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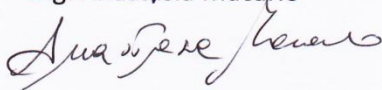
***Preparation and characterization of hybrid  
nanospheres containing Lipase for  
chiral drug biotransformation***

*Settore Scientifico e Disciplinare: ING-IND/27- Chimica Industriale e Tecnologica*

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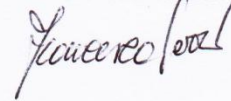
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## ABSTRACT

Lipase are able to catalyze esterification reactions in organic solvents displaying higher enantioselectivity than that showed in hydrolytic reactions. Among the high number of lipases described in the literature, only the enzymes belonging to a few species have been demonstrated to have adequate stability and biosynthetic capabilities to allow routine use in organic reactions and, hence, their applications as industrially relevant enzyme. Because of these properties, Lipases have been widely used for the production of enantiomerically pure compounds, resolving racemic alcohols and organic acids. There is an increasing trend toward the use of optically pure enantiomers for drugs because they are more specific targets and have fewer side-effects than racemic mixtures. Among 1800 drugs currently available, about half are chiral mixture. Racemic drugs have usually the desired therapeutic activity mainly in only one of the enantiomers. In this sense, 2-aryl propionic acids (profens), that make up an important group of non-steroidal anti-inflammatory drugs (NSAIDs) used in the treatment of arthritis and related diseases shows mainly the pharmacological activity by their corresponding (S)-enantiomer, 100-fold more active than its antipode. Therefore, since pharmacological studies have indicated that gastrointestinal problems are the most frequent side effects associated with profens consumption, important efforts to synthesize pure enantiomers of 2-aryl-propionic acids are currently in progress. Ibuprofen, 2-(4-isobutylphenyl) propionic acid, is a racemic carboxylic acid, showing each enantiomer a physiologically different behavior, being the (S)-enantiomer the form that exhibits an anti-inflammatory property. In recent years, lipase have been used for the chiral resolution of (R,S)-ibuprofen through mainly direct enantioselective esterification in organic media of its chemically synthesized racemic ester. Among different lipases,

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immobilized *Rhizomucor miehei* lipases is able to catalyze the esterification reaction.

Particularly, in this work, the Lipase from *Rhizomucor Miehei* has been immobilized into hybrid nanospheres containing a liposomal core, where enzyme is confined. The organic phase (Liposome + Lipase) has been protected by inorganic silica matrix obtained with and without surfactant. The influence of several experimental factors in the synthesis of immobilized lipase from *Rhizomucor miehei* in liposomal phase, such as silica/liposome weight ratio and mixing time between liposome and lipase, have been studied. All samples have been characterized, by base and advanced analytic techniques, in order to check the best procedure to obtain perfect hybrid nanosphere where lipase is perfectly in the core of the liposome phase. The optimized procedure has been used to prepare hybrid nanosphere with different silica shell. In particular, the silica cover has been prepared with or without surfactant. When surfactant has been used, hexadecylamine was the organic.

The optimized heterogeneous catalysts have been used in the enantioselective esterification of racemic ibuprofen to evaluate their catalytic performance. Particularly, the influence of several catalytic parameters on the activity of hybrid nanospheres, such as type of the solvent (a-polar solvent:isooctane; polar solvent: dimethylformamide), type of the alcohol (primary alcohol with different chains length: methanol; 1-propanol; 1-butanol) and reaction temperature (27, 37, 50 and 80 °C), has been investigated. The best catalytic performance of heterogeneous biocatalyst have been showed at 37°C, using iso-octane as solvent and 1-propanol as alcohol (ester yield value ranging between 78 and 93%). A strong iper-activation of immobilized enzyme, with respect to the free form, has been observed: in the same reaction conditions the ester yield of free Lipase is only the 25%. The reusability (turnover number (TON) and turnover number of frequency (TOF)) and the stability of heterogeneous

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biocatalysts were also determined for a potential industrial application. The stability of the prepared biocatalyst is very high: up to 9 reaction cycles with a TOF number 16-fold higher than that of free lipase.

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## SOMMARIO

Le Lipasi sono adatte a catalizzare reazioni di esterificazioni in solventi organici, mostrando elevata enantioselettività rispetto a quella mostrata nelle reazioni di idrolisi. Rispetto al grande quantitativo di Lipasi descritte in letteratura, solo gli enzimi derivanti da poche specie hanno dimostrato di avere una adeguata stabilità e capacità di biosintesi che hanno permesso di utilizzarle in reazioni organiche e, quindi, la loro applicazione come enzimi di interesse industriale. Grazie a tali proprietà, le Lipasi sono stati ampiamente utilizzate per la produzione di composti enantiomericamente puri, nella risoluzione di alcool racemici e acidi organici. Vi è una tendenza sempre più in crescita verso l'uso di farmaci in forma di enantiomeri otticamente puri perché hanno target più specifici e hanno meno effetti collaterali rispetto alle miscele racemiche. Tra 1800 farmaci attualmente disponibili, circa la metà sono miscele chirali. Solitamente l'attività terapeutica di un farmaco viene mostrata solo da uno solo degli enantiomeri. In questo senso, l'attività farmacologica dei profeni, che costituiscono un importante gruppo di farmaci anti-infiammatori non steroidei (FANS) utilizzati nel trattamento di artrite e di malattie correlate, viene mostrata dal rispettivo (S) -enantiomero, che mostra un'attività 100 volte maggiore rispetto al suo antipode. Pertanto sforzi importanti per sintetizzare enantiomeri puri dei profeni sono attualmente in corso. Ibuprofene, è un acido carbossilico racemico, di cui ciascun enantiomero mostra un comportamento fisiologicamente diverso, essendo l'enantiomero (S) la forma che presenta una proprietà anti-infiammatoria. Negli ultimi anni, le lipasi sono stati utilizzati per la risoluzione chirale di (R,S)-Ibuprofene tramite esterificazione enantioselettiva, principalmente diretta in mezzi organici, per l'ottenimento del rispettivo estere racemico.

Tra le diverse lipasi, la Lipasi da *Rhizomucor miehei* è in grado di catalizzare reazioni di est. In particolare, in questo lavoro, la lipasi da

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Rhizomucor miehei è stato immobilizzato in nanosfere ibridi contenenti un nucleo liposomiale, in cui l'enzima è confinato. La fase organica (liposomi + lipasi) è stata protetta da una matrice di silice inorganica, creando così una struttura nanoparticellare (biocatalizzatori eterogenei).

L'influenza di importanti fattori sperimentali nella procedura di sintesi dei biocatalizzatori ibridi sono stati studiati: il rapporto in peso tra la quantità di silice e di liposoma utilizzate, e il tempo di miscelazione tra liposoma e lipasi. Tutti i campioni sono stati caratterizzati mediante note ed avanzate tecniche analitiche, al fine di mettere a punto una procedura di sintesi ottimale per l'ottenimento di un biocatalizzatore eterogeneo morfologicamente omogeneo in cui lipasi è perfettamente immobilizzata all'interno della fase liposomiale, a sua volta perfettamente ricoperta dal guscio di silice inorganica. La procedura ottimizzata è stata utilizzata per la preparazione di due tipologie di nanosfere ibride, in cui il guscio di silice inorganica è stato sintetizzato con e senza l'utilizzo del surfattante (Hexadecylamine). I catalizzatori eterogenei ottimizzati sono stati successivamente utilizzati come catalizzatori, nel processo di esterificazione enantioselettiva dell' Ibuprofene racemico, allo scopo di valutarne la prestazione catalitica. In particolare, è stata studiata l'influenza di diversi parametri sulla performance catalitica dei biocatalizzatori eterogenei ottimizzati:

- natura del solvente (un solvente polare: isoottano; solvente polare: dimetilformammide);
- tipologia di alcool (alcool primario con differente lunghezza della catena : metanolo; 1 -propanol; 1-butanolo);
- temperatura di reazione (27, 37, 50 e 80 ° C).

La migliore prestazione catalitica per il biocatalizzatore eterogeneo, è stato mostrato a 37 ° C, utilizzando isoottano come solvente e 1-propanolo come alcool (valore di rendimento estere compreso tra 78 e

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93%). Inoltre, è stata osservata una forte iper-attivazione dell'enzima immobilizzato, rispetto alla forma libera: nelle stesse condizioni di reazione la resa in estere della Lipasi Libers risulta essere solo del 25%.

Inoltre, il riutilizzo (numero di turnover (TON) e numero di turnover di frequenza (TOF)) e la stabilità dei biocatalizzatori eterogenei sono stati determinati per una potenziale applicazione industriale. La stabilità dei biocatalizzatori ottimizzati sintetizzati è molto elevata: fino a 9 cicli di reazione con un numero TOF 16 volte più alto di quella della lipasi libera.

# **INTRODUCTION**

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Drug chirality is now a major theme in the design, discovery, development, launching and marketing of new drugs. Stereochemistry is an essential dimension in pharmacology. In past decades, the pharmacopoeia was dominated by racemates and due to the emergence of new technologies in the 1980s that allowed the preparation of pure enantiomers in significant quantities, the awareness and interest in the stereochemistry of drug action has increased.

Chiral molecules are constituents of a large proportion of therapeutic agents. The separation of enantiomers is of great interest to the pharmaceutical industry since more than half of pharmaceutically active ingredients are chiral. Chiral chemistry was discovered by Louis Pasteur (a French chemist and biologist) when he separated by hand, for the first time in 1848, the two isomers of sodium ammonium tartrate.

Chiral compounds exist in two enantiomeric forms, which have identical molecular formula but whose structural arrangement form non-superimposable mirror images. However, it needed to wait about a century to find that the phenomenon of chirality plays a key role not only in the life of plants and animals but also in pharmaceutical, agricultural and other chemical industries. Most biomolecules, such as enzymes, proteins, hormones, nutrients, sugars, fats, and many others are chiral. Many drugs, such as food additives, flavors and fragrances or agrochemicals are chiral and often the properties of the two enantiomers are very different. When one enantiomer is responsible for the activity of interest, its paired enantiomer could be inactive, be an antagonist of the active enantiomer or have a separate activity that could be desirable or undesirable. For example, the enantiomers of chiral drugs such as omeprazole and ibuprofen, exhibit different pharmacological and pharmacokinetic activities because they interact with enzymes and receptors consisting of amino acids and other chiral biomolecules. In many cases, one enantiomer is the active pharmaceutical ingredient while the other can be benign or even toxic.

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In pharmaceutical industries, 56% of the drugs currently in use are chiral products and 88% of the last ones are marketed as racemates consisting of an equimolar mixture of two enantiomers. Nowadays, there is an increasing effort in industrial research to develop new methods for chiral drug production. The current developed methods are:

- ✓ Chiral pool synthesis
- ✓ Laboratory-invented chiral building blocks (“new chiral pool”)
- ✓ Asymmetric synthesis
- ✓ Resolution of racemates
- ✓ Biotransformations.

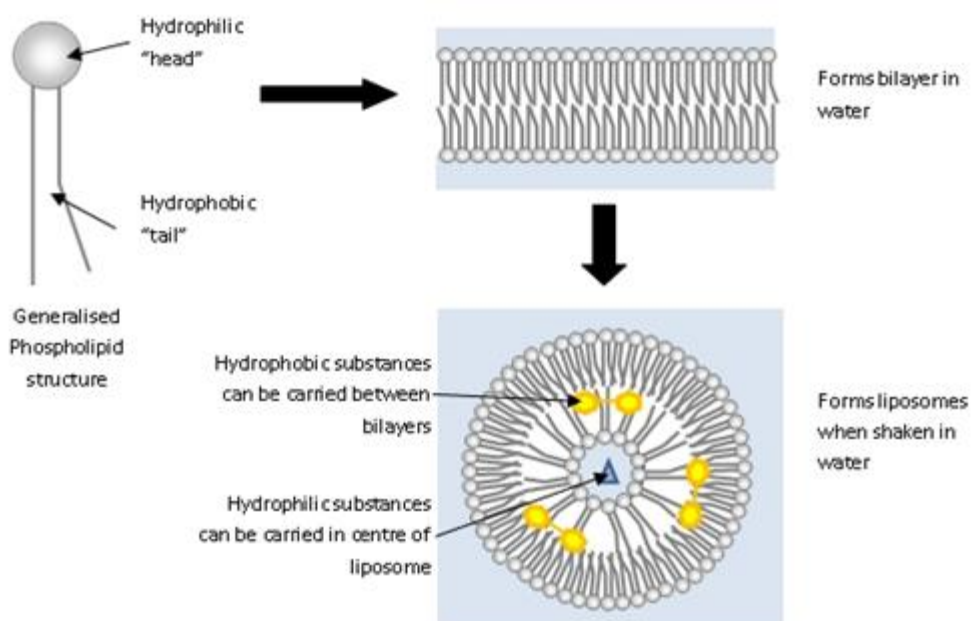
Each of them posses different advantages and disadvantages. Particularly, the *Biotransformation method* employs biocatalyst for organic synthesis of racemates. This method presents many noble features in the field of Green Chemistry and the synthesis of enantiopure drugs. The application of enzymes in organic synthesis provides advantages in comparison with conventional chemical methods, such as a mild reaction condition, high regio-, chemo- and enantio-selectivity, high catalytic efficiency and high product purity and quality. The main disadvantage in the enzyme use is the high cost of biocatalyst that can be overcome by confining it in a specific space of reactor. By immobilization procedure, the enzyme can be used for more than one reaction cycle, strongly reducing the cost related to its use. Sometimes, the immobilization could also activate the catalytic center of the enzyme, especially for Lipases species.

The work of the thesis has been developed in this scenario. Particularly, the Lipase from *Rhizomucor Miehei* has been immobilized into hybrid nanospheres containing a liposomal core, where enzyme is confined. The organic phase (Liposome+Lipase) has been protected by inorganic silica matrix obtained with and without surfactant.

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Liposomes have been chosen as organic protection of lipase because they represent a very suitable micro-environmental of enzymes, that can preserve the enzyme structure and life.

Liposomes are small vesicles with a diameter of 50-500 nanometers. Their structure is characterized by the presence of one or more external phospholipid bilayers, delimiting the inside of a heart in which the hydrophilic material is located in the aqueous phase (**Figure 1**).



**Figure 1.** Structure of liposome

The optimized heterogeneous biocatalyst has been used for enantioselective esterification of (R,S)-ibuprofen. Ibuprofen is a racemic carboxylic acid showing in each enantiomer a physiologically different behavior, being the (S)-enantiomer the form that exhibits an anti-inflammatory property.

