass wool, or use a double gooseneck; inject less; decrease injector temperature and increase head pressure (higher flow rate into column).

Sample preparation

All sample were diluted to a concentration present in the calibration curve done for menthol, described before, in methods "PART 1", we used n-hexane or ethyl acetate depending on the experiment itself.

The chromatograph used was a TermoQuest Trace GC 2000 Series equipped with an autosampler Thermo Finnigan As2000. The detector was a flame ionization detector (FID), the compounds were separated in a Cyclodex B (chiral b-cyclodextrin; J&W Scientific) capillary column (0.25 mm I.D. x 30 m with 0.25- μm film).

This column is intended for separation of esters and alcohols, it contains only a single enantiomer of a chiral compound rather than being achiral. The two enantiomers of the same analyte compound differ in affinity to the single-enantiomer stationary phase and therefore they exit the column at different times. Chiral b-cyclodextrin columns make it possible to separate chiral compounds without derivatization – enantiomers and positional isomers are separated by slight differences associated with forming reversible inclusion complexes in the cavities of the functionalized cyclodextrin [110].

The compounds inserted in the column are eluated accordingly to molecular weight, when they have no alcohol or ester group, otherwise, compounds with these functional groups have more affinity to the column and take more time to pass and allow their chiral separation. The only exception exists with the product of vinyl decanoate and menthol reaction, menthyl decanoate cannot be separated by this column and the chiral compound appears mixed in only one peak.

We used two different methods for analysis, one for the reaction with anhydrides and the other for the reaction with vinyl esters.

When we used anhydrides, we used a program with a total duration of 60 minutes, beginning with an oven temperature of 90ºC during 10 minutes (isothermal), after which we had a ramp to 140ºC at a rate of 1ºC/min. The injector and the detector were set at 200ºC and 250ºC respectively. The other conditions are showed in the next page figure.

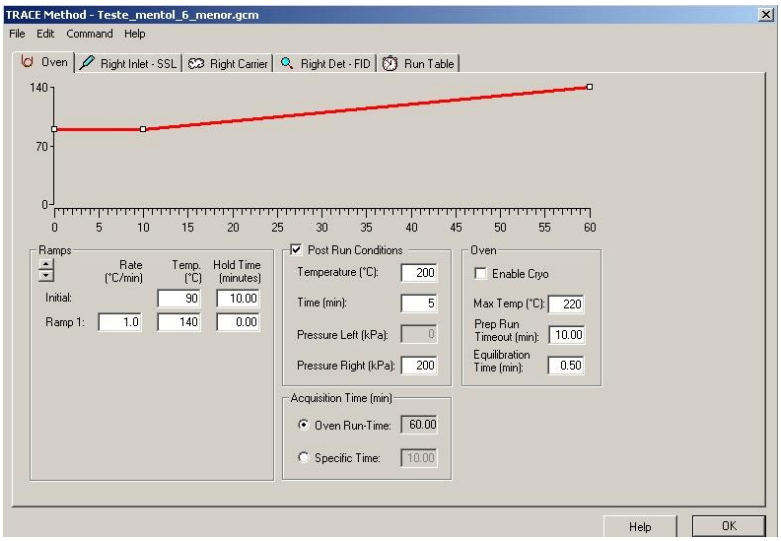

Figure II.6 - Trace GC method for reactions with acid anhydrides.

On the other hand, when vinyl esters were used, the program was as follow:

Total duration of 100 minutes, beginning with an isothermal of 10 minutes at 90ºC followed by a ramp to 180ºC at a rate of 1ºC/min. The injector and the detector were set at 200ºC and 250ºC respectively. The other conditions are showed in the figure II.7.

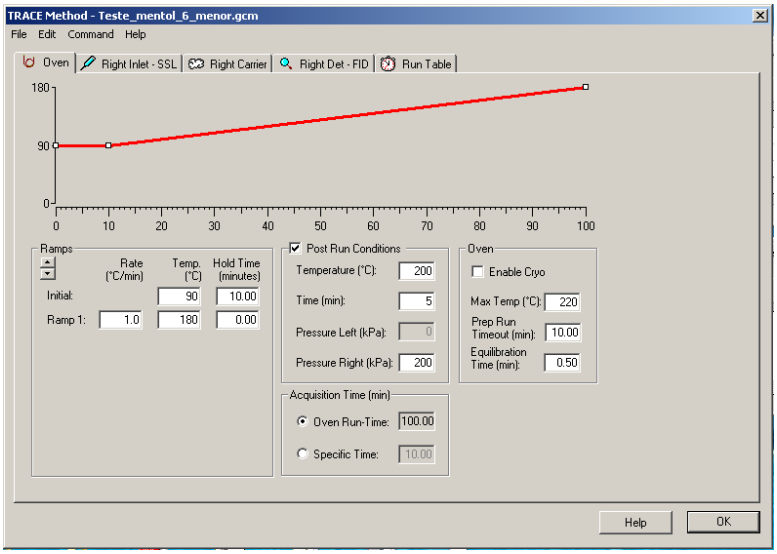

Figure II.7 - Trace GC method for reactions with vinyl esters.

The carrier-gas used in both programs was helium with a stable flow of 1 ml/min during the entire program. All peaks were identified recurring to Chrom-Card Data System program by Thermo Fisher Scientific and using tridecane as internal standard.

f. Sample analysis and peak identification:

Through the use of Chrom-Card Data System program, we were able to identify the peaks present in the samples. An image of a typical chromatogram for each reaction is provided below. Chromatogram for the reaction with anhydrides:

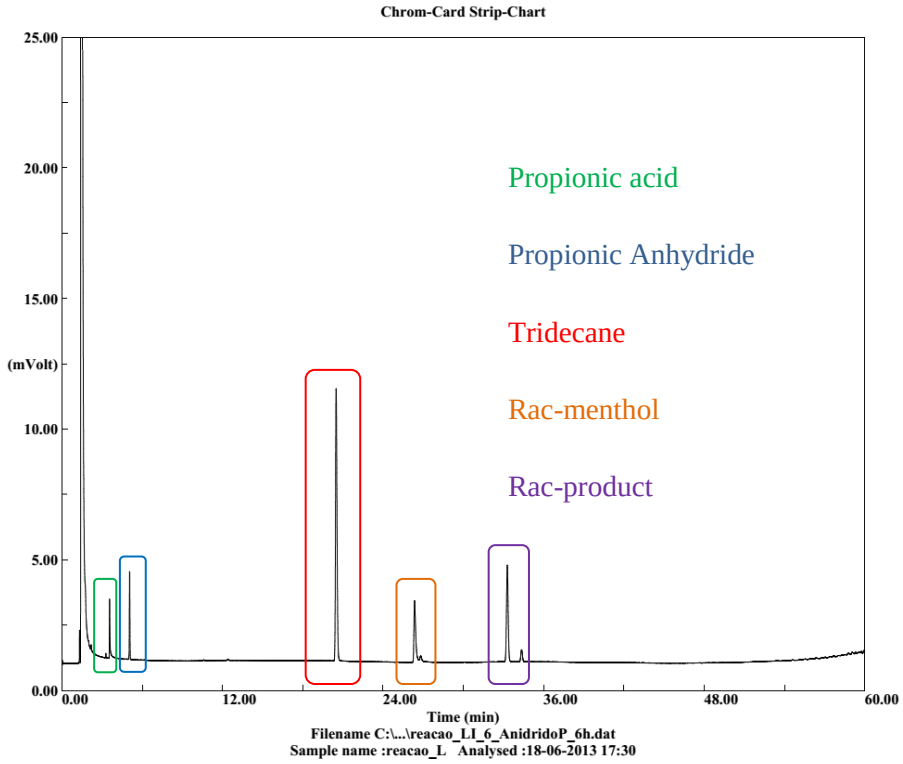

Figure II.8 - Chromatogram for the reaction with propionic anhydride, with identification of each peak.

After the solvent peak (unidentified on the figure above) we have propionic acid, product of the reaction between menthol and the propionic anhydride, which is the next peak on the figure. Then, we have the internal standard, the racemic menthol and the racemic product. The later peaks are separated by the program and their separated areas are used for conversion and enantiomers excess. Later, we also provide a figure with identification of each racemic peak. In the next table we have the retention time of each peak for the used conditions.

Table II.1 - Retention time of the different compounds when using the method for acid anhydrides.

Compound	Retention time (min)
Propionic acid	3.48
Propionic Anhydride	5.06
Tridecane	20.35
Rac-menthol (both peaks)	25.70-26.30
Rac-product (both peaks)	33.30-34.50

Next, we address the reaction with vinyl esters and present a Chromatogram for the same reaction:

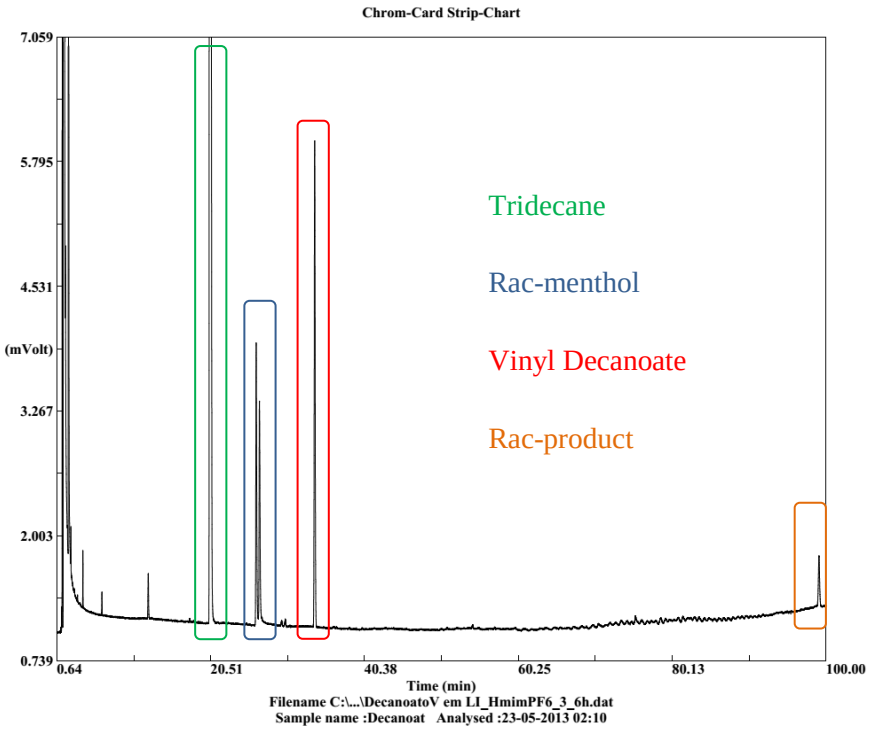

Figure II.9 - Chromatogram for the reaction with vinyl decanoate, with identification of each peak.

As before, we compressed the retention time for each peak in the next table using the conditions enunciated before.

Table II.2 - Retention time of the different compounds when using the method for acid anhydrides.

Compound	Retention time (min)
Tridecane	20.35
Rac-menthol (both peaks)	25.70-26.30
Vinyl decanoate	33.96
Rac-product	99.09

After the solvent peak (unidentified on the figure above) we have the internal standard, then the racemic menthol and the racemic product almost in the end of the program.