In details, the thesis work has been structured in 6 Chapters.

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In the CHAPTER I has been given an overview on the topic of chirality, the tendency and development of chiral drugs and an outline of the methods of enantioselective synthesis.

CHAPTER II has been focused on enzymes (general aspects, structures and classification) and the basic principles of enzyme catalysis. Subsequently, much attention has been paid on the enzyme Lipase, its industrial applications and, especially on the Lipase used in the present work, as catalyst in the resolution of chiral compound: Lipase from *Rhizomucor miehei* (RML).

The advantages of the use of immobilized enzyme in catalysis has been discussed in CHAPTER III.

After a brief introduction of the experimental work of the thesis, reported in the CHAPTER IV, the experimental details relating to materials, methods and equipment used for the construction of the experimental work, have been described in CHAPTER V.

Finally, CHAPTER VI has been dedicated exclusively to the discussion of the obtained experimental data.

# **CHAPTER I**

## *Overview of Chirality*



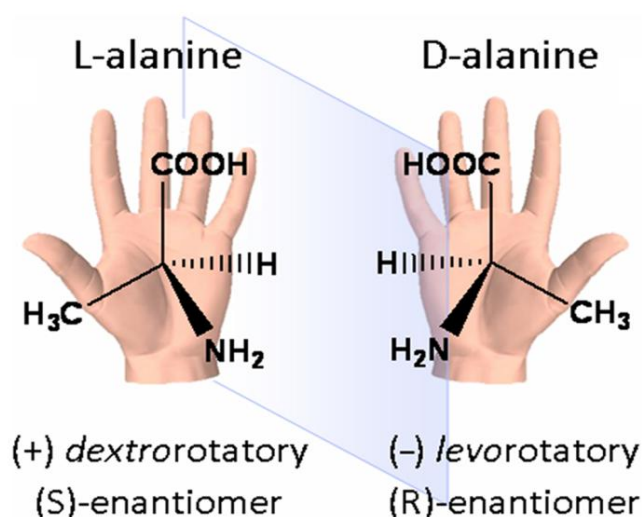


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## 1.1 INTRODUCTION OF CHIRALITY

The word "**Chiral**" is derived from the Greek word "*cheir*", which means "*handedness*", and is a fundamental property of a three-dimensional object. The concept was introduced for the first time by Sir L. Thompson, Lord Kelvin (1824-1907) who said: «*I call any geometrical figure, or group of points, chiral, and say that it has chirality, if its image in a plane mirror, ideally realized, cannot be brought to coincide with itself*».

Chirality is often illustrated with the idea of left- and right-handedness (**Figure 1.1**): a left hand and right hand are mirror images of each other but are not overlapping. The two mirror images of a chiral molecule are termed *enantiomers*. Like hands, enantiomers come in pairs. Both molecules of an enantiomer pair have the same chemical composition and can be drawn the same way in two dimensions, but in chiral environments such as the receptors and enzymes in the body, they can behave differently.



**Figure 1.1** Mirror images (enantiomers) of Alanine molecule.

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The origin of the chirality resides in stereoisomerism. Stereoisomers are compounds that have the same molecular formula and the same functional groups bonded together, but which differ in the three-dimensional arrangement of their atoms or groups. The condition for the creation of a stereoisomer is the presence of at least one asymmetric carbon atom in the molecule. A chirality occurs whenever a carbon atom has four different atoms or groups attached to it. It is also said to be chiral and such an asymmetric atom is called *chiral center*. Carbon is not the only atom that can act as an asymmetric center. In fact, sulfur, phosphorus and nitrogen can sometimes form chiral molecules such as omeprazole, cyclophosphamide and methaqualone, respectively<sup>1</sup>. A chiral molecule is not super-imposable with its mirror image and hence there is a possibility of two mirror image forms, called enantiomers. Such space isomers are known as enantiomers. The enantiomers of a chiral compound exhibit similar physicochemical properties in an achiral environment, but they rotate the plane of polarized light in opposite directions and react at different rates with a chiral compound. Isomers that are not enantiomers are called diastereomers. These have the same structural formula, but are not mirror images of one another, and generally exhibit different physical properties, even in an achiral environment. An equimolar mixture of the two enantiomers of a chiral compound is called a *racemic mixture* that does not exhibit optical activity.

The designation of different stereoisomers is somewhat complicated since there are at least three different systems to classify them and all are used more or less accidentally. The first system of stereoisomer nomenclature is the (*d/l*) or (+/-) system that is based on the direction in which the compound rotates plane-polarized light. Enantiomers that rotate plane-polarized light to the right are termed dextrarotatory which is indicated by d- or (+)- before the name of the compound, and those that rotate light to the left are termed levorotatory and are designated by a l- or

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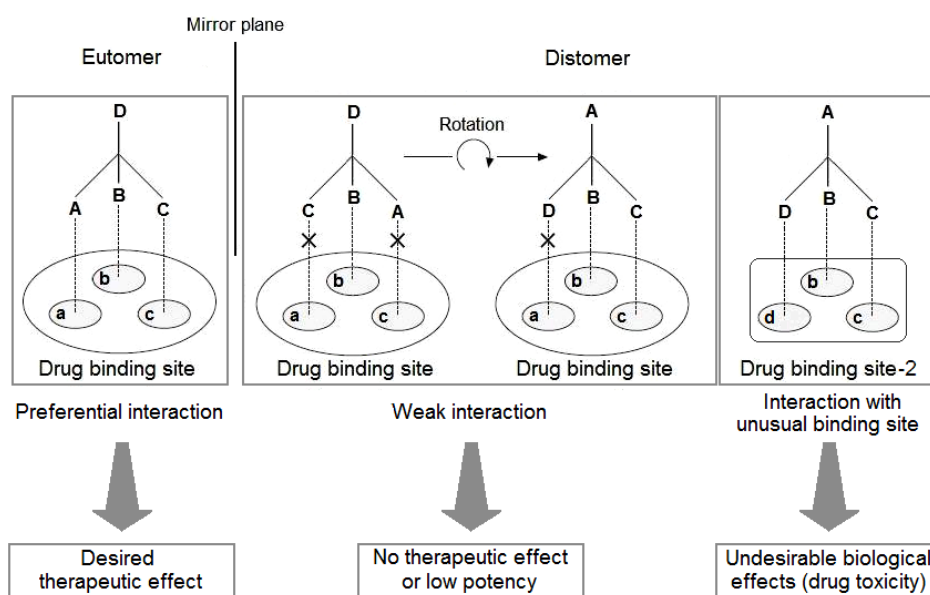
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(-)- prefix. Racemic mixture is denoted with sign (+/-) or (*d/l*) or with prefix *rac*<sup>2</sup>. This system has been used in chemistry for a long time, but the rotation of plane-polarized light is not an absolute property of a compound but is affected by many factors, such as the solvent used.<sup>3</sup> The current system of denotation of stereoisomers is based on the sequence of atoms or groups around its chiral centre and has a conventional character. It is known as the Cahn-Ingold-Prelog (CIP) system or (R,S)-system<sup>4</sup>. The configuration is indicated by use of prefixes *R* (Latin: *Rectus* means right) and *S* (Latin: *Sinister* means left) CIP system criteria: a higher atomic number or a higher atomic mass is given higher priority; when the proximate atom of two or more of the substituents are the same, the atomic number of the next atom determines the priority; double bonds or triple bonds are counted as if they were split into two or three single bonds, respectively; *cis* is given higher priority than *trans*; lone pair electrons are regulated as an atom with atomic number 0; and proximal groups have higher priority than distal groups. If counting from the highest priority (highest atomic number or highest mass) to the lowest one, goes in a clockwise direction, the configuration is designated as *R*; otherwise if counting goes in a counter clockwise direction, the configuration is designated as *S*. A racemate is designated as *RS*. Depending upon the direction of plane polarized light towards right (+) or left (-), each *R*- and *S*-enantiomer is designated as *R* (+) or *R*(-) and *S*(+) or *S*(-)<sup>2</sup>.

## 1.2 CHIRAL DRUGS IN A BIOLOGICAL SYSTEM

The enantiomers of a chiral drug display different types of biological behavior in a chiral living system. These variations may lead to differences in biological activities. Indeed, biological systems can recognize the two enantiomers as two different substances, and their interaction with each

other will therefore elicit different responses. The *R*-enantiomer of a drug will not necessarily behave the same way as the *S*-enantiomer of the same drug, when taken by a patient. To explain this we consider a hypothetical interaction between a chiral drug and its chiral binding site (**Figure 1.2**). The portions of the drug labeled A, B, and C must interact with the corresponding regions of the binding site labeled a, b, and c for the drug to have its pharmacological effect. The active enantiomer of the drug has a 3-dimensional structure that may bind precisely to the target sites (a, b, c), while the inactive enantiomer cannot bind in the same way no matter how it is rotated in space. Although the inactive enantiomer possesses all of the same groups A, B, C, and D as the active enantiomer, they cannot all be simultaneously aligned with the corresponding regions of the binding site.



**Figure 1.2.** The Hypothetical Interaction Between the two Enantiomers of a Chiral Drug and Its Binding Site

Thus, the difference in their 3-dimensional structures allows the eutomer to exert a desired pharmacological effect while preventing the distomer from exerting the same desirable pharmacological effect<sup>1</sup>. In some cases, the portion of a molecule containing the chiral center(s) may be in a

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region that does not play a role in the molecule's ability to interact with its target. In these instances, the individual enantiomers may display very similar or even equivalent pharmacological behavior at their target site. Even in these cases, the enantiomers may differ in their metabolic profiles as well as their affinities with other receptors, transporters, or enzymes. Therefore, for a given chiral drug, it is appropriate to consider the two enantiomers as two separate drugs with different properties unless otherwise proven experimentally. The examples of differential enantiomer potency of chiral drugs are reported.

**1. Racemic drugs with one major bioactive enantiomer.** In this group, there are a number of cardiovascular drugs, agents widely used for the treatment of hypertension, heart failure, arrhythmias, and other diseases. Among these are the  $\beta$ -adrenergic blocking agents, calcium channel antagonists and angiotensin-converting enzyme (ACE) inhibitors. Levorotary-isomer of all  $\beta$ -blockers is more potent in blocking  $\beta$ -adrenoceptors than their dextrorotary-isomer, in the same way as S(-)-propranolol is 100 times more active than its R(+)-antipode<sup>5-6</sup>. However, it has been demonstrated that *d,l*- and *d*-propranolol can inhibit the conversion of thyroxin (T4) to triiodothyronin (T3), contrary to its *l*-form<sup>7-8</sup>. Therefore, single *d*-propranolol might be used as a specific drug without  $\beta$ -blocking effects to reduce plasma concentrations of T3 particularly in patients suffering from hyperthyroidism in which racemic propranolol cannot be administered because of counter effects for  $\beta$ -blocking drugs<sup>5</sup>. In neurology and psychiatry, many of the drugs used are chiral compounds and most of them are marketed as racemates. Hypnotics such as hexobarbital, secobarbital, are racemic compounds and for all of these, only *l*-isomer is hypnotic or sedative, the other is either inactive or excitative. For example, S(-)-secobarbital is more potent as an anesthetic than R(+)-secobarbital i.e. it causes a smoother more rapid anesthetic effect<sup>9</sup>.

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**2. Both enantiomers having equal therapeutic properties.** There are only some racemic drugs that could belong to this group such as cyclophosphamide (antineoplastic), flecainide (antiarrhythmic), fluoxetine (antidepressant)<sup>10</sup>.

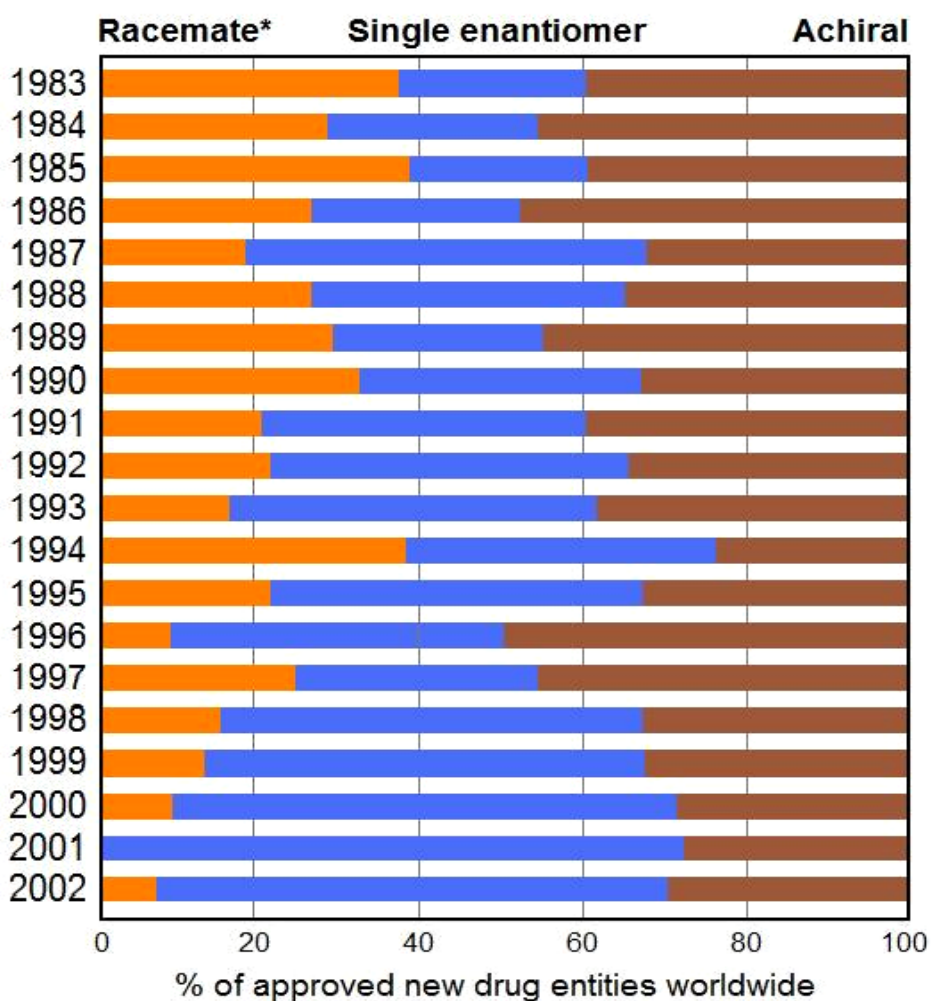
**3. Racemic drugs with chiral inversion.** Directional inversion was described with 2-arylpropionate nonsteroidal anti-inflammatory drugs (NSAID), such as ibuprofen, ketoprofen, etc. For this group, only S-enantiomer is active and has an analgesic and anti-inflammatory effect. For example, S-ibuprofen is over 100-fold more potent as an inhibitor of (R)-ibuprofen.