Now, we show the resolution and identification of the racemic peaks with a zoomed in view of a chromatogram from a reaction between propionic anhydride and menthol:

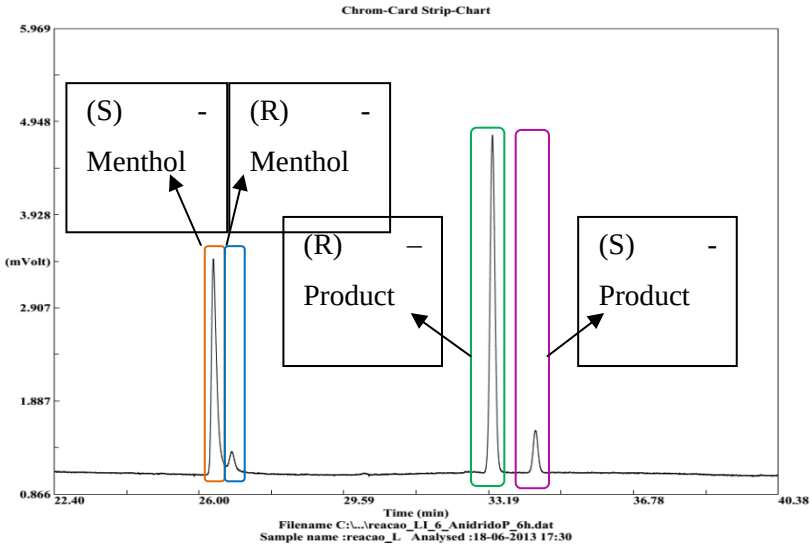

Figure II.10 - Chromatogram for the reaction with propionic anhydride, with identification of each peak.

Peak separation is good and concentration of each one is possible through the integration of all peaks, using calibration standards and recurring to the calibration curve, as described in methods.

g. Calculations

For each sample, conversion and enantiomeric excess (*ee*) must be calculated. The calculation can be by two methods, first through equation 1, which correlates the enantiomeric excess for substrate and product to obtain the conversion. This method can only be used with in reactions with acid anhydrides

$$Equation\ 1: Conversion = \frac{ees}{(eep + ees)}$$

Secondly, the conversion may be determined by recurring to a calibration curve (appendix) and calculating the concentration by adding an unreactive internal standard to the reaction medium with a known concentration and extrapolating the value for concentration and the value of conversion, equation 2:

$$Equation\ 2: Conversion = \frac{initial\ concentration\ (Ci) - concentration}{initial\ concentration\ (Ci)}\ X\ 100$$

The calculation of *ee* is made directly through the GC peak area using equation 3:

$$Equation\ 3: e.e. = \left|\frac{(R-S)}{(R+S)}\right| \text{ X } 100$$

The *ee* gives is a measure of how pure it is in a racemic mixture. The increase of the *ee* demonstrates an increase of one enantiomer versus the other.[111]

The measured values of *ee* and percent conversion were used to calculate the enantioselectivity, E, which indicates the degree to which the enzyme prefers one enantiomer over the other [106] and it can be calculated for substrate and product, as demonstrated by equation 4 and 5:

$$Equation\ 4:\ E(s) = \frac{\ln[(1 - Conversion)(1 - ees)]}{\ln[(1 - Conversion)(1 + ees)]}$$

$$Equation\ 5:\ E(p) = \frac{\ln[1 - Conversion(1 + eep)]}{\ln[1 - Conversion(1 - eep)]}$$

Chapter III

Results

In this section a detailed description of the major experimental findings is presented. The results are presented in two subsections describing the results of the experimental work for the modification/reaction of the menthol enantiomer. In the first part we describe the reaction of menthol with acid anhydrides and in the second we describe the reaction with vinyl esters.

III. Results

a. Menthol reaction with acid anhydrides.

The objective of this work is the development of a separation method which would allow the separation of racemic menthol using enantioselective enzymatic reactions. A successful separation of the menthol enantiomers were experimented by the selective modification of one of the enantiomers, making that same enantiomer different from the starting molecule. In this experiment, we use selective transesterification/ acylation of the (-) – menthol using an acid anhydride. This separation aimed to modify the menthol molecule, giving it different physical and chemical properties rendering the separation possible through simple processes. An example of this reaction is presented in figure I.11 (page 31).

This reaction can be followed by sampling and analysis by GC where values for conversion and enantioselectivity, and *ee* can be obtained. The *ee* of a substance is a measurement of how pure a substance is. In our case the desired product is the (-) product, being the unwanted the (+) product. The value of *ee* is obtained through equation 3 (page 52) both for the substrate (ee_S) and for the product (ee_P).

The conversion can be calculated by recurring to the *ee* of the product and of the substrate, using equation 1 or in cases where ee_P cannot be calculated, using calibration curve (used for the calculation of the conversion of racemic menthol through the method described in the appendix).

To evaluate be best parameters and conditions for this reaction to occur, a set of experiments were realized, were various parameters were tested, such as, temperature, acylating agent, solvent, etc.

i. Choosing the most appropriate anhydride as an acylating agent.

To choose the best anhydride to modify the (-) menthol, we experimented 3 different molecules that would turn the menthol molecule significantly different from the staring material: acetic anhydride, propionic anhydride and butyric anhydride. Our goal with this experiment was to find the molecule that would react more effectively with menthol resulting in the highest conversion and *ee*. For the effective separation of the reaction products is important that they have significantly different structure and high purity.

The reactions were performed at 310 K with 100 mg of CRL, 333 mM of each anhydride and of racemic menthol and in a water bath with heating to maintain the temperature stable.

We present the conversion figure for the reaction of menthol with these 3 molecules below:

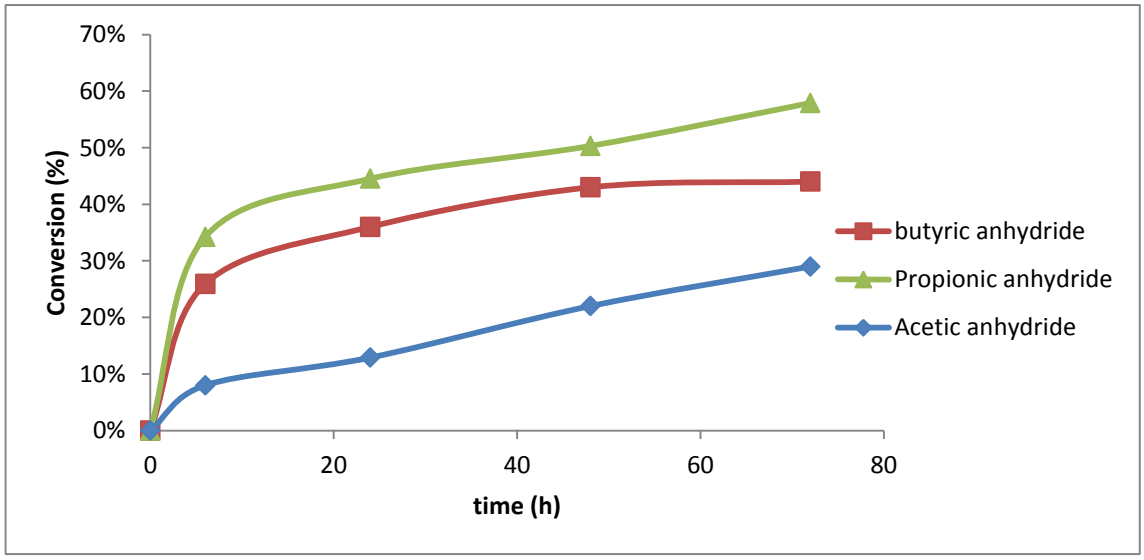

Figure III.1 - Conversion in the reaction of racemic menthol (333 mM) until 72h with three different acylating agents (333 mM) at 310 K, with 100 mg of enzyme in 5 ml of n-hexane.

By the evaluation of the figure above, we may see that propionic anhydride offers higher conversion values additionally to higher initial velocity to the conversion.

The conversion rate may be caused by the structure of the molecule itself and the way it interacts with the protein. But to prove that this is the best acylating agent we must observe the results for the enantiomeric excess of the substrate (ee_S) and the enantiomeric excess of the product (ee_P). As the results for the acetic anhydride produced lower values than the other two,

we will hereinafter compare the values for *ee* of butyric anhydride and propionic anhydride. The results for the *ee* of the reaction are presented in figures III.3 and III.4.

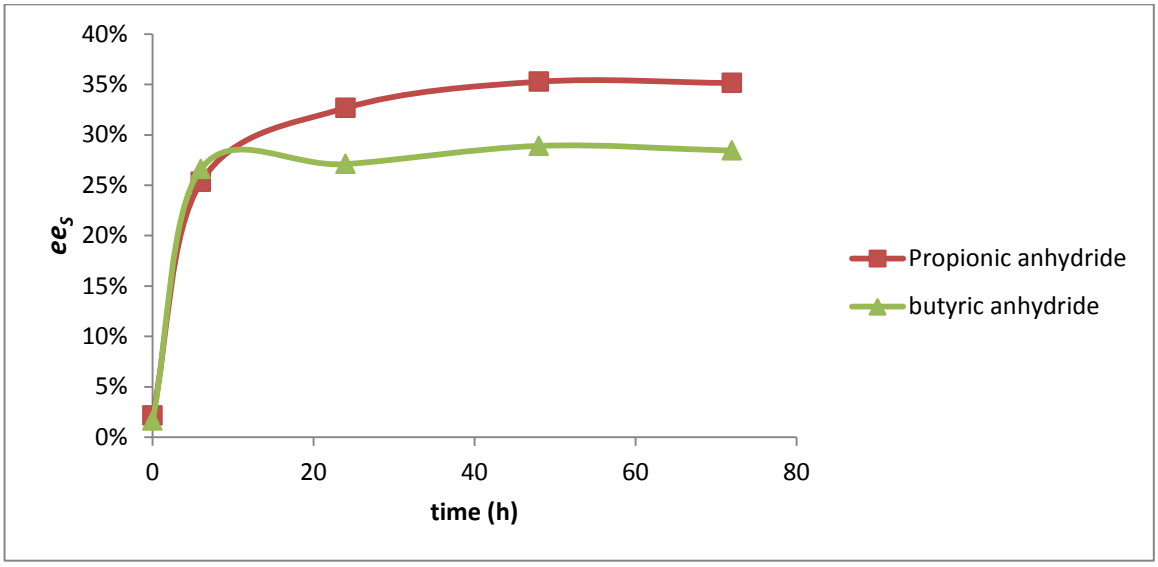

Figure III.2 - ee_S in the reaction of racemic menthol (333 mM) until 72h with three different acylating agents (333 mM) at 310K, with 100 mg of enzyme in 5 ml of n-Hexane.

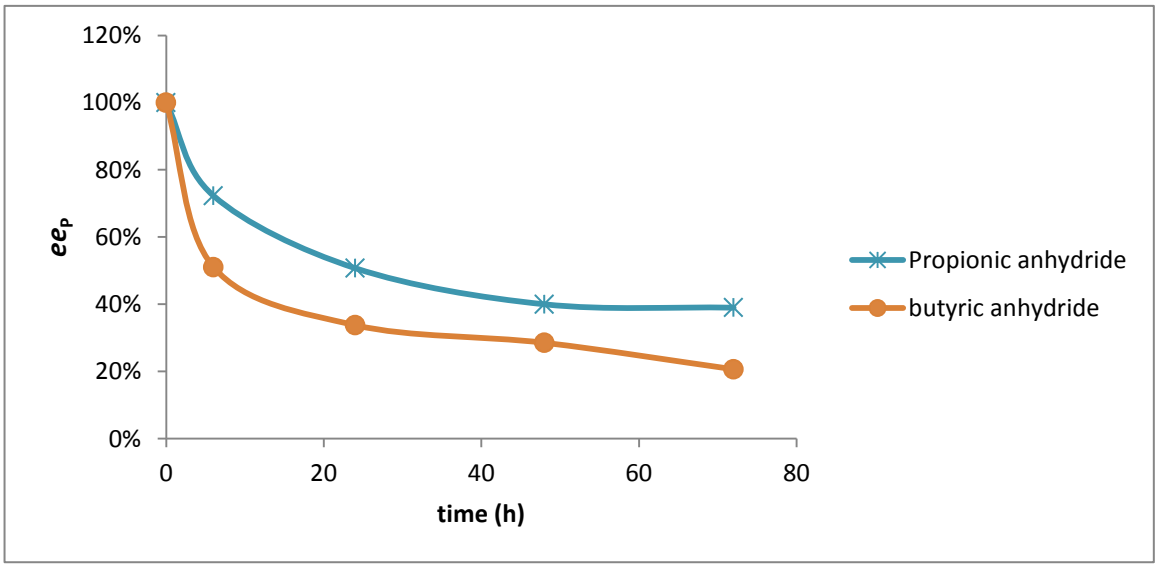

Figure III.3 - ee_P in the reaction of racemic menthol (333 mM) until 72h with three different acylating agents (333 mM) at 310K, with 100 mg of enzyme in 5 ml of n-Hexane.

From the figures above we observe that propionic anhydride has the best *ee* resulting in 33% of (+) menthol excess at 24 hours. The values for *ee* are low and require increase for a successful separation. This increase could be made, by adjusting the concentration of enzyme or

by adjusting other parameters, such as the temperature. An increase in concentration of the enzyme would speed the reaction, achieving a faster reaction without changing the enzyme selectivity. An adjustment in temperature would result in a variation of the conversion rate, as presented further. During all experiments presented in this thesis, control reactions are made separately, with the same concentration of the substrates, internal standard, volume, temperature and stirring speed, but without catalyst. By the observation of the control reactions, we observe an elevated uncatalyzed reaction between menthol and all anhydrides which are due to the elevated reactivity of the anhydrides, producing unselective products. This behavior will also be taken into account and attempted to minimize in the next experiments with the intention to produce a more selective process. The enantioselectivity of enzyme was calculated demonstrating that the enantiomeric ratio for the propionic anhydride is 2,5 times better than for butyric anhydride.

ii. Experiments with different amounts of enzyme (100, 200 and 400 mg of lipase from Candida rugosa) at constant temperature.

To study the effect of the enzyme, several reactions were performed using different amounts of enzyme, with substrate concentrations of 333 mM and 277 K.

Table III.1 shows the comparison of the reactions conversion and *ee*. with 100, 200 and 400 mg of enzyme.

Table III.1 - Comparison of the values obtained at 48 h for reactions preformed with different amounts of enzyme, at 277 K and with an initial concentration of 333 mM for racemic menthol and propionic anhydride in 5 ml of n-Hexane.

	Chemical Conversion (%)		**Total Conversion (%)**		ee_S **(%)**	ee_P **(%)**
Amount of enzyme (mg)	-	+	-	+		
100	2	2	67	8	44	91
200	2	2	87	4	79	92
400	2	2	100	2	≥99	90

Table III.2 - Comparison of the values obtained for enzymatic reaction and total enzymatic conversion for both enantiomers.

	Enzymatic Conversion (%)		***ee*$_S$ (%)**
Amount of enzyme (mg)	-	+	
100	65	6	83
200	85	2	95
400	98	0	≥99

The results presented in the tables above (III.1 and III.2) show that the amount of catalyst has a significant effect on the total conversion of the (R/S) - menthol and in the ee_S. This significant effect is more evident with 400 mg of enzyme, since we obtain a total conversion and an ee_S of 100 %. We can also observe that above 200 mg of enzyme, no benefit is achieved in $\boldsymbol{ee_S}$ within the error margin of the measurements by adding more enzyme.

It is also important to highlight that the chemical reaction (control - without enzyme), allowed us to understand the performance of the enzyme in the system. In the case of the ee_P we can observe that the values are very similar for all the experiments, as expected.

We choose the low temperature for this comparison, because the chemical reaction extent is reduced to a larger extent than the enzymatic reaction, which is very important because we want to inhibit the chemical reaction to a negligible value of conversion, as we noticed previously.

These results indicate that besides the reaction increase in conversion, as we increase the amount of enzyme, it also increases the value of *ee* of the substrate due to the increase of the conversion in a shorter time interval. The enhanced selectivity in hydrophobic solvents, as n-Hexane, may be due to the rigidity of the lipase caused by the reduced hydration of the enzyme surface, as it was mentioned before.

With the decrease of the temperature the reactions diminishes their rate due to the movement of the particles and the vibration of the molecule making the protein structure more rigid, which may lead to a more selective reaction. Furthermore, through the values for the ee_P, we observe that an increase in enzyme concentration only increases the reaction rate, maintaining the selectivity.

iii. Influence of the temperature in the reaction of *rac*-menthol with propionic anhydride with the same amount of catalyst.

To study the effect of the temperature in conversion and selectivity, several reactions were performed using different temperatures, but maintaining the previous conditions of substrate (333 mM) and enzyme concentrations (40 mg/ ml of n-Hexane) and stirring speed.

By GC and data analysis, we observed that the reaction reached a plateau at 48 hours In the tables III.3 and III.4 is showed the comparison of the reactions at 48h with 200 mg of enzyme in terms of chemistry, enzymatic and total conversion and ee_S *and* ee_P:

Table III.3 - Comparison of the values obtained at 48 h, for the reaction preformed at different temperatures with racemic menthol (333 mM) and propionic anhydride (333 mM) with 200 mg of enzyme in 5 ml of n-Hexane.

	Chemistry Conversion (%)		**Total Conversion (%)**		ee_S **(%)**	ee_P **(%)**
Temperature (K)	-	+	-	+		
277	2	2	87	4	79	92
298	12	12	81	17	62	73
310	18	18	63	26	32	34

Table III.4 - Comparison of the values obtained at 48 h for enzymatic and total conversion, with the same conditions as the previous table.

	Enzymatic Conversion (%)		ee_S **(%)**
Temperature (K)	-	+	
277	85	2	95
298	69	5	86
310	45	8	70

By assessment of the tables shown above, we can observe that the influence of the temperature is evidenced in these experiments. To better understand these results, we calculate the conversion for each enantiomer in both cases, with and without catalyst, to notice in which the role of temperature is higher of the chemical reaction (control). The chemical reaction is unselective, producing a racemic product and decreasing the overall selectivity of the reaction. In

the second table presented (table III.4) the contribution of the enzymatic reaction to the total reaction is obtained by subtracting the value of the chemical reaction from the total conversion.

As we can observed the lower temperature (277 K) seems to have the smallest contribution of the chemical reaction for the reaction, once it only converts 2% of each enantiomer. However at the highest temperature (310 K) we can observe the main contribution for the conversion of each enantiomer (18 %) in the chemistry reaction.

Once, our major concern is to obtain a preferential conversion by the enzyme, with a high conversion value for one enantiomer and the lowest possible to the other. This result should be followed by a high *ee* of the substrate and also the product. So the most promising result is at lower temperature, 277 K.

Temperature can affect enzymes in two ways: increasing the rate and denaturating the enzyme duo to excess of vibration and consequent lead to denaturation. The enzyme used in this assay is not immobilized, being referenced in the literature that it has an optimum temperature of 310 K [112].

The increase of temperature renders improved rates as will be discussed further on, but in this case, the inhibition of the chemical reaction by performing the reaction at lower temperatures improves the selectivity overall as we can see on the *ee* value.

A further increase of temperature leads to a decrease of conversion in all the experiments. An increase of temperature from 293 K to 313 K results in an increase of the solubility of water in n-Hexane from 0.0101 wt% to 0.0317 wt%[113]. As a consequence the stripping effect in the hydrophobic solvent increases with the increasing of the temperature, giving the enzyme a more rigid structure, thus increasing the selectivity. However, the solubility of water in *n*-hexane is extremely low, and therefore the later effect should not be very pronounced. The balance between this increase and the contribution of the chemical reaction, is negative thus, to improve the reaction selectivity, a lower temperature is more appropriate [38,114,115].

iv. Reaction between Propionic anhydride and menthol in different solvents

This set of experiments proposed to demonstrate the effect of solvent in conversion and *ee* for the reaction between racemic menthol and propionic anhydride using CRL at 310 K. This experiment was preformed recurring to two widespread organic solvents: n-Hexane and tetrahydrofuran (THF) and a mixture (1:1) of both of them. Experiments were carried out in order

to determine to influence of the solvent in conversion, *ee*., and enantioselectivity of the transesterification reaction. There are several affects that effect enzyme activity in terms of solvent, such as water affinity, polarity, etc. *N*-Hexane has a low capacity to solubilize water whereas THF is miscible with water[116]. To evaluate the content of water in the used solvents we calculated it recurring to Karl Fischer titration. The values of water content obtained are depicted below:

Table III.5. - Average content of water in different solvents

water content			
Average	n-Hexane (mg/g)	THF (mg/g)	n-Hexane:THF
	0,03	0,21	0,12

As we can see, *n*-Hexane is greatly hydrophobic. It should be noted that this solvent becomes saturated with water during manipulations, and the value given in the table must reflect that.

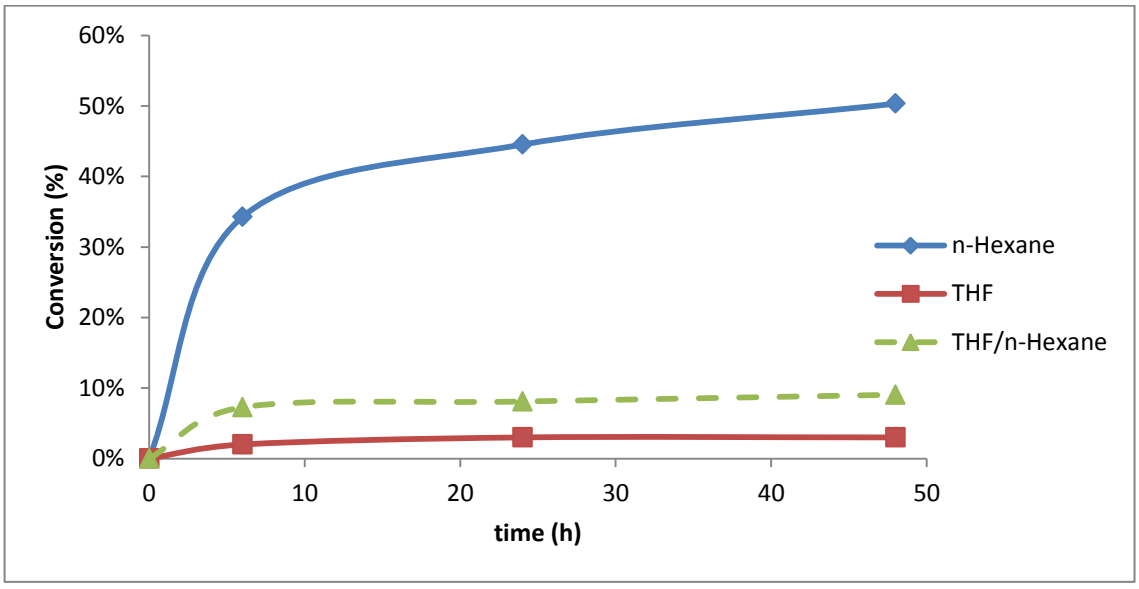

Figure III.4 - Comparison of the conversion values obtained for the reaction between racemic menthol (333 mM) and propionic anhydride (333 mM) in different solvents, at 310 K with 100 mg of enzyme in 10 ml of solvent.