### **1.3 TENDENCE AND DEVELOPMENT OF CHIRAL DRUGS**

The authorities in many countries began to issue regulatory guidelines on chiral drugs in the mid-1980s owing to the accessibility of enantiomerically pure drug candidates and the accumulation of knowledge on chiral drugs.

The first policy statement regarding the development of new stereoisomeric drugs was published by The United States Food and Drug Administration in 1992<sup>11</sup>. In 1994, guidelines concerning investigations into any chiral active substance were issued by a commission of European countries. With the stiffening of regulations, demand for chiral raw materials, intermediates, and active ingredients has grown dramatically since 1990<sup>12</sup>. An overall look at the 20-year period from 1983–2002 (**Figure 1.3**), shows that the single enantiomers drug segment has become an important part of the overall pharmaceutical market (39% of worldwide

approved drugs). Single enantiomers reached 50% of all approved drugs for the first time in 1998, rising to >60% between 2000–2001, probably due to the directives of the major regulatory agencies which favored and encouraged the development of single-enantiomer drugs over racemates<sup>13</sup>.



\*Data includes the diastereomeric mixture.

**Figure 1.3.** Emergence of single enantiomeric drugs: A worldwide scenario for 20 years; single enantiomers have dominated racemates since 1990<sup>14</sup>

According to estimates from Technology Catalyst International and IMS Health (**Table 1.1**), single enantiomers therapeutics had sales of \$225 billion in 2005, representing 37% of the total formulation pharmaceutical market of \$602 billion.

Besides the development of new drugs in single enantiomeric form, chiral technology has a crucial role in the re-evaluation and remarketing of single enantiomeric forms of existing racemic drugs ("**Racemic switching**": a reprocessing and reformulation of a racemic mixture, into its single enantiomer product) which permits additional years of market exclusivity . Besides drugs, 'chirality' is receiving attention from several business sectors such as biochemicals, agrochemicals, aroma and flavour compounds, dyes and pigments and polymers. The industrial demand for enantiopure chemicals is therefore expected to show explosive growth in the coming years<sup>15</sup>.

**Table 1.1.** Worldwide sales of single-enantiomer pharmaceutical products final formulation<sup>16</sup>

<b>Therapeutic Category</b>	<b>2000 Sales (in \$ billions)</b>	<b>2004 Sales (in \$ billions)</b>	<b>2005 Sales (in \$ billions)</b>	<b>CAGR (%)* 2000-2005</b>
<i>Cardiovascular</i>	27.650	34.033	36.196	6
<i>Antibiotics and antifungal</i>	25.492	32.305	34.298	6
<i>Cancer therapies</i>	12.201	21.358	27.172	17
<i>Hematology</i>	11.898	20.119	22.172	13
<i>Hormone and endocrinology</i>	15.228	20.608	22.355	8
<i>Central nervous system</i>	9.322	17.106	18.551	15
<i>Respiratory</i>	6.506	12.827	14.708	18
<i>Antiviral</i>	5.890	11.654	14.683	20
<i>Gastrointestinal</i>	4.171	11.647	13.476	26
<i>Ophthalmic</i>	2.265	3.063	3.416	9
<i>Dermatological</i>	1.272	1.486	1.561	4
<i>Vaccines</i>	1.427	2.450	3.100	17
<i>Other</i>	7.128	10.400	13.268	13
<b>Total</b>	<b>130.991</b>	<b>199.056</b>	<b>225.223</b>	<b>11</b>

\* CAGR is compound annual growth rate



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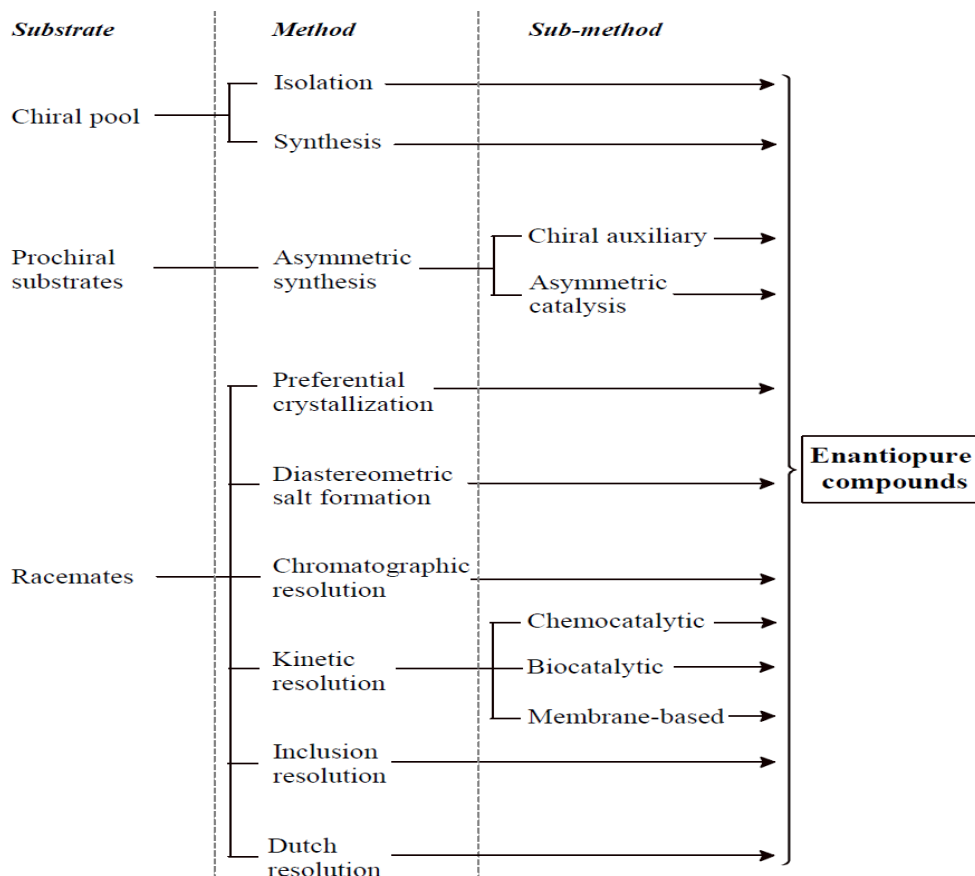
## 1.4 METHODS OF ENANTIOSELECTIVE SYNTHESIS.

The therapeutic effect of a chiral drug is attributed to a single enantiomer (eutomer) and the other enantiomer may be inactive and/or produce undesirable effects (distomer). Nowadays there is growing interest in the chiral switch process by which the distomer of a chiral drug is converted to its active eutomer. There has been an increasing effort in industrial research to develop new methods and the methods developed to date are revealing their own advantages and disadvantages.

For pharmacological studies of such drugs, there is therefore a need for an effective means of separating and quantifying the enantiomers in biological samples. The chiral switch process (racemate to single enantiomer) has resulted in a number of agents being re-marketed as single enantiomer product.

There are several ways to obtain chiral molecules in commercially pure form (**Figure 1.4**):

- ✓ Chiral pool synthesis
- ✓ Laboratory-invented chiral building blocks (“new chiral pool”)
- ✓ Asymmetric synthesis
- ✓ Resolution of racemates
- ✓ Biotransformations.



**Figure 1.4.** Methods for synthesizing enantiopure compound

**Synthesis from chiral pool:** Chiral natural products and raw materials with high enantiomeric purity are abundantly available in nature, frequently at low cost. Some of them include proteinogenic L-amino acids, ascorbic acid, dextrose, ephedrine, limonene, quinidine and quinine. The chiral natural product is incorporated into a chemical reaction where stereoselective transformation occurs and results in an enantiopure end-product. This methodology is useful in the preparation of (S) - enantiomer of a key precursor in the manufacture of neuroleptics such as remoxipride.

**Laboratory-inverted chiral building blocks :** In this laboratory method chiral molecules are used as precursors to prepare enantiopure products. Up to now, a lot of man-made chiral molecules have been developed which allow the enantioselective preparation of alcohols, amines

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and amino acids. For example, Eliel's chiral oxathiane will enable the preparation of enantiopure hydroxyl aldehydes and tertiary carbinols<sup>17</sup>

**Synthesis from prochiral substrate (Asymmetric Synthesis):**

pharmaceutical compounds are prepared from the precursors of complex molecular architecture containing one or more stereocentres. During the preparation of a desirable enantiomer, there will be wastage due to production of unwanted enantiomers. Asymmetric synthesis is one of the important methods used in the preparation of a desirable enantiomer by preventing unwanted enantiomers. In this method enantiomerically pure starting materials are used. The asymmetric synthesis can be achieved either by means of a chiral auxiliary or using an asymmetric catalyst.

1. Asymmetric synthesis via chiral auxiliary: The chiral auxiliary is incorporated in the prochiral substrate, which converts the enantiotopic ligands and faces of substrate to that of diastereotopic type and then to the transition states. This will create new stereogenic centers of diastereoisomeric nature in the substrate, which leads to stereoselective reaction. For example, Evan's chiral oxazolidinone acts as a chiral auxiliary, which induces high stereoselectivity in the reactions of N-acylated derivatives<sup>18-19</sup>. Chiral auxiliaries became very popular 30 years ago due to their efficiency in the generation of stereoselectivity, affording 100% pure enantiomers after the separation of the diastereoisomers. However, the need for using stoichiometric quantities of the chiral auxiliary and the disadvantage of requiring two additional synthetic steps, introduction and removal of the auxiliary, prompted synthetic chemists to divert their attention to the asymmetric or enantioselective catalysis.

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2. Asymmetric synthesis via chiral reagents: In this method chiral reagents are used, which will distinguish enantiotopic ligands and double-bond faces and this in turn will lead to the efficient formation of stereogenic centers.
  3. Asymmetric synthesis with chiral catalysts: In this method each molecule of catalyst induces the asymmetric formation of multiple numbers of chiral molecules, which leads to high product enantioselectivities. For example, Noyori's catalyst (ruthenium or rhodium complexes of chiral phosphine- substituted binaphthyls e.g., BINAP) catalyzes the enantioselective formation of (S)- naproxen and (R)-carnitine<sup>20</sup>.

Starting from the racemate (Resolutions): The separation of the enantiomers of a compound from the racemic mixture is termed as resolution. There are three general methods for the resolution of racemic mixtures, that depend on the nature of the substrate:

- ✓ Direct crystallization
- ✓ Resolution by preparative chiral chromatography
- ✓ Kinetic resolution

- Direct Crystallization: Crystallization is the predominant resolution technique and is applicable only when the racemate is a conglomerate. *Preferential crystallization* method does not require a resolving agent and the stereo-specific growth of each individual isomer occurs in two different crystallizers from solution. In the *Catalytic kinetic resolution* method, the resolving agent reacts with each enantiomer of a racemate at a different rate and thus resolution occurs. On the contrary, the *Diastereoisomeric*

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*crystallization* method requires a resolving agent, which interacts with racemate and produces two diastereoisomeric derivatives. They have different physical properties and can be separated by fractional crystallization, distillation or chromatographic separation. After the separation, the derivatives can be decomposed easily by changing the pH, and the resolving agent is removed and recycled. This methodology has been used in the resolution of naproxen, ibuprofen,  $\alpha$ -methyl-L-dopa, methadone, asparagines, glutamic acid, and others. Racemic ibuprofen can be resolved to its enantiomers by using a resolving agent, L-lysine.

- *Resolution by preparative chiral chromatography:* The use of chromatographic techniques in the resolution of enantiomers to obtain large quantities of enantiomerically pure drugs and drug intermediates is a growing field. Chromatographic separation relies on a difference in affinity between a chiral stationary phase and a mobile phase. In chiral chromatography, the enantiomers of a racemate are discriminated depending on their efficiency concerning the binding of a complexant, which has been added to either the mobile phase or the stationary phase (usually porous silica gel). If the chiral selector is continuously added to the mobile phase the mode of chromatography is called chiral mobile phase chromatography (CMP) and if it is immobilized on the surface of the stationary phase, it is called chiral stationary phase chromatography (CSP). This method is used for quantitative work and along with a polarimetric detector, is also used for qualitative analysis. The accuracy of the method is acceptable and the detection limit for the individual enantiomers is lower than for most other techniques. The chromatographic technique uses two approaches, indirect and direct. ***Indirect method:*** In this method

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the sample (racemate) is derived with an optically pure agent, which results in a second chiral center in the molecule. Thus the enantiomers get converted to diastereoisomers, which possess different physical and chemical properties and so are separable on a non-chiral environment. There is a very low availability of optically pure derivating agents and of the derivated product required to be transformed into the original enantiomers after the chromatographic run. **Direct method:** In this method a chiral environment is created in the chromatographic system by using the chiral stationary phase or by adding chiral additives to the mobile phase. The mechanism of separation is based on the reversible formation of diastereoisomeric complexes of different stability between the enantiomers of the racemate and the chiral stationary phase. This will lead to different elution times of the enantiomers and hence the required enantiomer is separated. The most frequently used preparative chiral chromatographic techniques for the resolution of racemate are Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC).

There are basically two options for chiral **HPLC** analysis, namely the direct and the indirect approach. The molecular interactions between the solute and the chiral phase are favored thanks to lower operating temperatures. There is a broad choice of stationary phases and mobile phases<sup>21</sup>. In **indirect chiral HPLC**, drug enantiomers are derived from an enantiopure chiral reagent to form a pair of diastereomers, which may then be separated on a conventional chromatographic column, since diastereomers exhibit different physicochemical properties. In the **direct method**, transient rather than covalent diastereomeric complexes are formed between the drug enantiomers and a chiral selector

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present either added to the mobile phase (CMPA) or coated/bonded to the surface of a silica support (CSP).

As a GC column contains a higher amount of theoretical plates, it offers more efficiency in the separation of enantiomers when compared to HPLC and TLC. But for analysis with GC the analyte needs to be sufficiently volatile and thermostable. The derivation products of the indirect GC method are often less volatile and less thermostable, which limits the use of this method. In spite of this, much work has been done in the field of indirect separations with GC. For example, menthylchloroformate as a derivation agent shows good potential for the derivation of primary and secondary amines (amphetamine, methamphetamine), amino acids and alcohols<sup>22-23</sup>, (+) or (-) amphetamine as a deriving agent used for the separation of anti-inflammatory 2-arylpropionic acids<sup>24-25</sup>. In direct GC separation method various chiral stationary phases are used. The earliest chiral GC columns are based on amino acids. The separation is based on hydrogen bonding between the solute and the amino acids present in the stationary phase. These columns are mostly used to separate amino acids and also for drugs such as captopril<sup>26</sup> and ifosfamide<sup>27</sup>. Another frequently used chiral GC column is based on inclusion chromatography and it uses cyclodextrin derivatives coupled to the stationary phase<sup>28-29</sup>.

In recent years, Simulated Moving Bed Chromatography (SMBC) has become an alternative approach for the separation of enantiomers in quantities ranging from grams to several hundred kilograms<sup>30-31</sup>. SMBC is a continuous chromatographic multi-column separation process where in six to eight columns are run in series.

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- Kinetic resolution. Kinetic resolution can separate two enantiomers on the basis of their different reaction rates with a chiral entity. The chiral entity can be a chemocatalyst (metal complex or an organic chiral catalyst) or a biocatalyst (an enzyme or a microorganism)<sup>32</sup>. The maximum theoretical yield for a kinetic resolution is 50% for each enantiomer and one of them is chemically modified. Of particular interest is *dynamic kinetic resolution* that permits the total conversion of a racemic mixture into a single enantiopure product. This racemization can be spontaneous or can be induced by using a racemization catalyst<sup>33</sup>. Enantioselective membranes can be employed for the kinetic resolution of enantiomers. These membranes are able to resolve optical isomers because of their chiral recognition sites. They act as selective barriers in the resolution process, and they selectively transport one enantiomer due to the stereospecific interaction between the enantiomer and chiral recognition sites, thereby producing a permeate solution enriched with one enantiomer. The separation of two enantiomers could result, due to one or a combination of the following mechanisms viz. hydrogen bonding, hydrophobic, Coulombic forces, van der Waals interactions and steric effects with the chiral sites<sup>34</sup>.

**Biontransformation:** In the last decade, an alternative to the conventional chemical methods which employs biocatalysts (enzymes) for organic synthesis was evaluated. Biocatalysis presents many noble features in the field of Green Chemistry and the synthesis of chiral building blocks causes the enantiopure drugs or food additives to synthesize: gentle reaction conditions with good regio-, chemo-, enantio-, diastereo-, and prochiral selective acylation/deacylation reactions. The application of enzymes in organic synthesis provides advantages in comparison with conventional

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chemical methods, such as a mild reaction condition, high selectivity, high catalytic efficiency, and high product purity and quality<sup>35</sup>.

Many pharmaceutically important enantiopure compounds are obtained by biocatalysis, like Aminophosphonic acids or Ketoprofen. Aminophosphonic acids, considered as structural analogues of amino acids, exhibit inhibitory activity towards different enzymes, especially towards proteinases such as HIV protease, thrombin, aminopeptidases, and human collagenase. *Cunninghamella echinulata* is a fungal strain and is used to resolve racemic mixtures of 1-aminoethanephosphonic acid with 42% of enantiomeric excess (ee) of R-isomer by L/D amino acid oxidase activity<sup>36</sup>. Ketoprofen (2-(3-benzoylphenyl) propionic acid) is widely used clinically as a nonsteroidal anti-inflammatory drug similar to other 2-arylpropionic acids like naproxen and ibuprofen. The anti-inflammatory activity of ketoprofen was previously believed to reside in its (*S*)-enantiomer, as is also the case for the other 2-arylpropionic acids. The (*R*)-ketoprofen has several advantages as an anti-periodontal, analgesic and antipyretic. The yeast, *Citeromyces matriensis*, produces esterase enzyme, which has the capability of enantioselective hydrolysis of (*R*)-Ketoprofen ethyl ester to (*R*)-Ketoprofen<sup>37</sup>.

In spite of promising indications, the industrial applications of many enzymes are hindered because of two inherent limitations which are exhibited by almost all enzymes: lack of operational and storage stability and high cost. The immobilization of an enzyme is simple but is an equally effective method for overcoming these limitations.

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# CHAPTER II

## *Enzymes*

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## 2.1 INTRODUCTION TO ENZYME

Enzymes are considered as Nature's biocatalysts, with high stereoselectivity, and having a protein structure and considered as potential chiral catalysts for a wide range of processes. The history of enzymes and applied biocatalysts goes back thousands of years to ancient Egypt where enzymes were obtained from microorganisms and used in alcohol production, cheese making, cooking, baking and brewing. This can be seen in an old Egyptian papyrus showing methods used to preserve alcoholic drinks and food. The same methods were used in ancient Greece. The first enzyme, *diastase*, was discovered in 1833 in France by Anselme Payen. A few decades later, during studies concerning the fermentation of sugar into alcohol using yeast, Louis Pasteur came to the conclusion that this fermentation was catalyzed by a vital force contained within the yeast cells that he called "ferments", which were thought to function only within living organisms. He wrote that "alcoholic fermentation is an act correlated to the life and organization of the yeast cells, not to the death or putrefaction of the cells<sup>1</sup>".

To describe this process, in 1877, Wilhelm Kühne used, for the first time, the term "enzyme", which comes from the Greek ἐνζυμοῦν, "leavened". The word *enzyme* was used later to refer to nonliving substances such as pepsin and the word *ferment* was used to refer to the chemical activity produced by living organisms. In 1897 Eduard Buchner submitted his first paper describing the ability of yeast extracts, that lacked any living yeast cells, to ferment sugar. In a series of experiments he found that the sugar was fermented even when there were no living yeast cells in the mixture<sup>2</sup>.

Having shown that enzymes could function outside a living cell, the next step was to determine their biochemical nature. Many early workers noted that enzymatic activity was associated with proteins, but several scientists argued that proteins were merely carriers for the true enzymes and

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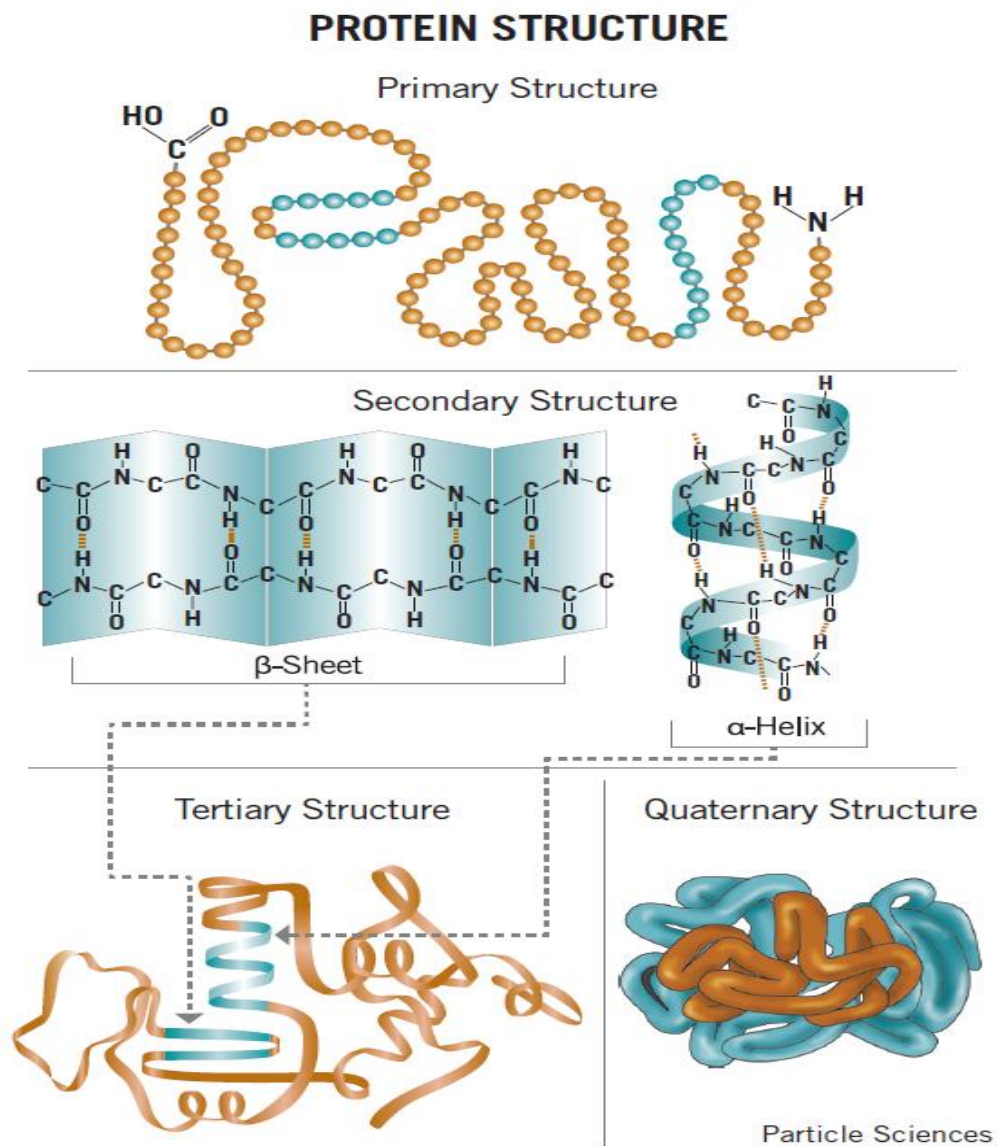
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that proteins were incapable of catalysis<sup>3</sup>. However, in 1926, Sumner showed that the enzyme *urease* was a pure protein and crystallized. In 1937 he did likewise with the enzyme *catalase*. The conclusion that pure proteins can be enzymes was definitively proved by Northrop and Stanley, who, in 1930, worked on the digestive enzymes *pepsin*, *trypsin* and *chymotrypsin*. This discovery that enzymes could be crystallized eventually allowed their structures to be solved by x-ray crystallography. This was first done for *lysozyme*, an enzyme found in tears, saliva and egg whites that digests the coating of some bacteria; the structure was solved in 1965<sup>4</sup>. This high-resolution structure of lysozyme marked the beginning of the field of structural biology and the effort to understand how enzymes work at an atomic level in detail.

## 2.2 ENZYME CATALYSTS

### 2.2.1 Enzyme Structure

Enzymes are generally globular proteins in solution that take on a conformation similar to a sphere. Their molecular weight can differ from 10,000 to millions of Daltons. They are made from simple molecules called amino acids that contain both a basic amino group and an acidic carboxyl group. There are 20 different standard L- $\alpha$ -amino acids used by cells for protein construction. The amino acids differ in structure by the substituent on their side chains. These side chains confer different chemical, physical and structural properties to the final peptide or protein. In simple cases the enzyme molecule is formed by a single polypeptide chain containing a hundred amino acid residues. The sequence of amino acids in the polypeptide chain is the *primary structure* (**Figure 2.1(1)**), specific for each protein. Moreover, the type, the sequence and the position of the amino acids, affect the spatial configuration and the overall shape of the molecule, which the biological properties depend on.



**Figure 2.1.** Protein structure<sup>5</sup>

Stretches or strands of proteins or peptides have distinct characteristic local structural conformations or *secondary structure* (**Figure 2.1(2)**), dependent on hydrogen bonding. The two main types of secondary structure are the  $\alpha$ -helix and the  $\beta$ -sheet. Because of the rigidity of the junctions

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between the peptide bonds, it is possible to have a very limited number of regular secondary structures: the  $\alpha$ -helix and the  $\beta$ -sheet. In the  $\alpha$ -helix, the carboxylic group of an amino acid forms a hydrogen bridge with the amino group of the subsequent fourth amino acid<sup>6</sup>.

An important aspect is that the stability of the  $\alpha$ -helix depends not only on the hydrogen bonds that form between the hydrogen atoms and the nitrogen of the peptide bonds, but also on the amino acid sequence, in fact not all peptides can form a stable  $\alpha$ -helix. The hydrogen bonding in a  $\beta$ -sheet is between strands rather than within strands. The sheet conformation consists of pairs of strands lying side-by-side. The carbonyl oxygens in one hydrogen strand bond with the amino hydrogens of the adjacent strand. The two strands can be either parallel or anti-parallel depending on whether the strand directions (N-terminus to C-terminus) are the same or opposite. The anti-parallel  $\beta$ -sheet is more stable due to the more well-aligned hydrogen bonds.

The interactions of secondary structures form a *tertiary structure* (**Figure 2.1(3)**). These interactions also form the basis of the catalytic cleft of the protein for the activity of enzymes: satisfying requirements with the solvent, maximizing hydrophobic interactions, and forming salt bridges. Since exposure of these residues (e.g., phenylalanine, tyrosine, etc.) to a native hydrophilic environment would be unfavourable, a core of hydrophobic amino acids is given to enzymes by this tertiary structure. The tertiary structures also tend to yield basic or acidic amino acids on the surface due to their activity to hydrogen bond<sup>7</sup>.

The protein molecule will bend and twist in such a way as to achieve maximum stability or lowest energy state. Although the three-dimensional shape of a protein may seem irregular and random, it is fashioned by many stabilizing forces due to bonding interactions between the side-chain groups of the amino acids. This structure is specific and individual amino acid residues are found in well-defined positions which are essential in order for

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the enzyme to carry out its specific biological activity. The tertiary structure of a polypeptide chain, formed by enzymes belonging to more chains, represents a substructure, in that the chains formed from independent subunits aggregate in oligomeric form called a quaternary structure. The ***quaternary structure*** (Figure 2.1(4)) refers to how these protein subunits interact with each other and arrange themselves to form a larger aggregate protein complex. The final shape of the protein complex is once again stabilized by various interactions, including hydrogen-bonding, disulfide-bridges and salt bridges.

Most of the characteristics of enzymes as catalysts derive from their molecular structure. Catalysis takes place in a small portion of the enzyme called the *active site*, which is usually formed by very few amino acid residues, while the rest of the protein acts as a scaffold. The substrate is bound to the enzyme at the active site and so, changes in the distribution of electrons in its chemical bonds are produced that cause the reactions that subsequently lead to the formation of products.

### **2.2.2. Enzyme flexibility**

An important feature of enzymes is that their structure is flexible. The origin of these properties is due to the weakness of ionic and hydrophobic interactions responsible for the specification of the spatial conformation polypeptide chains.