In Fig III.5 it is possible to observe by this figure that there is a significant interference by the solvent in conversion. Reactions performed in THF and in n-Hexane/THF have a significant lower conversion. These results are in accordance with the literature[49], stating that

reaction performed in THF with CRL would yield a lower conversion rate. This difference might be because to the hydrophilic nature of THF, being proved that in controlled environments it will dehydrate the enzyme, by removal of the water layer surrounding the enzyme, lowering its mobility, flexibility and conversion rate[117] or by the increase in polarity which causes an increase of selectivity, but a severe decrease in conversion by the enzyme[117]. These results are shown for both experiments using THF as a solvent or cosolvent. The conversion value is not the only one to suffer a change; in the next figures (III.6 and III.7) we present the enantioselectivity excess for both substrate and product in these experiments:

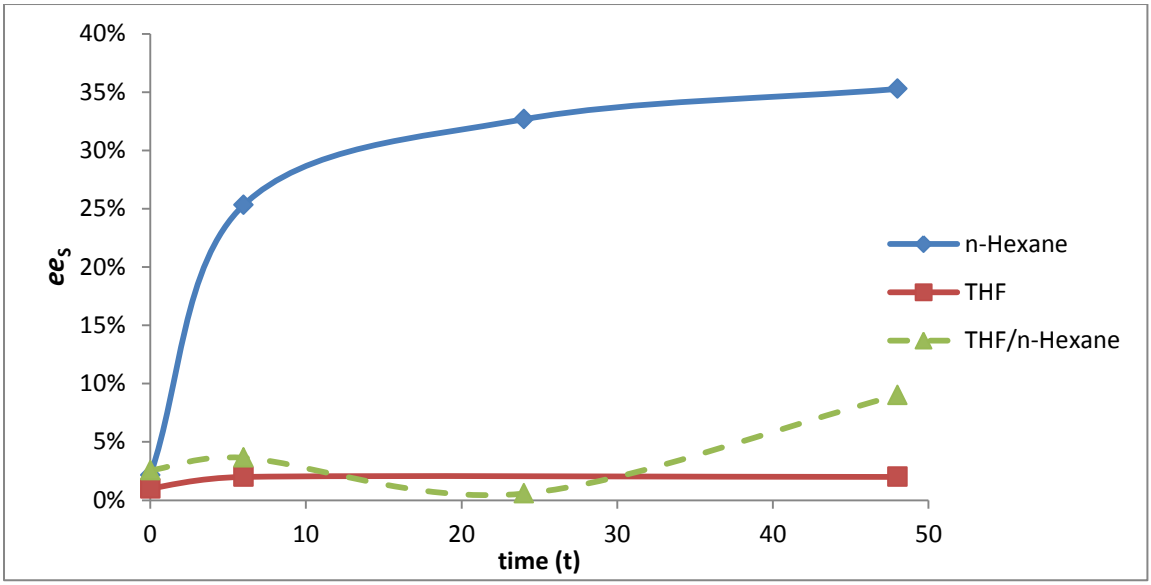

Figure III.5 - Comparison of the ee_S values obtained for the reaction between racemic menthol (333 mM) and propionic anhydride (333 mM) in different solvents, at 310 K with 100 mg of enzyme in 10 ml of solvent.

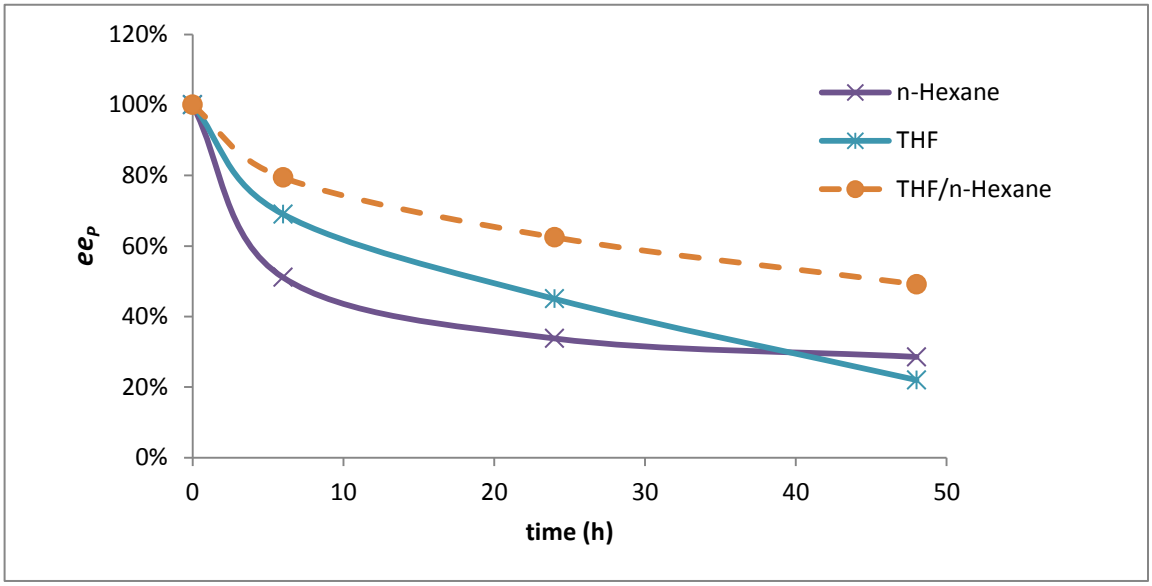

Figure III.6 - Comparison of the ee_P values obtained for the reaction between racemic menthol (333 mM) and propionic anhydride (333 mM) in different solvents, at 310 K with 100 mg of enzyme in 10 ml of solvent.

From the evaluation of figures presented, we can observe that the reactions obtained high selectivity and conversion only in the case of n-Hexane. As for the reaction with a mixture of n-Hexane with THF and pure THF, it rendered a minimum conversion accompanied with an increase in ee_S, not observed in these results due to the low conversion of racemic menthol, but depicted in the results for ee_P and as referenced in literature[117]. Proving THF is not a very effective solvent or co solvent for this reaction, rendering a low conversion rate. Besides, the control reaction showed no difference between them, proving the solvent properties affect only the enzyme catalysed transesterification by CRL.

v. Reaction of propionic anhydride and racemic menthol in supercritical carbon dioxide ($scCO_2$).

An experimental reaction between racemic menthol and propionic anhydride in $scCO_2$ was performed as a method to evaluate the influence of the solvent in the reaction. We know, by the literature, that $scCO_2$ influences the catalytic activity of CRL and with this experiment we aim in quantifying that influence. The conditions of this experiment were maintained with the exception of the amount of enzyme, we used 500 mg in a cell of 10 ml with a pressure of 150 bar.

The results for conversion of rac-menthol with propionic anhydride are presented in the figure below as well as the results for *ee*:

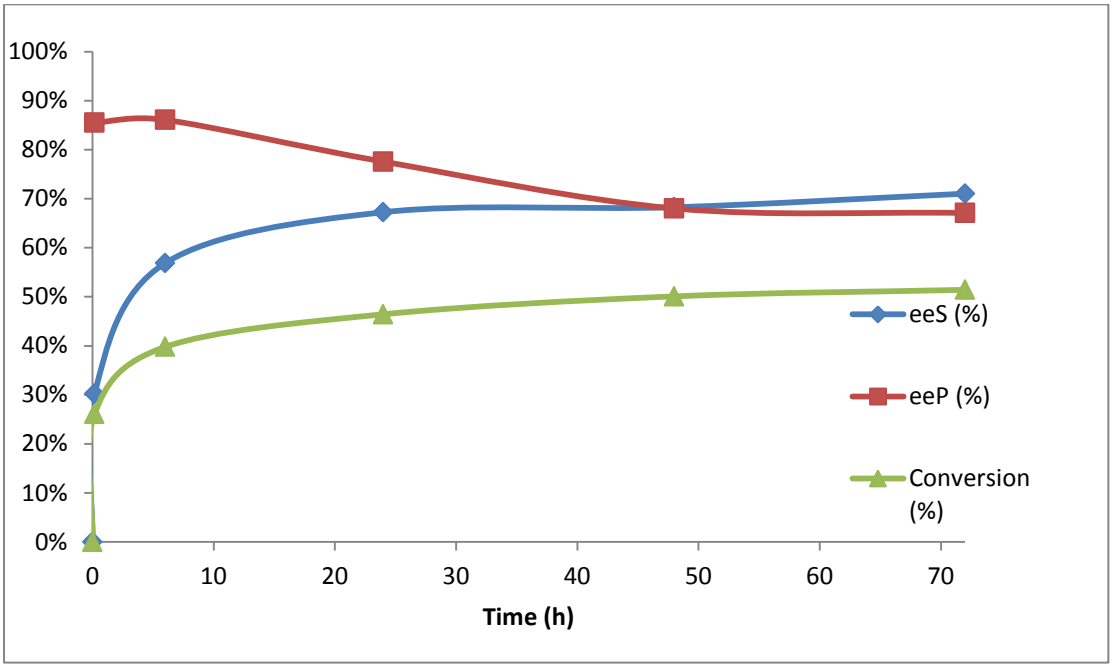

Figure III.7 - Results for the *ee* and total conversion in $scCO_2$ with 333 mM of racemic menthol, 333 mM of propionic anhydride at 310 K and with a volume of 10 ml of $scCO_2$ at 150 bar.

By the assessment of the figure shown above we observe a lower reaction rate due to loss of activity by CRL at 48 hours, these values are in accordance with the values demonstrated in literature which referred a loss activity by CRL[118]. The decreased relative activity of lipases in $scCO_2$ was attributed to the interactions between CO_2 and the enzyme and to the partitioning of water between the enzyme and its surroundings[118]. It was established in the literature that CO_2 may form covalent complexes with the free amino groups on the surface of the enzyme resulting in a charge removal at lysine residues resulting in inhibition of the enzyme.

The high diffusivity and low viscosity of the $scCO_2$ at 150 bar, should improve the catalytic activity providing higher values of *ee*, but the negative effects of scCO2 on the free lipase prevail. At 48 hours, the value of conversion reaches 50% with an ee_S of 68% and $\boldsymbol{ee_P}$ of 68%. When compared with reactions carried out in n-hexane the $\boldsymbol{ee_S}$ obtained under $scCO_2$ was lower, as presented in table III.6.