The interactions include ionic salts bonds between groups of opposite charge and hydrogen bonds. When these bonds are formed between groups of the polypeptide chain exposed to water, the hydrogen bonds are unstable and break very easily restoring the original interactions with water. Hydrogen bonds are more stable if they are formed in hydrophobic regions where there is an exchange process with water. For this reason it is reasonable to assume that its structure is not fixed, and that there exists a mixture of tautomeric forms in equilibrium among

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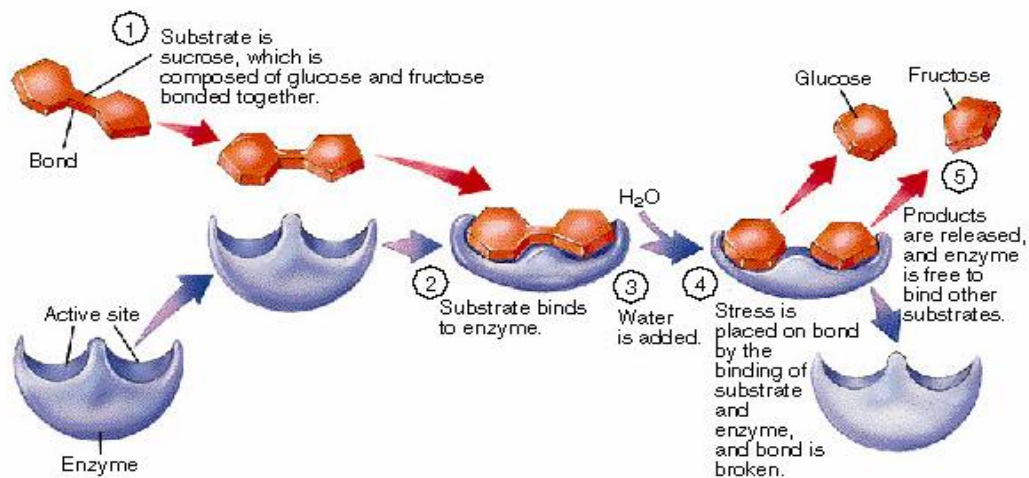
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themselves. When a substrate binds preferably with one of these forms, it prevails on another: in this case we speak of an induced structural change which usually has a positive impact on the catalytic activity.

### **2.2.3 Mechanism of enzymatic catalysis.**

One of the fundamental advances of the age of Molecular Biology (1950 - 1970) was the description of the mechanism of enzymatic catalysis. In the 19th century, the English chemist Adrian Brown first observed that the rate of sucrose fermentation in yeast was independent of sucrose concentration. Later, in 1902, Brown suggested that this apparent independence could be explained by assuming that invertase, the key enzyme for sucrose hydrolysis in the yeast, formed a binary complex with its substrate and that, under the conditions observed by Brown, the sucrose concentration was high enough to be saturating.

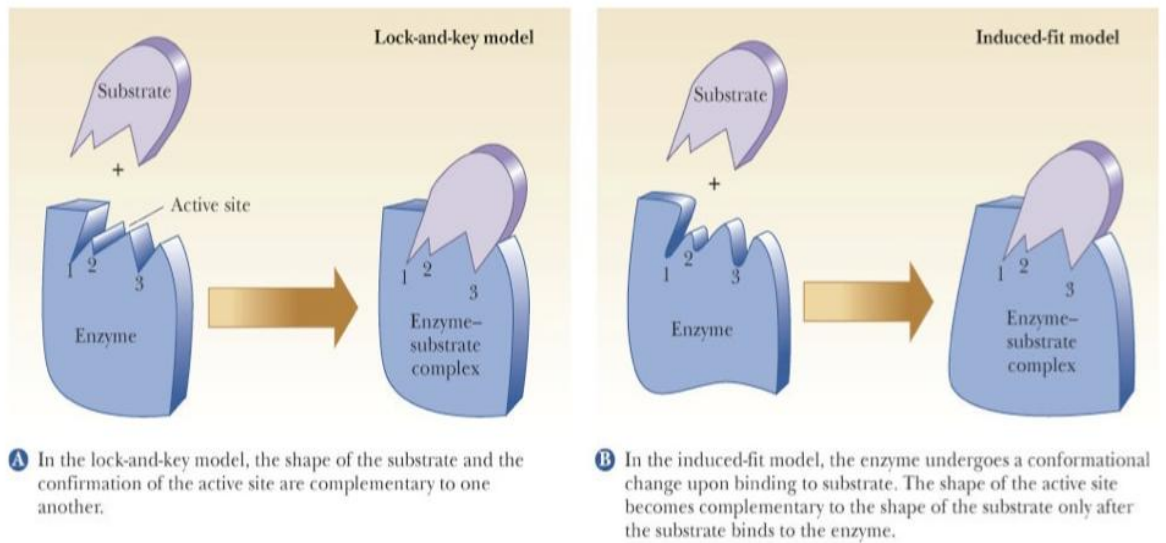
Despite a similar contentious claim by the French chemist Victor Henri (1903), Brown's concept was the first to provide a clear physical chemical. The first studies concerning the kinetics of enzymes were put forward by Victor Henri in 1903, who established that the enzymes are combined with the first substrate to form a complex and then release the product. This idea was extended into a general theory of the action of enzymes in particular by Leonor Michaelis and Maud Menten in 1913<sup>8</sup>. They hypothesized that the enzyme was first combined with the substrate, forming the enzyme-substrate complex in a relatively fast and reversible stage. The formation of this complex puts the specific functional groups of the enzyme in the correct orientation necessary for the catalysis of the reaction. After the formation of the complex, according to the theory, it undergoes a slower second stage, in which it decomposes producing a free enzyme and a reaction product P (**Figure 2.2**):



**Figure 2.2.** Sample of Michaelis-Menten mechanism<sup>9</sup>

Catalysis takes place in a small portion of the enzyme called the *active site*, which is usually formed by very few amino acid residues, while the rest of the protein acts as a scaffold. Due to their unique structure, all the enzymes have a high specificity towards the substrate. Hendrickson, said: "there is only one enzyme for each metabolic reaction"<sup>10</sup>. The enzymes, in fact, are not only able to discriminate on the basis of the chemical identity of the substrate, but also on the basis of its geometrical configuration and stereochemistry.

The specificity of the enzyme-substrate binding can be explained by the *lock-and-key* theory. In this model described by Fisher, the active site has anchor points for specific functional groups of the substrate (**Figure 2.3 A**). However, the catalytic power of an enzyme can be explained by the **adaptation induced Koshland model**. In this model, the enzyme has a site in which it can stay in a specific way connected to a substrate, but this site becomes complementary to the substrate only in the transition state and only through a conformational change of the enzyme (**Figure 2.3B**).



**Figure 2.3** . Representation of lock-and-key model (A) and Induced-fit model (B)<sup>11</sup>

In order to understand the mechanism of an enzymatic reaction it is important to know the native structure of the enzyme and all of the complexes that are formed during the reaction. So, it is possible to differentiate between the amino acids which are linked with the substrate and the amino acids responsible for catalysis.

There are some enzymes called "**allosteric**", that have multiple active sites. One site is dedicated to the substrate, a second one (third, etc..) is dedicated to binding with an "allosteric effector" which can be:

- the same substrate;
- a coenzyme;
- the product of reaction;

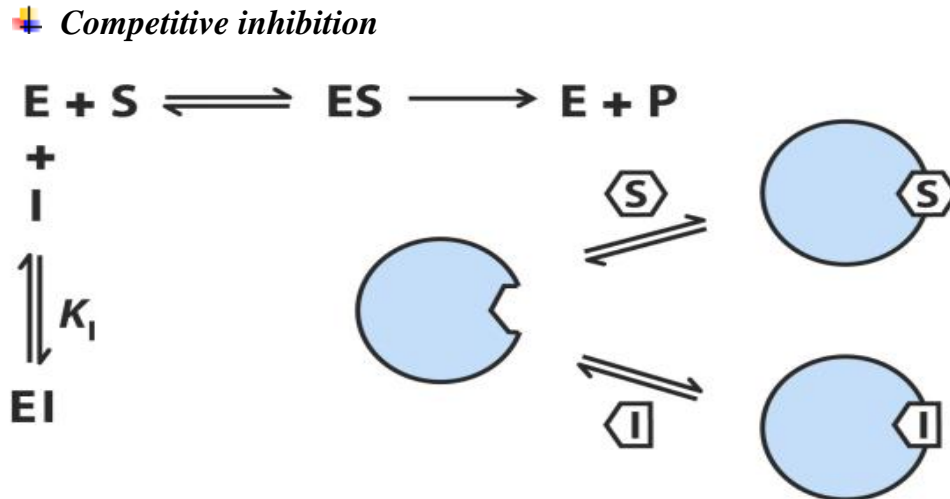
The link between the enzyme and its allosteric factor can activate (positive cooperation) or inactivate (negative cooperation) the same enzyme. The binding "enzyme - allosteric factor" changes the quaternary structure of the enzyme molecule and, consequently, the structure between the active site and the substrate. For these enzymes the substrate

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concentration is related to its presence/absence. For these enzymes the substrate concentration is related to the presence / absence of the corresponding allosteric effector.

Many, but not all, enzymes require small molecules to perform as catalysts. These molecules are termed *coenzymes* or *cofactors*. The term *coenzyme* is used to refer to small molecular weight organic molecules that associate reversibly to the enzyme and are not part of its structure; coenzymes bound to enzymes actually take part in the reaction and, therefore, are sometimes called *cosubstrates*, since they are stoichiometric in nature. Coenzymes often function as intermediate carriers of electrons, specific atoms or functional groups that are transferred during the reaction.

There are, also, the other molecules, called **inhibitors**, that deactivate or reduce the rate of reaction of an enzyme. There are two types of inhibition: competitive (**Figure 2.4**) and uncompetitive (**Figure 2.5**).



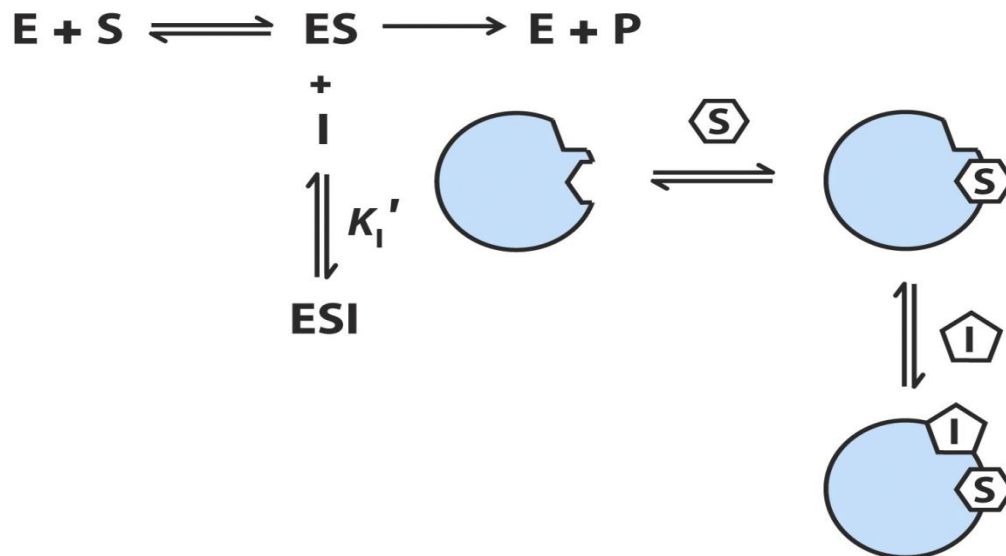
**Figure 2.4.** The scheme of competitive inhibition<sup>12</sup>

The inhibitor competes with the substrate for binding to the active site of the enzyme. The inhibitor (I) is placed on the active site occupying and thus rendering, the presence of the substrate useless. A competitive

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inhibitor decreases the rate of the catalytic reaction by decreasing the fraction of the enzyme molecules available for binding with the substrate. The inhibition can be removed by increasing the concentration of the substrate.

**+** *Uncompetitive inhibition*



**Figure 2.5.** The scheme of uncompetitive inhibition <sup>12</sup>

In the uncompetitive inhibition, the inhibitor attaches to the enzyme in a different area from the active site, creating the complex ESI. The deformation of the structure of the enzyme also affects active site function: it receives the substrate, but at a very slow speed (instead of the ES complex, the complex ESI is formed). Non-competitive inhibition actually decreases the presence of the enzyme (engaged in the inactive complex ESI) and thus apparently decreases the speed of the reaction.

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### 2.2.4 Performance of enzyme activity

The speed of a catalyzed reaction is influenced by the following factors:

#### Substrate concentration

The kinetics of the reactions catalyzed by enzymes is characterized by the phenomenon of saturation by the substrate (**Figure 2.6**): at low concentrations of substrate the reaction rate is proportional to its concentration (first order kinetics), but if it increases the concentration of the substrate, the reaction rate tends to increase to a lesser extent and is no longer proportional to the concentration of substrate (zero-order kinetics). By further increasing the concentration, the reaction rate becomes constant and independent of substrate concentration. At this point the enzyme is saturated (fully bonded) by the substrate; to the state of saturation, the factor that limits the speed of the reaction is represented only by the concentration of the enzyme. The initial velocity of a reaction catalyzed by an enzyme is put in relation to the single substrate concentration via two constants:  $K_m$  and  $V_{max}$ . The first one is a Michelis-Menten constant, and represents the affinity between enzyme and substrate and also the ability to form the product P.  $V_{max}$  is at a maximum speed that is reached when the substrate saturates the enzyme.

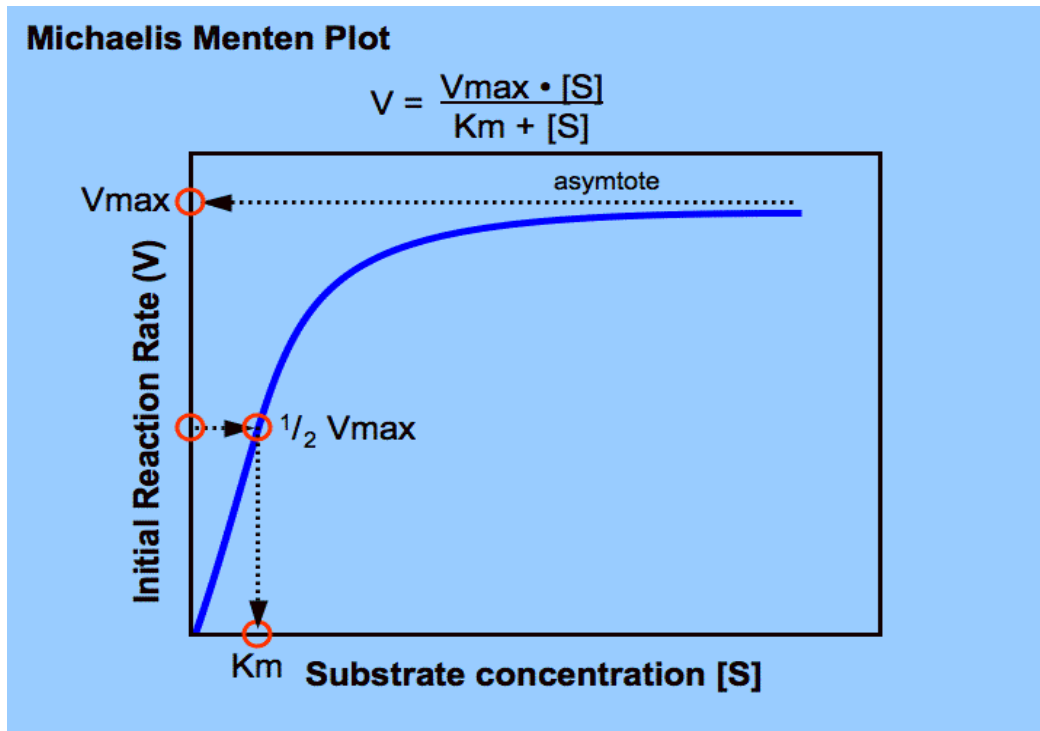


Figure 2.6 . Michaelis -Menten cinetic <sup>13</sup>

▣ Enzyme concentration

The maximum velocity of an enzymatic reaction depends on the enzyme concentration: in the presence of a high concentration of substrate, all enzyme molecules are linked to molecules of the substrate and by increasing the enzyme concentration, the amount of product also increases. (Figure 2.7).

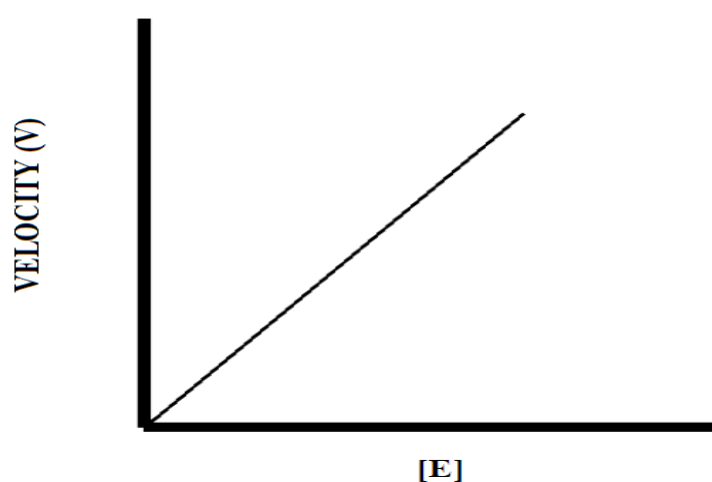


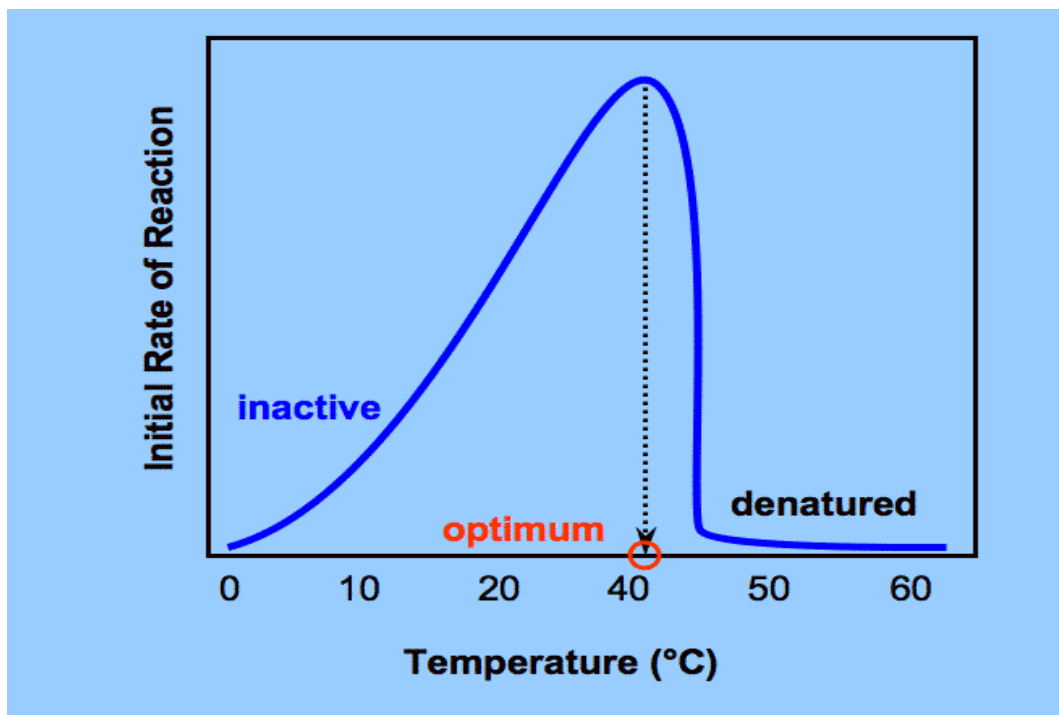
Figure 2.7 . Representation of relationship between enzyme concentration and velocity of catalytic reaction<sup>13</sup>



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## Temperature

The speed of the enzymatic reactions varies with the increase of the temperature according to **Figure 2.8**. The temperature range over which enzymes show activity is limited between the melting point (0°C) and boiling point (100°C) of water. If a temperature is too low, there can be no noticeable reaction rate since the enzyme is operating at a temperature far below its optimum. If the temperature at which the enzyme is operating at is well above 100°C, then thermal deactivation can occur. Thermal deactivation of enzymes limits their useful lifetime in processing environments. Therefore, design and manufacturing levels in many processes are important to reach the correct reaction temperature. If the reaction temperature is too high, the enzymes will eventually deactivate in an irreversible way and thus prevent the reaction from taking place. For many enzymes, the optimum temperature is 37°C, but deactivation can occur at temperatures as low as 45 to 55°C. Deactivation of enzymes may be irreversible or reversible.



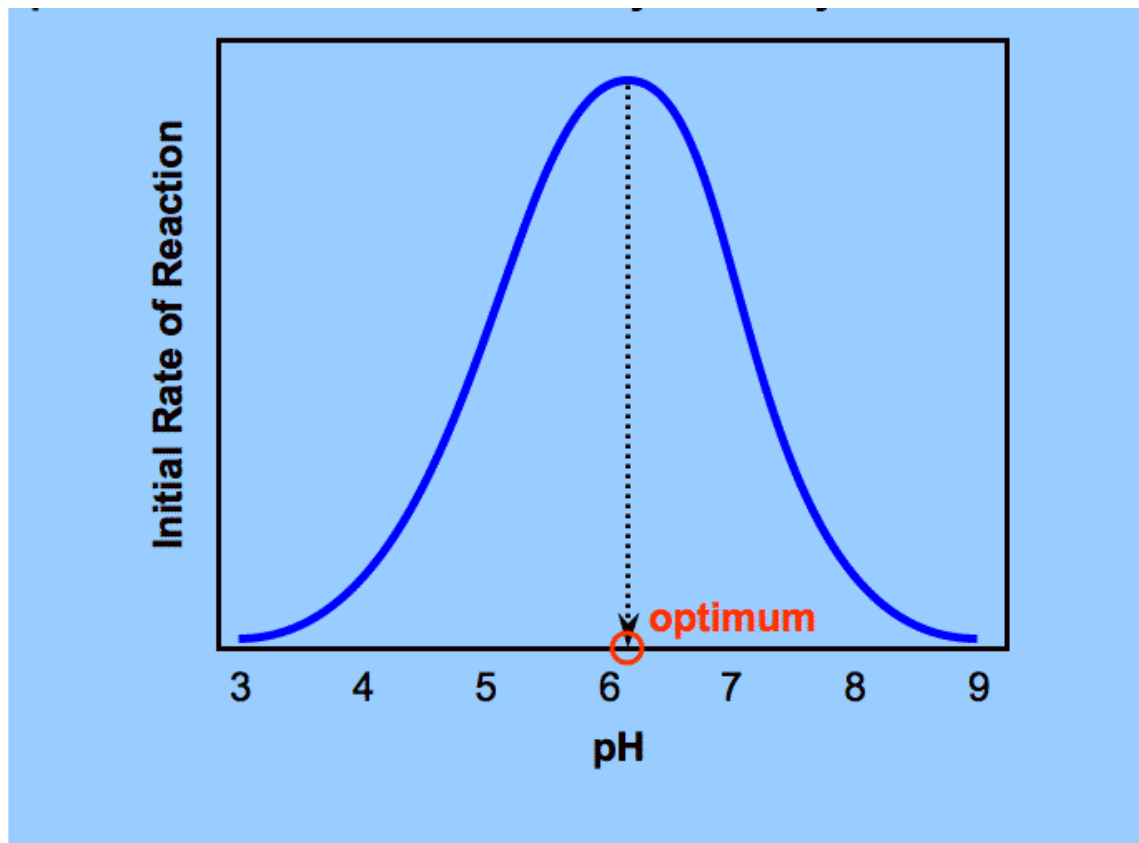
**Figure 2.8.** Temperature influences the rate of enzyme-catalyzed reaction<sup>13</sup>

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## pH

Most proteins, and therefore enzymes, are active only within a narrow pH range usually between 5 and 9 (**Figure 2.9**). Several factors are influenced directly by the pH in which the reaction takes place.

- the binding of substrate to the enzyme
- the ionization states of the amino acid residues involved in the catalytic activity of the enzyme.
- the ionization of the substrate
- variation in the protein structure at extreme pH.



**Figure 2.9.** pH influences the rate of enzyme-catalyzed reaction<sup>13</sup>

The graph of pH against the reaction rate is a bell shaped curve. The curve represents the ionization of a certain amino acid residue that must be

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in a specific ionization state to permit enzyme activity. The inflection point of the curve is called the pK of the reaction and can identify amino acid residues essential to enzymatic activity.

### 2.3 OVERVIEW OF ENZYME CLASSES (EC NUMBERS) AND RELATED REACTION.

Although there are about 25000 enzymes existing in nature, approximately 4000 enzymes have been recognized by The International Union of Biochemistry and Molecular Biology (IUBMB) and categorized into six categories, according to the types of reactions they catalyze: hydrolases, oxidoreductases, isomerases, transferases, lyases, and ligases. (Figure 2.10 and Table 2.1)

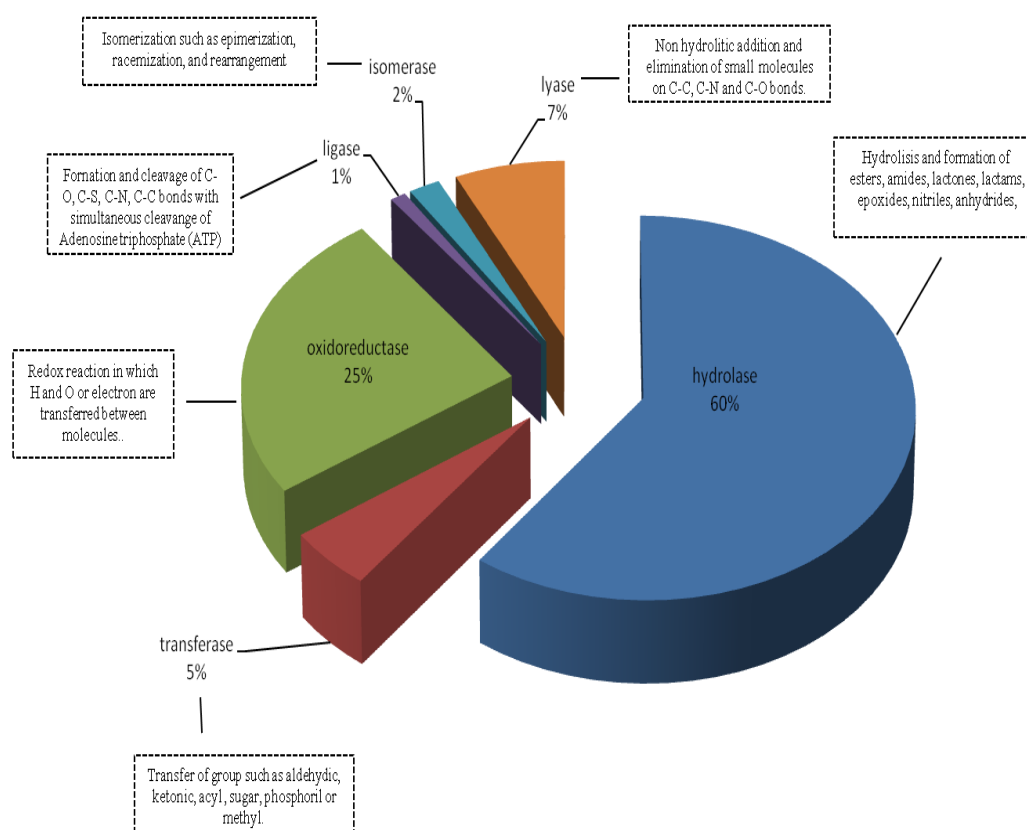


Figure 2.10 . Classification of enzymes<sup>14</sup>

Among these types, oxidoreductases and hydrolases are the most widely applied biocatalysts in biotransformation reactions. For example, between 1987- 2003, around 85% of enzyme research studies were carried out on oxidoreductases and hydrolases (25% and 60%, respectively).

**Tabella 2.1.** Broad classification of enzyme catalyzed reactions <sup>15,16</sup>

Enzyme Class	EC Number	Reactions catalyzed	Examples
<i>Oxidoreductase</i>	1	Oxidation or reaction of substrates	Dehydrogenases Oxidases Peroxidases Oxygenases
<i>Transferases</i>	2	Transfer of a group from one molecule to another	Methyltransferases Glycosyltransferases Acyltransferases
<i>Hydrolases</i>	3	Bond cleavage while water is added	Esterases Glycosidases Peptidases
<i>Lyases</i>	4	Non-hydrolytic cleavage of Bonds	Decarboxilases
<i>Isomerases</i>	5	Conversion of one isomer to another (intramolecular rearrangements)	Racemases Epimerases Intramolecular lyases
<i>Ligases</i>	6	Joining of two molecules at the expense of chemical Energy	DNA ligase

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Each enzyme is given an **E.C.** number (enzyme commission number) consisting of 4 parts. The first number indicates the class of the enzyme; the second number indicates the type of bond on which the enzyme acts; while the third number indicates the subclass of the type of bond on which the enzyme acts; and the fourth indicates a serial number.