Table III.6 – Comparison of the results obtained for the reaction in different solvents at 48h with equal concentration of enzyme, 333,33 mM of menthol and 333,33 mM of propionic anhydride, at 310,15 K.

solvent	ee_S (%)	ee_P (%)	Total Conversion (%)	E s (enantioselectivity)
n-Hexane	79%	92%	46%	88
$scCO_2$	68%	68%	50%	11

From the table presented above, we observe that for equal concentration of enzyme, the reaction preformed in n-Hexane, presents a better *ee*, indicating an improved selectivity by the enzyme in n-Hexane. This argument is supported by the presentation of the enantioselectivity (E) values, which indicates the degree to which the enzyme prefers one enantiomer over the other. This value can be obtained for both the substrate and the product and it is given by equations 3 and 4 (page 52). The observed results for E demonstrate the improved selectivity of the enzyme in n-Hexane which gives a result 6-8 times higher.

vi. Reaction of *Rac*-menthol with Propionic anhydride in Ionic liquids:

The main goal of this work consisted in the use of new solvents which would eliminate the need to use VOC's as solvents in transesterification reactions allowing biotransformations. There has been increasing reports of works where the use enzymes in IL maintained very high stability and were able to maintain catalytic activity[49]. Solvent selection for enzymatic reactions can be severely restricted due to the inherent ability of solvents to deactivate enzymes, as is the case with hydrophilic solvents[48,119]. Due to those restrictions, the choice of IL was restricted to the use of hydrophobic ILs. Our choice was set upon the use of ILs with anions that would coordinate less molecules of water striping the protein from its essential water content[49]. For that reason we performed the reaction on [Bmim][PF_6], [Bmim][BF_4], [Bmim][Tf_2N], [Omim][PF_6] and [Hmim][PF_6], one of those selected IL is hydrophilic ([Bmim][BF_4]) and serve as a contrast. The reactions were performed in 2 ml of dried IL with 200 mg of CRL and at 310 K. The concentration of racemic menthol and propionic anhydride was the same as in other reactions (333 mM). All IL presented very high conversions, near 50% or above with different *ee*. The results for all reactions and respective controls are displayed in the next page:

Results obtained:

In the next table, we present the values for conversion of all ILs during this experiment:

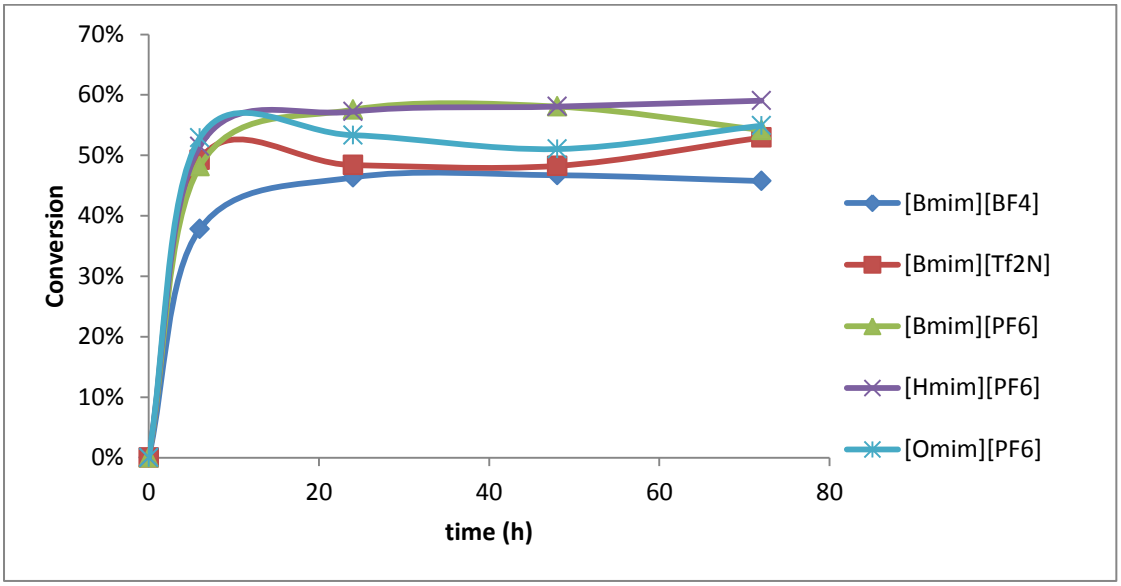

Figure III.8 - Comparison for the values of conversion obtained in the reactions for all the ILs at 310K, 333 mM of rac.menthol and propionic anhydride and in 2 ml of each solvent.

In the next table, we summarize the results at the 48 hours for the different ILs with enzymatic reaction:

Table III.7 - Comparison for the values obtained in the reactions for all ILs experienced by 48 hours at 310 K, 333 mM, of rac-menthol and propionic anhydride and with 200 mg of enzyme in 2 ml of each solvent.

Ionic Liquid (IL)	ee_S (%)	ee_P (%)	Total Conversion (%)	E s (enantioselectivity)
[Bmim][BF4]	55%	75%	47%	8
[Bmim][Tf2N]	67%	78%	48%	12
[Bmim][PF6]	79%	54%	58%	9
[Hmim][PF6]	74%	65%	58%	7
[Omim][PF6]	89%	74%	51%	42

The control experiment evidenced a low reaction with no ee_S or ee_P and preference for any of the enantiomers, at 48H. The average values for the control gave a conversion of 16% with 1% of ee_S and of ee_P and an enantioselectivity of 1. Further, when the enzyme is present and increase of viscosity can be viewed in the reaction vial, which might affect the non-enzymatically reaction slowing her. By following the reaction through GC analysis, we conclude that 48 hours is an appropriate time for comparison.

After comparison of the values obtained at 48 hours, for conversion considering the respective values for *ee* which elucidate to the selectivity of the reaction performed, we conclude that the most appropriated ILs for this reaction are [Omim][PF_6] and [Bmim][Tf_2N] which gave a conversion near 50% with the highest combination ee_S and ee_P of all the others. We can also assess that all IL presented similar values for conversion, including [Bmim][PF_6] presenting a conversion of 58% and an ee_S of 79% and an ee_P of 54%. And we can also observe that anion selection had much greater influence on CRL esterification activity than cation choice. This is due to the nucleophilic properties of the anion, ILs with more nucleophilic properties could easily interact with lipase and consequently there might be conformational change which might be the cause of the activity variances. In the case of the PF_6 anion, it has lower hydrogen-bond basicity minimizing the interference with the internal the internal hydrogen bonds of the enzyme. In the case of IL with BF_4^- anion, the effects are stronger in the protein, caused by its higher hydrogen bonding[120].

These high values for the conversion in combination the values obtained for preference of one enantiomer to the other gives certainty about the best choice for media for these reactions to be performed in future biphasic systems. This conclusion are in accordance with the finding done by others investigators, which stated that dried IL would performed with an increased yield on comparison to less polar organic solvents such as n-Hexane, acetonitrile, and tetrahydrofuran[49]. Other finding in accordance to the identified in literature is the increase in conversion and *ee* with the hydrophobicity of the ions present in the IL, this is evidenced for the three IL with the same PF_6 anion, as the alkyl chain increases in length, the IL becomes more hydrophobic, resulting in improved results[49].

vii. Comparison of the results obtained for the reaction of *Rac*-menthol with propionic anhydride

After the presentation of the results for the experiments using propionic anhydride as acylating agent, a comparison must be made to understand the most advantageous conditions for the reaction between racemic menthol and the propionic anhydride in terms of conversion, *ee* (substrate and product), temperature and amount of catalyst.

By the results obtained and discussed previously we observe that the best conditions for the reaction on organic solvent obtained at different temperatures and concentration of enzyme, using propionic anhydride as acylating agent. Subsequently, we will compare and evaluate the results obtained in different solvents to determine the most advantageous conditions:

Table III.8 - Comparison of the values obtained for conversion, ee_S and ee_P, at 48h for the reaction of racemic menthol (333 mM) with propionic anhydride (333 mM) altering the paramets: temperature, concentration of enzyme and solvent.

Solvent	Enzyme (mg)/ml	Temperature K	Total Conversion (%)	ee_S (%)	ee_P (%)
n-Hexane	40	310	50%	35%	40%
n-Hexane	40	277	46%	79%	92%
$scCO_2$	50	310	50%	68%	68%
[Omim][PF_6]	100	310	51%	89%	74%
[Hmim][PF_6]	100	310	58%	74%	65%
[Bmim][PF_6]	100	310	58%	79%	54%

By the observation of the table III.8, we observe that the best conditions for the reaction in organic solvent are lowest temperature obtaining higher values for conversion through enzymatic reaction as well as for selectivity. Nevertheless, this temperature is not appropriate for the use in supercritical condition due to the properties of $scCO_2$ (critical point). When we compare the reactions made in at the same temperature, we observe and increase in conversion rate in $scCO_2$ and a further increase when compared to the reaction preformed in [Omim][PF_6].

We conclude the most appropriate solvent for the reaction is the IL [Omim][PF_6], rendering a high conversion accompanied with the highest *ee*.

The results have an increase in enzyme concentration, in one case is to counter the effects of $scCO_2$ in enzyme activity, in the other is because of the high viscosity difference between organic solvents and the IL which would interfere with the diffusion slowing the rate of

diffusion[121]. Through these results we support our initial proposal for an alternative "greener" solvent for the reaction to take place. A biphasic system using ILs to support the reaction and $scCO_2$ to facilitate the separation and increase the enzyme activity is supported by the literature and will be addressed in the conclusions chapter[122].

b. Menthol reaction with vinyl esters.

A successful separation of the menthol enantiomers were experimented recurring to the selective modification of one of the enantiomers, making that same enantiomer different from the starting molecule. In this experiment, we use selective transesterification/ acylation of the (-) – menthol recurring to a vinyl ester. This separation aimed to modify the menthol molecule, giving it different physical and chemical properties rendering the separation possible thru simple processes. An example of this reaction is presented in figure I.12 (pag 31).

This reaction can be followed by sampling and analysis by GC where values for conversion and enantioselectivity, and *ee* can be obtained. A brief explanation of the concepts and formulas was given in the end of the second chapter.

To evaluate be best parameters and conditions for this reaction to occur, a set of experiments were realized, were various parameters were tested, such as, temperature, acylating agent solvent, etc.

i. Choosing the most appropriate vinyl ester as an acylating agent.

To choose the best vinyl ester to modify the (-)-menthol, we experimented 3 different molecules that would originate significant differences to the initial menthol molecule: vinyl laurate (C12), vinyl decanoate (C10) and vinyl stearate (C18). Note, we only account for the atoms that will be attached to the menthol molecule, so we don't count with the ones on the vinyl part of the molecule. As result of this reaction, we intend to produce a pure enantiomeric product plus a vinyl alcohol molecule which undergoes keto–enol tautomerization converting in acetaldehyde. This reaction is irreversible. Our goal with this experiment was to find the molecule that would react with menthol resulting in the highest conversion and *ee*. For the effective separation of the reaction products is important that they have significantly different structure and high purity.

The reactions were performed at 310 K and in a water bath with heating to maintain the temperature stable. The results for the conversion are showed below:

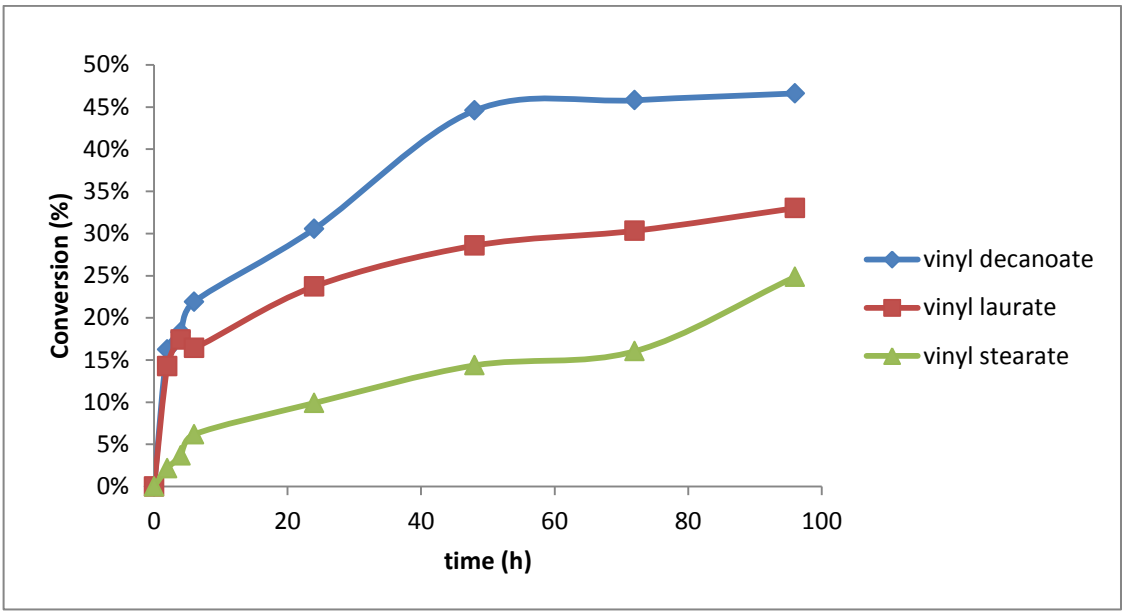

Figure III.9 - Conversion of racemic menthol until 96h with three different acylating agents (333 mM) and racemic menthol (333 mM) at 310 K with 100 mg of enzyme in a volume of 5 ml of n-Hexane until 96H.

Using these three acylating agents, we can see that the vinyl decanoate is the one that renders the highest conversion. The difference in conversion rate may be caused by the structure

of the molecule itself and the way it interacts with the protein active site. The active site of the enzyme is very specific to the substrate and it has a very precise shape that must be matched by the substrate for the reaction to occur. These results may also be due to the interactions of the decanoate molecule with the enzyme, its catalytic center and the loop guarding the catalytic center. We also observed, by GC analysis that the control reaction did not show any reaction at all, showing that the enzyme is essential for the reaction to occur. But to prove that this is the best acylating agent we must observe the results for the ee_S presented in the next page:

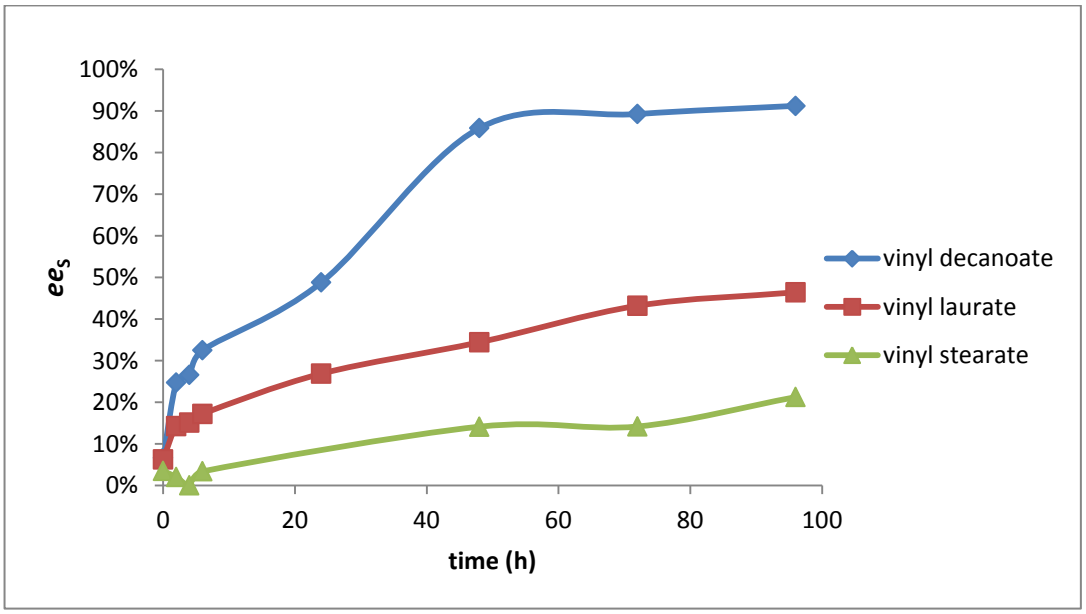

Figure III.10 - *ee* for (+) - menthol for the reaction of racemic menthol (333 mM) with each of the different acylating agents (333 mM) with 100 mg of enzyme in 5 ml of n-Hexane at 310K until 96H.

From figure III.12 we see that vinyl decanoate has the best *ee* meaning that almost all of the (-) - menthol was consumed in this reaction leaving only the (+) - menthol. Without the ee_P we cannot estimate exactly the range of (+) menthol that reacted, but we can see by the calculated conversion of that (+) – menthol did not react extensively. With these results we can also observe that vinyl decanoate gives the best results both for conversion and for *ee*. After 48h, no changes were observed in the reaction ratio reaching a plateau, with a conversion of *ca*. 45% of (-) menthol with an *ee* of 86%. The values of enantiomeric ratio for vinyl decanoate are also superior in comparison to the others, at 48 hours, vinyl decanoate had 85, vinyl laurate had 19 and vinyl stearate had 13.

ii. Reaction between racemic menthol and vinyl decanoate at different temperatures in n-Hexane.

In this essay, different temperatures were tested to evaluate their effect in selective conversion of rac-menthol by CRL.

The catalytic reaction was performed at 3 different temperatures: 310 K, 323 K and 277 K and the results for conversion are presented below:

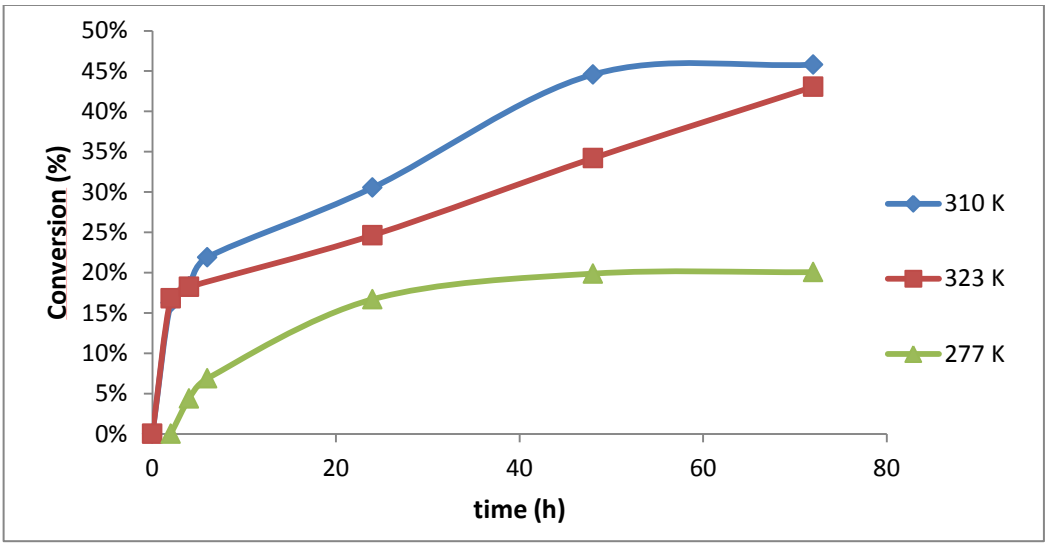

Figure III.11 – Conversion of rac-menthol (333 mM) with vinyl decanoate (333 mM) at different temperatures with 100 mg of enzyme in 5 ml of n-Hexane.

The results obtained for the catalytic activity by CRL are in accordance with the literature: being 310.15 K the optimum temperature. The increase of conversion can be explained by the temperature dependency of the reaction rate. The conversion is expected to reach the same value for all conditions. A further increase of temperature leads to a decrease of conversion. Besides possible denaturation effects this decrease could be also ascribed to a higher solubility of enzyme bound water in the organic solvent if higher temperatures are applied [123,124].

Another factor that we must be evaluated to assess the effect of temperature in catalysis is the *ee* obtained:

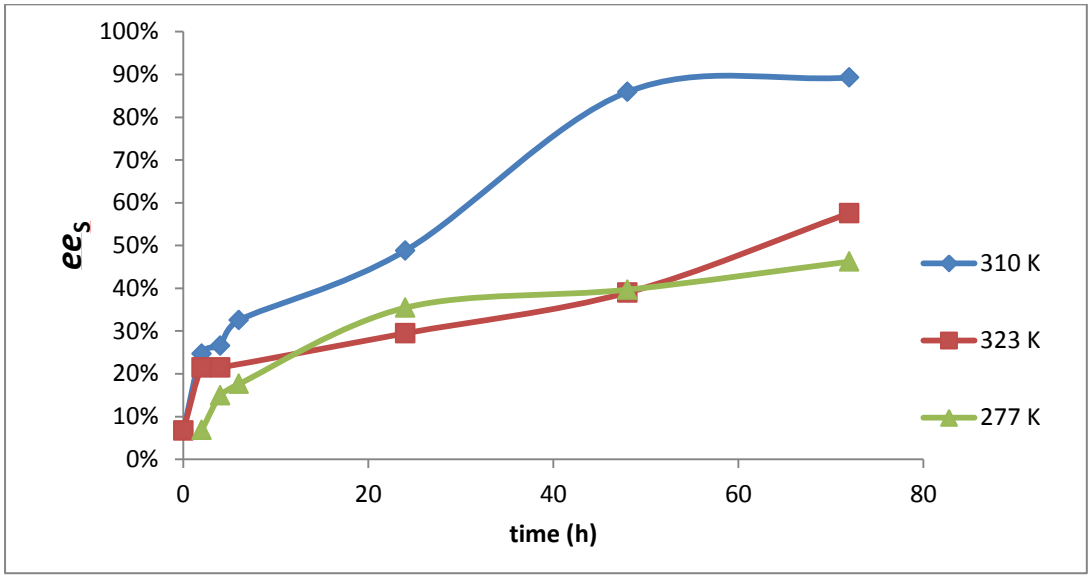

Figure III.12 - ee_S of rac-menthol (333 mM) with vinyl decanoate (333 mM) at different temperatures with 100 mg of enzyme in 5 ml of n-Hexane.

The influence of temperature on the *ee* varies severely depending on the reaction under investigation. Considering a hydrolysis reaction[125], it was found an increase in enantioselectivity with increasing temperature, whereas for esterifications and transesterifications a decrease in enantioselectivity with increasing temperature was often observed[114,115]. By the following table, we may observe the increased enantiomeric ratio for the temperature of 310 K, proving that 310 K is the most appropriate temperature for this reaction:

Table III.9 – Enantiomeric ratio of the enzyme at different temperatures.

E			
Time (h)	310 K	323 K	277 K
0	1	1	1
2	15	41	2
4	19	69	3
24	31	26	5
48	85	10	6
72	111	13	5

iii. Comparison of the catalyzed reactions between menthol and vinyl decanoate when we use different enzymes for the purpose.