With oxidoreductases (**EC 1**) many successful reduction and oxidation processes have been carried out successfully. Redox biocatalysts are easily classified based on the nature of the oxidizing reactant (the electron acceptor) and the reaction products. The advantage in using enzymes as catalysts in redox processes is their excellent selectivity and the possibility of using molecular oxygen as a cheap and sustainable oxidizing agent. The best known biocatalysts, thanks to their high redox potentials, are the *fungal peroxidases* and the *microbial peroxidases*. Another type of oxidative enzyme, which is used as a biocatalyst in organic reactions is named *Laccase*. These enzymes have been obtained in prokaryotes, insects, higher plants, and fungi, and are used in textile and paper industries. The use of laccase enzymes is attractive in that they are essentially 'green' catalysts, which operate with air and generate water as a byproduct, making them more generally useful for scientists. *Laccase enzymes* have been studied in various fields, such as applicability in molecular genetics, genetic transcription, genetic expression, and cloning<sup>17</sup>.

Representatives of enzyme class **EC 2**, so-called *Transferases*, are other versatile catalysts for organic synthetic transformations. These enzymes can shift functional groups from one molecule (donor) to another one (acceptor). In particular, *transaminases* have attracted widespread attention with interesting applications for the synthesis of amino acids and amines. Due to some disadvantages, coupling reactions can occur, equilibrium processes usually do not achieve a high scale of products, and the group transferring molecules are very costly or their products cannot be recycled easily, *transferase* was not widely used in industrial processes.

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Nevertheless, high stereo- and region selectivity in reactions that are catalyzed by transferases are the main causes for their increasing usefulness<sup>15</sup>.

*Lyases*, which are summarized in enzyme class **EC 4**, catalyzed the cleavage of C–N, C–O, and C–C bonds in diverse hydrolysis processes, usually leaving double bonds which can be exploited in more processes.

Enzyme class **EC 5** consists of those enzymes capable of catalyzing isomerization reactions). These types of biocatalysts are a small group that can catalyze structural and geometric converts within a single molecule and make it suitable to apply cheaper molecules to obtain high-value products.

Whereas enzymes from enzyme classes EC 1 to EC 5 are already widely used as catalysts in organic synthesis and have enabled a broad range of highly efficient synthetic processes, the application range of *Ligases* (**EC 6**) is still narrow. Since in situ regeneration of the cofactor ATP is still a challenge, *Ligases* have found limited use as catalysts for in vitro applications in organic syntheses.

The main social concerns about energy resources and environmental quality have increasingly made enzymatic methods desirable to pharmaceutical industries<sup>18</sup> Although these six types of biocatalysts are found in nature, more than 80% of industrial synthesized enzymes are *Hydrolases* (**EC 3**), of these, approximately 50% act on carbohydrates, and also provide most food industry related enzymes. The high use of *Hydrolase* is due to the following facts: many hydrolases are commercially available, often within an attractive price range; their direct and often simple use without the additional cofactor and cofactor regeneration methods; their suitability (particularly in the case of lipases) for reactions in pure organic media; the large-scale production of fatty acid esters starting from the fatty acid and an alcohol moiety<sup>19,20</sup>

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These enzymes, especially **Lipases**, exhibit various advantages over other enzymes, as they have the desired activity in aqueous and non-aqueous organic solvents, allowing the transformation of non-aqueous soluble molecules; they need no cofactors for their catalytic activities. Hydrolytic enzymes, especially proteases and lipases, which have successful applications in organic syntheses, can be generally used in synthetic processes by employing high concentrations of reactants or removing as much water as possible from the reaction system<sup>21</sup>.

## 2.4 MICROBIAL LIPASES

The main sources for obtaining lipases are microorganisms, produced by higher eukaryotes (plants and animals). Both eukaryotic microorganisms (yeast and fungi) and prokaryotic (bacteria, including actinomycetes ) are lipase producers and their properties vary according to the origin<sup>22,23</sup>. Lipases of microbial origin, mainly bacterial and fungal, represent the most widely used class of enzymes in biotechnological applications and organic chemistry.

Generally, *bacterial lipases* are glycoproteins but some extracellular bacterial lipases are lipoproteins. The production of lipases from bacteria is often dependent on nitrogen and carbon sources, inorganic salts, presence of lipids, temperature and availability of oxygen. It was reported that the enzyme production in most bacteria is affected by certain polysaccharides. Most bacterial lipases are non-specific in substrate specificity and a few are thermostable<sup>24</sup>. A number of lipase-producing bacterial sources are available but only a few are commercially exploited as wild or recombinant strains. Among these, the important ones are: *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Chromobacterium* and *Pseudomonas*. The lipases from *Pseudomonas* bacteria are widely used for a variety of biotechnological applications<sup>25</sup>. Normally *bacterial lipases* have neutral or alkalyne optima pH, with the exception of *P. fluorescens* SIK W1 lipase,

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which has an acidic optimum at pH 4.8<sup>26</sup>. Bacterial Lipases show stability over a wide range, from pH 4 to 11<sup>27,28</sup>.

Work on *fungal lipases* started as early as the 1950s and comprehensive reviews have been presented by Lawrence<sup>29</sup> and subsequently by Brockerhoff and Jensen<sup>30</sup> who discussed various aspects of these enzymes. Since then, many workers have exploited fungi as valuable sources of lipase due to the following properties: thermal stability, pH stability, substrate specificity and activity in organic solvents. Fungal lipases have advantages compared with bacterial ones due to the fact that technology favors the use of batch fermentation and low cost extraction methods. In this regard, a good number of fungi have been screened for lipase production. The Chief producers of commercial lipase are *Aspergillus niger*, *A. terreus*, *A. carneus*, *Candida cylindracea*, *Humicola lanuginosa*, *Mucor miehei*, *Rhizopus arrhizus*, *R. delemar*, *R. japonicus*, *R. niveus* and *R. oryzae*<sup>31</sup>. The fungal Lipase has an optimum pH range of 4-8<sup>32,33</sup> with some exceptions, like the *Aspergillus niger* NCIM 1207 lipase<sup>34</sup> and the *Aspergillus Carneus* lipase<sup>25</sup> which showed an optimum pH of 2.5 and 9, respectively. Most fungi have an optimum lipase activity at temperatures between 25-30°C except for some thermophilic fungi which are more active at high temperatures (45-75 °C)<sup>35</sup>.

## **2.5 INDUSTRIAL APPLICATIONS INVOLVING MICROBIAL LIPASE**

The application of biotechnological strategies based on the use of enzymes plays an increasingly important role in the industrial sector so that, over the past ten years, there has been a 12% increase in the volume of enzymes produced, with a growth of exponential type. Consequently, the global demand for these enzymes has grown each year, with over 90% of its



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trade involving the United States, Europe and Japan, where there is an expectation of growth in this market, and for the year 2012, spending reaching more than 27 billion is expected, with predictions of a future increase of about 4%. More than 4000 enzymes are known and approximately 200 are used commercially, the vast majority having microbial origin. At least 75% of all industrial enzymes are hydrolases and of these 90% are produced by microorganisms through fermentation processes. After proteases and carbohydrases, lipases are the third largest group in sales worldwide<sup>22,36</sup>. The advent of enzymology represents an important advance in the biotechnology industry, with the worldwide usage of enzymes reaching nearly U.S. \$ 1.5 billion in 2000<sup>37</sup>. The major share of the industrial enzyme market is occupied by hydrolytic enzymes, such as proteases, amylases, amidases, esterases and lipases. In recent times, lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) have emerged as key enzymes in the swiftly growing field of biotechnology, owing to their multifaceted properties, which find usage in a wide array of industrial applications<sup>38,39,40</sup>.

The great interest in lipases is mainly due to their properties in terms of enantioselectivity, regioselectivity, and broad substrate specificity<sup>40,41</sup>. The use of lipases as biocatalysts for the production of biomolecules has many more potential benefits for future developments besides their specificity. The most important characteristics are:

- ▶ efficiency under mild reaction conditions ;
- ▶ utility in "natural" reaction systems and products;
- ▶ reduction of environmental pollution;
- ▶ possibility to improve lipases by genetic engineering.

For these reasons, many nutritional and functional molecules, with high added value, have been produced enzymatically.

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There has been a tremendous increase in the significance of the biotechnological application of lipases since the middle of the last century<sup>42,43</sup>, due to the versatile catalytic behavior of lipase<sup>44</sup>.

Lipases are part of the family of hydrolases that act on carboxylic ester bonds. The physiological role of lipases is to hydrolyse triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. In addition to their natural function of hydrolyzing carboxylic ester bonds, lipases can catalyze esterification, interesterification, and transesterification reactions in nonaqueous media. This versatility makes lipases the enzymes of choice for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries<sup>45</sup>.

#### ■ FATS AND THE OIL INDUSTRY

This is one of the prime areas in food processing that demand new economic and green technologies. Lipases allow us to modify the properties of lipids by altering the position of fatty acid chains in the triglycerides and by replacing one or more of these with new ones. For this reason, a relatively inexpensive and less desirable lipid can be modified to a higher value fat. Pogori et al.<sup>46</sup> have used a *Rhizopus Japonicus* lipase to produce hard butter by the interesterification of palm oil with methyl stearate.

*Rhizomucor miehei* lipase and Lipozyme IM20 were also used for the modification of tallow by interestification of its stearine fractions with liquid oils and acidolysis of its olein fractions with karanja stearine. Interesterification using fungal lipases can also be used to produce oils and fats containing nutritionally important polyunsaturated fatty acids (PUFAs), that play a role in human health<sup>47-51</sup>. Lipases are also used to modify the fatty acid chain lengths and to accelerate cheese ripening and the lipolysis of butter, fat and cream. It is well known that, *Mucor miehei*, *Aspergillus niger* and *Aspergillus oryzae* are the lipases most used in this type of application.

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## ■ DETERGENT INDUSTRY

Lipases have a major use as an additive in the detergent industry thanks to the ability to hydrolyze fats. In fact their function is to remove fatty residues and clean clogged drains. Lipase should be thermostable, tolerant to an alkaline environment, capable of functioning in the presence of various components of washing powder formulas<sup>52</sup>.

Enzymes can reduce the environmental impact of detergent products, since they save energy by enabling a lower washing temperature to be used and allow the amount of other often less desirable chemicals in detergents to be reduced. They are also biodegradable, and do not present a risk to aquatic life. Ever since the discovery of lipases, they have become part and parcel of the detergent industry along with proteases, amylases, and cellulases. Nowadays they are extensively used in household detergent, industrial cleaners and leather processing. Several fungal lipases like *Aspergillus oryzae*, *Candida* sp., *Rhizopus oryzae* and *Humicola lanuginosa* are known to produce lipases under standardized conditions suitable for detergent applications<sup>53</sup>.

## ■ AGROCHEMICALS

Fine and chemical makers underline the importance of new products and processes for the pesticide industry via lipases, in view of the potential for reducing costs and environmental contamination<sup>54</sup>. A variety of pesticides having optically active compounds are made using lipases<sup>55,56</sup>. One of the most important application of lipases has been in the organic synthesis of pesticides for the production of optically active compounds<sup>57</sup>. Mitsuda *et al.* reported the preparation of (-)- $\alpha$ -ethynyl alcohol moieties of pyrethroid insecticides by lipasecatalysed enantioselective hydrolysis<sup>58</sup>. Hirohara *et al.* also reported the enzymic preparation of optically active alcohols related to synthetic pyrethroid insecticides<sup>59</sup>. In the field of

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pesticide biotechnology, much attention has been focused on the use of lipases as an enantioselective biocatalyst in organic media. The restricted flexibility of microbial lipases in organic solvents paved the way for new possibilities in the production of enantiomeric substances for the pesticide industry<sup>60</sup>.

### ■ BIOPOLYMERS

Lipases have become one of the most important groups of enzymes for their applications in organic syntheses. Lipases can be used as biocatalysts in the production of useful biodegradable compounds. Lipases can catalyse ester syntheses and transesterification reactions in organic solvent systems and have opened up the possibility of an enzyme catalysed production of biodegradable polyesters. Aromatic polyesters can be synthesized by lipase biocatalysis<sup>61</sup>. Lipases are also widely used for the enzymatic polymerization via polycondensation methods. For example *Mucor miehei* in immobilized form induced polycondensation of adipic acid and 1,4- butanediol in diisopropyl ether<sup>62</sup>.

Enantioselectivity for racemic lactones was carried out using lipase-catalyzed ROP of lactones to produce optically active polymers and optically active lactones that remained as unreacted material<sup>63</sup>.

### ■ BIODIESEL PRODUCTION

The use of lipases, however, also extends to the processes of production of biodiesel in which they are used as biocatalysts to catalyze esterification and transesterification reactions that, in an organic environment, become predominant compared to that of hydrolysis which is most favored in aqueous environments. The production of esters of fatty acids derives from these reactions which have biofuel properties, but are of plant origin<sup>64</sup>.

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The use of lipases as catalysts in the production of esters has several advantages compared to the traditional process, which is conducted in an alkaline or acid. Indeed, the process carried out using acid or basic catalysts proves to be considerably more expensive from the energy point of view, the efficiency of the process is reduced due to the formation of secondary reactions, such as saponification, which implies therefore the use of anhydrous reactants to avoid the production of soap. Moreover, a further disadvantage is the difficulty of recovery of the catalyst and its reuse, and especially in obtaining high purity products because of the difficulty of separation of the latter due to the presence of saponified byproducts. The use of the lipase, however, leads to high selectivity and purity, and the use of substrates which are purer and less expensive. Several research groups have reported the production of biodiesel through lipase catalysis<sup>65-68</sup>.

Literature about biodiesel production by immobilized enzyme, shows that most of the researchers have used lipases from different sources for this type of production (**Table 2.2**).