There have been several reports of enzymatic transesterification recurring to several enzymes, such as, Novozyme 435 (immobilized *Candida antarctica* lipase B)[126,127], *Pseudomonas cepacia* lipase[128], Lipozyme TL IM (immobilized *Thermomyces lanuginosus lipase*)[129,130] and Novozyme RM (*Rhizomucor miehei* lipase)[131] besides *Candida rugosa* lipase. To evaluate the catalytic ability of these enzymes to undergo the reaction intended in this work, we experimented them in equally conditions. The reactions were performed in n-Hexane, with 100 mg of enzyme, 333 mM of menthol and of vinyl decanoate and used tridecane as internal standard. Results obtain for conversion are presented below in figure III.16:

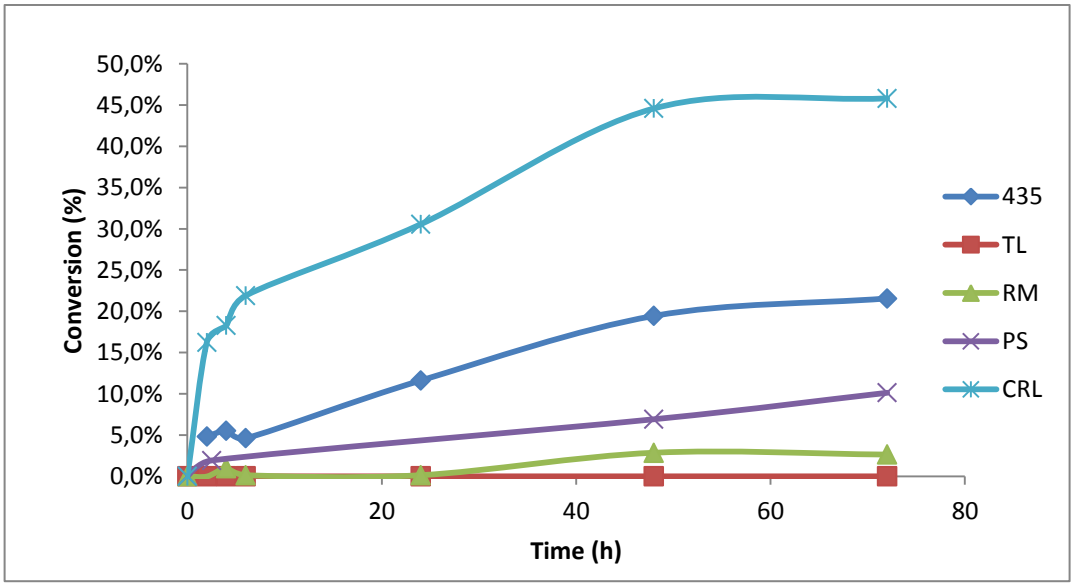

Figure III.13 - Conversion of rac-menthol (333 mM) with vinyl decanoate (333 mM) with different enzymes (100 mg of enzyme in 5 ml of n-Hexane) at 48 h and 310 K.

As it can be observed, CRL has reached a higher conversion than all the other enzymes in this study obtaining, at 48 h a conversion of 45%. The other enzymes were unable to convert efficiently menthol into the desired product or producing low levels of transesterification. Two of the enzymes tested in this study provided a very low or none conversion rate of rac-menthol (Novozyme TL and RM), while the other two enzymes (Novozyme 435 and Pseudomonas cepacia lipase) provided conversions between 7-20% at 48. Another factor involved is the

amount of immobilized enzyme in the support; this parameter fits only the case of the Novozyme enzymes. Nonetheless in figure III.7, we can observe their inability for selectively convert the racemic menthol:

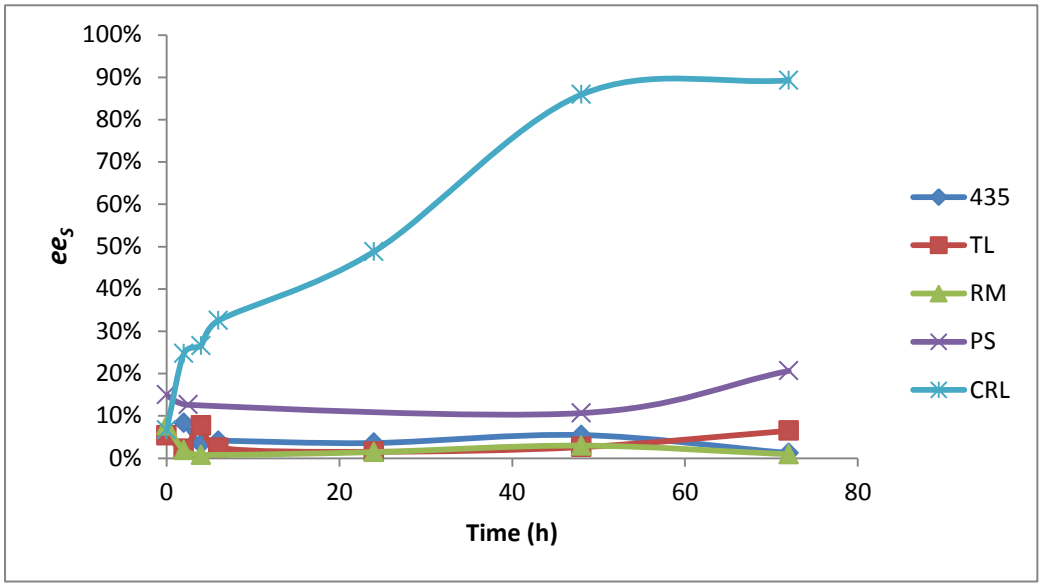

Figure III.14 - Conversion of rac-menthol (333 mM) with vinyl decanoate (333 mM) with different enzymes (100 mg of enzyme in 5 ml of n-Hexane) at 48 h and 310 K.

By the observation of the *ee* data, we can conclude that the only enzyme demonstrating selectivity is the CRL. Demonstrating that the CRL is the only viable enzyme to be used as a catalyst in this specific reaction. At 48 hours the results for enantioselectivity for these reactions with other enzymes are low, being clear that the CRL alone presents enantioselectivity for one enantiomer. At 48 hours, the values for enantioselectivity were: 85 (CRL), 17 (RM), 4 (PS), 1 (TL), 2 (435).

iv. Reaction of vinyl decanoate and *Rac*-menthol in supercritical carbon dioxide (scCO2).

To evaluate the behavior of the reaction between racemic menthol and vinyl decanoate in $scCO_2$, as already shown before for the reaction using propionic anhydride, we tried to evaluate the influence of the solvent in the reaction. This time the conditions used were maintained with the exception of the amount of enzyme, we used 1g (10ml cell) for the reaction and 150 bar.

The results for conversion of rac-menthol with vinyl decanoate are presented in the figure below as well as the results for *ee*:

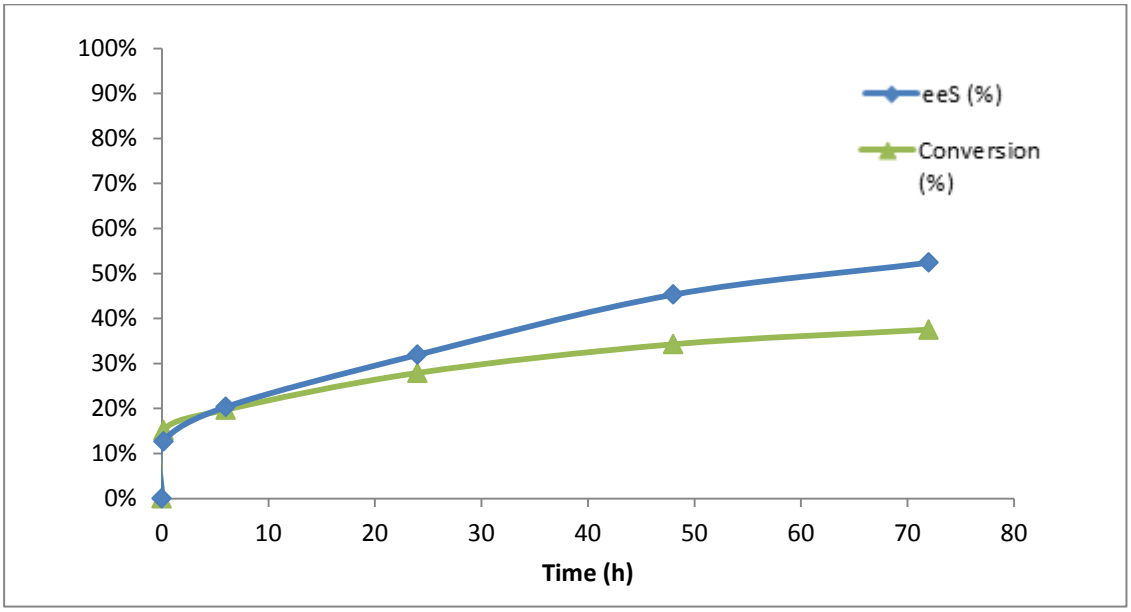

Figure III.15 - Figure displaying the values for conversion and *ee* for the reaction of rac-menthol (333 mM) and vinyl decanoate (333 mM) using 1 g of enzyme in 10 ml of $scCO_2$ at 150 bar.

Through the observation of the figure III.18 shown above, we observe a lower reaction rate due to loss of activity by CRL, this data are in accordance with the literature[118], which referred a loss of activity by CRL in $scCO_2$, as referred earlier[118]. The high diffusivity and low viscosity of the $scCO_2$ in supercritical conditions, should improve the catalytic activity providing higher values of *ee*, but the negative effects of $scCO_2$ on the free lipase prevail over those. At 48 hours, the value of conversion reaches 34% with an ee_S of 45%. When compared with reactions carried out in *n*-hexane the ee_S obtained under $scCO_2$ was lower, has presented in table III.10.

Table III.10 – Comparison of the results obtained for the reaction in different solvents at 48h with equal concentration of enzyme, 333 mM of menthol and 333 mM of propionic anhydride, at 310 K.

solvent	Amount of enzyme (mg/ml)	ee_S (%)	Total Conversion (%)	E s (enantioselectivity)
n-Hexane	20	86	45	85
$scCO_2$	100	45	34	23

At 72 hours, the value of conversion reaches 38% with an ee_S of 52% (not presented), this value of the ee_S presented is agreeing with the values presented for the reaction in n-Hexane at 310K in earlier reaction times. Also, this comparison is made between a reaction with 100 mg of enzyme in 5 ml of n-Hexane to this reaction in 10 ml of $scCO_2$ (150 bar) with 1 g of enzyme (the other parameters are equal). Indicating a decreased reaction rate in supercritical solvents and the need to increase the concentration of enzyme to reach the same conversion/ selectivity even at later hours. This argument is supported by the values enantioselectivity (E), which indicate the preference by the enzyme of one enantiomer over the other. This value at 48 hours is increasingly higher (3.7 times more) in the organic solvent than in $scCO_2$.

v. Reaction of *Rac*-menthol with Vinyl decanoate in Ionic liquids:

As discussed before, during the presentation and discussion of the results obtained for the reaction of propionic anhydride in IL, we intend to find a suitable media for the transesterification reaction with vinyl decanoate to occur, allowing multiphase biotransformations and without the negative impact on the environment done by organic solvents. For this experiment, we used the same ILs as in the similar experiment done with propionic anhydride. They are the best choice of media for these reactions, as explained before. Experiments performed using CRL and other enzymes in [Bmim][PF_6] reported that CRL exhibit the highest activity, being able to catalyze the reaction 1.5 times faster than in n-Hexane[49]. All the ionic liquids are hydrophobic with the exception of [Bmim][BF_4]. The reactions were performed in 2 ml of dried IL with 200 mg of CRL and at 310 K. The concentration of racemic menthol and vinyl decanoate was the same as in other reactions (333 mM). All IL presented very high conversions, near 50% or above with different ee_S. The results for conversion in all ILs during this experiment are presented in the next page.

Results obtained:

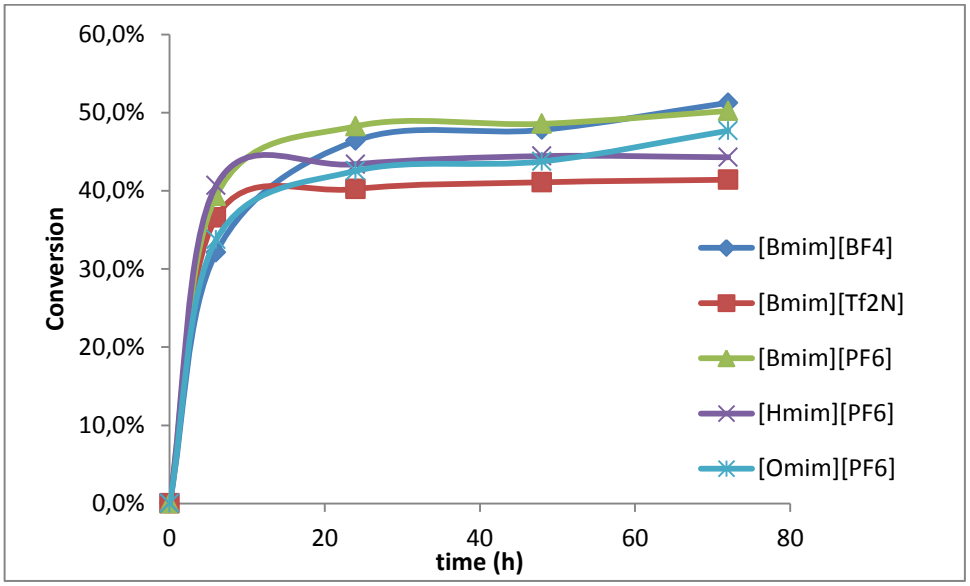

Figure III.16 - Comparison for the values of conversion obtained in the reactions for all the ILs at 48 h, 310K, 333 mM of rac.menthol and vinyl decanoate in 2 ml of solvent.

In the next table, a comparison of the different values for conversion, ee_S and E at 48 hours, are presented:

Table III.11 - Comparison for the values obtained in the reactions for all the ILs at 48h, 310K, 333 mM of rac.menthol and vinyl decanoate in 2 ml of solvent.

Ionic Liquid (IL)	Total conversion (%)	e.e subst (%)	E (enantioselectivity)
[Bmim][BF4]	48%	84%	90
[Bmim][Tf2N]	41%	83%	30
[Bmim][PF6]	54%	84%	16
[Hmim][PF6]	44%	91%	45
[Omim][PF6]	44%	83%	96

The control experiment evidenced almost no reaction, no *ee* and preference for any of the enantiomers, at 48H, giving a conversion of 4.5% with 1% of ee_S and an enantioselectivity of 1 (average values). On the other hand, for the experiments conducted with enzyme, we observe a high increase in conversion and *ee* since the beginning of the reaction, reaching values near 90% in the case of ee_S. During the experiment, we observed that by 48 hours most of the reaction has

reached equilibrium in al ILs, but due to problems in sampling and heterogeneity in the reaction media caused by the viscosity of the ILs tested, conversion values and enatioselectivity, show substantial uncertainty. The most reliable data remains the ee_S, the only value of enantioneric excess obtained directly by GC analysis, since the GC chiral column used to analyze the results could not separate the enantiomeric product, as referred before.

By the observation of table III.11, we can, also assess that all IL presented similar values for conversion, including [Bmim][PF_6] presenting a conversion over 50% and a ee_S of 84%. These results are consistent with the referenced work[49] stating [Bmim][PF_6] as an excellent media for reactions since it does not strip water from the enzymes, maintaining them catalytically active due to its weak water coordination by the PF_6 anion[120]. In this study, we observe another effect, due to the nature of the decanoate molecule, the conversion and *ee* values do not rise with the length of the alkyl chain. This findings suggest that the molecule of vinyl decanoate and the alkyl chain might interact between them by van der Waals interactions, being the IL responsible of solvating the reactant. The high values for the conversion in combination with the values obtained for the preference of one enantiomer to the other gives some confidence about the best choice for an IL media for these reactions to be performed in future biphasic systems.

vi. Comparison of the results obtained for the reaction of *Rac*-menthol with vinyl decanoate.

After the presentation of all the results for vinyl decanoate a comparison must be made to comprehend which are the most advantageous conditions for the reaction between racemic menthol and the vinyl decanoate in terms of conversion, *ee* and conversion rate.

By the results obtained and discussed previously we observe that the best conditions for the reaction on organic solvent is obtained at 310K, with 100 mg of enzyme, using vinyl decanoate as an acylating agent. Subsequently, we will compare and evaluate the results obtained in different solvents.

Table III.12 - Comparison of the values obtained for conversion and ee_S at 310K and 48h.

Solvent	Enzyme (mg)/ml	Total conversion (%)	ee_S (%)
n-Hexane	20	45.0%	86.0%
scCO2	100	34.3%	45.3%
[Omim][PF6]	100	44,4%	82,9%
[Hmim][PF6]	100	44,4%	90,7%
[Bmim][PF6]	100	54,4%	83,7%

Through the observation of the previous table (III.12), we observe that the most appropriate solvent for the reaction is the IL [Hmim][PF_6], rendering a high conversion accompanied with the highest *ee*. The results have an increase in enzyme concentration, in one case is to counter the effects of $scCO_2$ in enzyme activity, in the other is because of the high viscosity difference between organic solvents and the IL which would interfere with the diffusion slowing the rate of diffusion[121]. Thru these results we support our initial proposal for an alternative "greener" solvent for the reaction to take place. A biphasic system using ILs to support the reaction and $scCO_2$ to facilitate the separation and enzyme activity is supported by the literature and will be addressed in the conclusion chapter[122].

Chapter IV

Conclusion

IV. Conclusion

The objective of this work was the study of the transesterification reaction of *rac*-menthol using two different groups of acylating agents (acid anhydrides and vinyl esters), with particular emphasis on the influence of each one in the conversion of racemic menthol, ee_S and ee_P, also looking at the influenceof the solvent medium, concentration of the catalyst and temperature. Therefore, the main goal was to optimize a separation process of (-) menthol from the racemic mixture recurring to a selective enzyme and an appropriate acylating agent. This goal was partially achieved, being necessary further experiments to achieve a completely functional biphasic system that would separate the racemic compound in a continuous reactor without the use of organic solvents. Nevertheless, the most appropriate conditions and substrates for the reaction were found. The optimization of the biphasic system will be explored in the near future.

Amongst all the acylating agents tested propionic anhydride and vinyl decanoate are the most promising agents. High enantioselectivy and conversions were achieved using ionic solvents as media for this reaction. The best conditions for the reaction with propionic anhydride were obtained in [Omim][PF_6] at 310 K with 40 mg of enzyme/ ml with 333 mM of racemic menthol and propionic anhydride attaining a conversion of 51%, with 89% of ee_S and 74% of ee_P. in the case of vinyl decanoate were obtained in [Hmim][PF_6] at 310 K with 40 mg of enzyme/ ml with 333 mM of racemic menthol and propionic anhydride attaining a conversion of 44% with 91% of ee_S.

We have also compared the behavior of different biocatalysts of which *Candida rugosa* lipase (CRL) was the suitable candidate for this work, as it was the enzyme that exhibited the highest enantioselectivity in the reactions. The optimum temperature was also evaluated for each reaction. In the case of propionic anhydride the optimum temperature was achieved at 4 °C. Nevertheless when using a scCO_2-IL biphasic system a temperature higher than 31 °C must be used (CO_2 critical temperature).

The solvent selection is important due to the effects that each solvent characteristics could have in each particular system. The best solvent for the reaction to occur was found to be in each case an IL. In the particular case of propionic anhydride the best medium was [Omim][PF_6] and for the reaction using vinyl decanoate, the most appropriate medium was [Hmim][PF_6].

Another important aspect developed in this work was a strategy to minimize the contribution of the spontaneous reaction occurring with acid anhydrides and without the presence of a catalyst (chemical reaction). To overcome this problem, we tested the contribution of the spontaneous reaction increasing the amount of enzyme in the reaction media in addition to

different temperatures with the objective of achieving an improved selective reaction. We have achieved the best conditions at 277 K, with 40 mg of enzyme/ ml with 333 mM of racemic menthol and propionic anhydride obtaining 46% of conversion, ee_S 79% and ee_P 92%.

As future work, a biphasic system seems to be the most suitable process to reach a successful and advantageous process for the resolution of sec-alcohols, circumventing present limitations regarding the physical separation of the two enantiomers of racemic menthol. The biphasic system should be studied, evaluating the partitioning of all the intervening compounds, in the best conditions tested: for propionic anhydride at 310 K with 100 mg enzyme/ml in [Omim][PF_6]; in the case of vinyl decanoate at 310 K with 100 mg enzyme/ml in [Hmim][PF_6].

Chapter V

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V. Bibliography

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Chapter VI

Appendix

VI. Appendix

Calibration curve for (+) – menthol and (-) – menthol

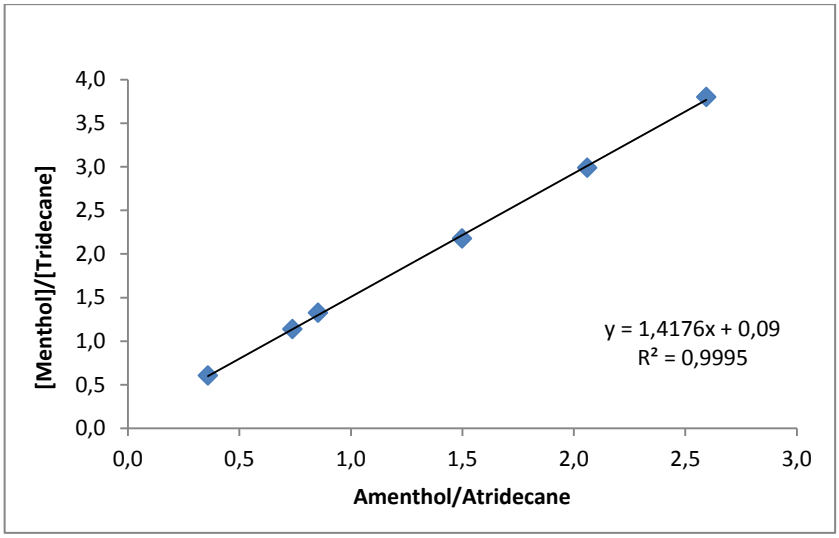

Figure VI.1 - Graphic for the calibration curve of (+) – menthol.

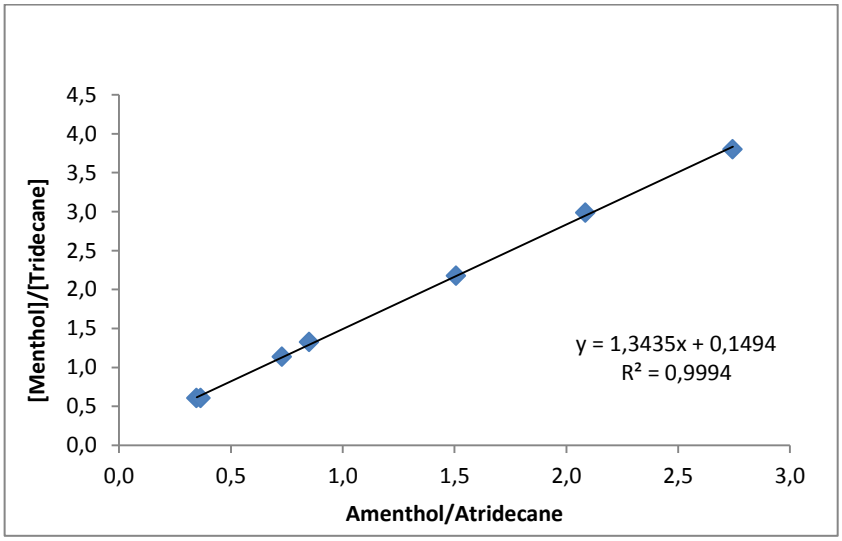

Figure VI.2 - Graphic for the calibration curve of (-) – menthol.

In the figures VI.1 and VI.2 is represented the calibration curve obtain for the (-) and (+) – menthol. The equation represented in both graphics was obtained requiring the calibration curve to pass on the origin.

As we can observe exist a linear correlation between the area and the concentration of each menthol enantiomers in all concentration range.

The concentration for each enantiomer was calculated through each calibration curve, replacing the values of the ratio between the area of each enantiomer and the internal standard in the x axis. The same procedure was used for the anhydride and acid, using the respective calibration curves.