Immobilized *Rhizopus oryzae* cells were used for the production of biodiesel by Hama *et al* A reactor of type "packed-bed" using whole cells of the fungus (30°C) was developed for the production of fuel from the methanolysis of soybean oil<sup>69</sup>. Martín-Hernández *et al* studied enzymatic biodiesel via alcoholysis synthesis for different vegetable oils (sunflower, olive, soya and borage) and used commercial lipases Novozym ® 435 (*Candida antarctica*) and Lipozyme ® TL IM (*Thermomyces lanuginosus*)<sup>70</sup>. Soetaert and Vandamme reported the use of *Mucor miehei* and *Candida antarctica* in the transesterification of various oils using hexane as solvent and found that the lipase from *Mucor miehei* is more efficient in converting primary alcohols (yield between 95% and 98%), whereas lipase from *Candida Antarctica* is better for the conversion of secondary alcohols (yield between 61% and 84%)<sup>71</sup>. Ban *et al.* investigated the enzymatic production of biodiesel fuel from plant oils using *Rhizopus oryzae* cells

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immobilized within biomass support particles (BSP) for the methanolysis of soybean oil<sup>72</sup>. Adachi *et al.* mentioned that an *Aspergillus oryzae* whole-cell biocatalyst which coexpresses *Fusarium Heterosporum* lipase (FHL) a mono- and di-acylglycerol lipase B (mdlB) in the same cell has been developed to improve biodiesel production<sup>73</sup>. Macario *et al.* used Lipase from *Rhizomucor miehei*, immobilized by different route (adsorption, electrostatic binding force and encapsulation in hybrid-liposome nanospheres) to catalyze the transesterification reaction of triolein with methanol to methyl esters, typical biodiesel mixture compounds<sup>74-77</sup>.

**Table 2.2.** Survey of the immobilized lipases used biodiesel production.

Source of Lipase	Immobilization support	Substrate	Reference
<i>T. lanuginosus</i>	Polyurethane foam	Canola oil and methanol	78
<i>C. antarctica</i>	Ceramic beads	Waste cooking oil	79
<i>M. juvanicus, C. rugosa, R. niveus, P. expansum</i>	Silica gel (resin D4020)	Waste oil	80
<i>T. lanuginosus</i>	Microporous polymeric matrix	Sunflower, soybean, waste cooking oil	81
<i>C. rugosa</i>	Chitosan	Rapeseed oil	82
<i>Lipase</i>	Hydrotalcite and zeolite	Waste cooking oil	83
<i>S. cerevisiae</i>	Mg-Al Hydrotalcite	Refined rape oil	84
<i>C. antarctica and Candida sp.</i>	Acrylic resin and textile membrane		85
<i>C. rugosa</i>	Activate carbon	Palm oil	86
<i>P. cepacia</i>	Polymeric matrix	Sapium sabiferum	87
<i>Rhizopus</i>	Polyurethane foam	Soyabean oil	88
<i>P. cepacia</i>	Hydrophobic sol-gel support	Soyabean oil	89
<i>Lipase</i>	Magnetic nanoparticles	Triolein and ethanol	90
<i>Rhizomucor</i>	Zeolites	Waste oil	91
<i>Candida sp.</i>	Fixed bed reactor	Waste cooking oil	92
<i>C. rugosa</i>	Calcium alginate beads	Oil and grease	93
<i>Rhizopus oryzae</i>	Biomass support particles	Jatropha oil	94
<i>Fusarium heterosporum</i>	Biomass support particles	Rapeseed oil	95
<i>Commercial lipase</i>	Macroporous polypropylene	Vegetable oil	96
<i>Thermomyces lanuginosus</i>	Aldehyde lewattit	Ethanol and soyabean oil	97

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## ■ COSMETICS

The overwhelming interest of industrialists in screening lipases for use in the cosmetic and the perfume industry has mainly been due to its activity in surfactants and in aroma production, the main ingredients in cosmetics and perfumes. Immobilized *Rhizomucor miehei* lipase was used by Unichem International (Spain) as the biocatalyst for the production of isopropyl myristate and isopropyl palmitate. The company claims that the use of the enzymatic process produces substance of a much higher quality, requiring minimum downstream refining.

Vitamine A and its derivatives have been used extensively in pharmaceutical products and cosmetics such as skin care products<sup>98,99</sup>. Immobilised *Candida antarctica* was successfully used by Rajasse *et al.* for the acylation of retinol by reverse hydrolysis, alcoholysis, and acidolysis<sup>100</sup>.

## ■ BIOSENSORS

Enzyme-based electrodes represent a major application of immobilized enzymes in medicine. The high specificity and reactivity of an enzyme towards its substrate are properties which are being exploited in biosensor technology. Biosensors possess advantages such as reliability, sensitivity, accuracy, ease of handling, and low-cost compared with conventional detection methods. These characteristics, in combination with the unique properties of an enzyme already mentioned, render an enzyme based biosensor ideal for biomedical applications. In **Table 2.3** some immobilized enzymes used as biosensors are indicated.

By screening various hydrolytic enzymes to fit the special demands, fungal lipases turned out to be the most practical<sup>101</sup>. Many authors have reported the use of *Candida rugosa* lipase biosensor as a DNA probe<sup>102,103</sup>. Kartal *et al.* developed a potentiometric biosensor based on *Candida*

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*rugosa* lipase that was used for the detection of both triglycerides and organophosphorous pesticides<sup>104</sup>.

Lipases may be immobilized onto pH/oxygen electrodes in combination with glucose oxidase, they function as lipid biosensors<sup>105</sup> and may be used in triglycerides<sup>106</sup> and blood cholesterol determinations<sup>107</sup>. Danilov and Egorov<sup>108</sup> reviewed the data on the creation of biosensors with bacterial bioluminescence in medicine. An important analytical use of lipases in the determination of lipids was for clinical purposes<sup>109</sup>. The basic concept was to use a lipase to generate glycerol from the triacylglycerol in the analytical sample and to quantify the released glycerol (or alternatively the nonesterified fatty acids) by a chemical or enzymatic method. As far as physicians were concerned, this principle enabled them to diagnose patients with cardiovascular complaints very precisely.

Krawczyk reviewed analytical applications of the inhibition of enzymatic reactions in the determination of environmental pollutants<sup>110</sup>. Numerous enzymes in immobilized form, including lipase, were used as biosensors, for the determination of fluoride. Wei *et al.* developed a method for the enzymatic determination of organophosphorous pesticides with a surface-acoustic-wave impedance sensor, which was based on lipase-catalyzed hydrolysis<sup>111</sup>.

Much remains to be done by enzyme technologists and biomedical engineers to achieve developments in this area.



**Table 2.3.** Survey of immobilized enzyme used as biosensor for the detection of some special compounds, pesticides and heavy metals.

Enzyme	Inhibitors	Immobilization matrix	Samples	References
<b>Biosensor for the determination of pesticides</b>				
Acetylcholinesterase	Paraoxon	Multiwell carbon nanotubes	Real water sample	112
Acetylcholinesterase	Paraoxon	Entrapment in TCNQ-graphite	Orange water sample	113
Acetylcholinesterase	Paraoxon	Multiwell carbon nanotubes	Spiked	114
Variants	Carbofuran	Polymer	River water sample	
Butyrylcholinesterase	Chloropyrifos-methyl, coumaphos, carbofuran	Cross-linking with BSA in GA vapour	Spiked grape juice	115
Choline oxidase	Pirimiphos-methyl	PB-SPE surface	Durum wheat	116
Parathion hydrolase	Parathion	CPE surface	Spiked river water	117
Catalase	Azide	Gelatine with GA	Fruit juice	118
<b>Biosensor for the determination of heavy metals</b>				
Urease	Hg <sup>2+</sup> , Cu, Cd	Entrapment in sol-gel matrix	Tap and river water	119
Urease	Hg (NO <sub>3</sub> ) <sub>2</sub> , phenyl mercury, HgCl <sub>2</sub> , Hg <sub>2</sub> (NO <sub>3</sub> ) <sub>2</sub>	Entrapment in sol-gel film	Water sample	120
Glucose oxidase	Hg <sup>2+</sup>	Cross-linking with GA and BSA	Spiked water	121
Glucose oxidase	Chromium (IV)	Cross-linking with GA and covering with alanine membrane	Soil sample	122
<b>Biosensor for the determination of the other chemical components</b>				
Butyrylcholinesterase	$\alpha$ - chaonine, $\alpha$ - solanine, solanide	Cross-linking with GA vapour	Potatoes	123
Butyrylcholinesterase	$\alpha$ - chaonine, $\alpha$ - solanine	Cross-linking with BSA and GA vapour	Agriculture	124
Butyrylcholinesterase	Tomatite	Cross-linking with BSA and GA	Tomatoes	125
Tyrosinase	Benzoic acid	Mixture of graphite, tyrosinase and teflon	Mayonnaise sauce, cola soft drink	126
Acetylcholinesterase	Anatoxin-a	Entrapment in PVA-SbQ	Fresh water	127
Glutathione S transferase	Captan	Entrapment in sodium alginate gel	Contaminated water	128

BSA: Bovine Serum Albumine, GA: Glutaraldehyde, PVA-SbQ: Polyvinyl Alcohol bearing Styryl pyridium group, PB-SPE: Prussian Blue Screen Printed Electrode, TCNQ: 7,7,8,8-tetracyanoquinone diaminomethane.

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## ■ BIOMEDICAL AND PHARMACEUTICAL APPLICATION

Because of their excellent capability for specific region-selective reactions in a variety of organic solvents with broad substrate recognition, lipases have emerged as an important biocatalyst in biomedical applications. Recently, Parmar *et al.* reviewed a variety of substrates accepted by hydrolytic enzymes, including lipases, to produce compounds in high enantiomeric excess, which can be used as chiral building blocks for the synthesis of compounds of pharmaceutical interest<sup>129</sup>. There are other reports regarding the application of microbial lipases to the hydrolysis of racemic esters, to transesterification and to racemization *in situ* to yield optically pure enantiomers for the manufacture of chiral synthons<sup>130,131</sup>. Efficient kinetic resolution processes are in vogue for the preparation of optically active homochiral intermediates for the synthesis of nikkomycin-B, non-steroidal anti-inflammatory drugs (naproxen, ibuprofen, suprofen and ketoprofen), the potential anti-viral agent lamivudine (which can be used to combat HIV) and for the enantiospecific synthesis of anti-tumour agents, alkaloids, antibiotics and vitamins<sup>132,133</sup>. During the past decade, many studies have shown that racemic drugs usually have the desired therapeutic activity residing mainly in one of the enantiomers and the other enantiomers might interact with different receptor sites, which can cause unwanted side effects. *Aspergillus niger* and *Penicillium urticae* lipases were highly enantiospecific, which frequently cleaved undesired enantiomers; *Candida rugosa* lipase, however, was highly (S)-specific<sup>132,134</sup>. Lipases from species of *Mucor* and *Rhizopus* were also (S)-specific with low enantiospecificity<sup>130,135</sup>. *Pseudomonas cepacia* lipase was found to be equally active towards both isomers<sup>136</sup>.

Profens, a class of nonsteroidal anti-inflammatory drugs, are active in the (s)-enantiomer form. Esterification of the profens is an effective way to prepare the prodrug, since the organism is rich in enzymes capable of hydrolyzing the resultant ester<sup>137</sup>. With a thoroughly neat selection of the

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alcohol moiety, the resultant ester prodrug can provide bioactivity comparable to that of the parent drug<sup>138-140</sup>.

Goto *et al.* also studied the enzymatic resolution of racemic ibuprofen by surfactant-coated lipases in organic media<sup>141</sup>. Enzymatic esterifications were performed in dry homogeneous organic media, and the effects of lipase origin (*M. miehei*, *C. rugosa*, *P. fluorescens*, *P. cepacia* and *A. niger*), alcohol alkyl chain length and reaction medium reactivity were investigated.

Xie *et al.* synthesized pure (S)-ibuprofen using *Candida rugosa* lipase catalyzed kinetic resolution via esterification<sup>142</sup>.

*Aspergillus niger* AC-54 has the great capability of preferably esterifying (R)-ibuprofen. This shows the best results in the esterification of the racemate using 1-propanol as an esterifying agent; isooctane as a solvent, 7% M/V of enzyme and a molar ratio propanol:ibuprofen equal to 2.41:1<sup>143</sup>. Also *Candida rugosa* lipase showed good selectivity in the resolution of (R,S)-ibuprofen<sup>144</sup>.

Enantioselective resolution of ibuprofen by Lipozymet IM20 (commercial *Rhizomucor miehei* lipase) has been carried out using isooctane as a solvent and butanol as an esterifying agent. Under these conditions, enantiomeric excess and total conversion values were 93.8% and 49.9%, respectively, and the enantioselectivity was 113 after 112 h of reaction. These conditions have been considered in the design of a continuous reactor to scale up the process<sup>145</sup>.

An enantioselective esterification process was developed for the synthesis of 2-N-morpholinoethyl (S)-ibuprofen ester prodrug from racemic ibuprofen by using *Candida rugosa* lipase immobilized on Accurel MP1000 in cyclohexane. Compared with the performance of Lipase MY, the immobilized lipase possesses a higher enzyme activity and thermal stability, but with a slightly suppressed enantioselectivity<sup>146</sup>.

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The enantiomers of glycidol and its derivatives have been widely used as intermediates for the synthesis of many interesting compounds, such as anticancer drugs, protein synthesis inhibitors and an antibacterial agent<sup>147</sup>. In the last year, boron-containing compounds have proved extremely useful considering that both achiral and chiral boron-compounds are used as building blocks in organic synthesis<sup>148</sup>. In addition, this compound has been used in important biological applications for example as new agents for cancer therapy.

Other important compounds are applied in organic synthesis, such as drug intermediates, and they are optically active. They are also used in peptide and lactam synthesis<sup>148</sup>. For example *Aspergillus niger* and *Rhizopus oligosporus* are used in the resolution of (R,S)-phenylethylamine with n-heptane or n-hexane. R-2b amide was obtained with conversion from 6 to 99%<sup>149</sup>.

Microbial lipases are also used in the synthesis of chiral intermediates for anticancer drugs, antiviral agents,  $\beta$ 3-receptor agonists, antihypertensive drugs, melatonin receptor agonists, anti-cholesterol drugs, and anti-Alzheimer's drugs. In the case of anticancer drugs what appears more interesting is the use of immobilized *Pseudomonas cepacia* and BMS lipase (extracellular lipase derived from the fermentation of *Pseudomonas* sp.SC13856) on accrual polypropylene for the enzymatic resolution processes for the preparation of chiral C-13 paclitaxel side-chain<sup>150</sup>. This is a precursor for the paclitaxel semi-synthetic process. The Paclitaxel is the only compound known to inhibit the depolymerization process of micro tubulin. Various types of cancers have been treated with paclitaxel and the results in the treatment of ovarian cancer and metastatic breast cancer are very promising.

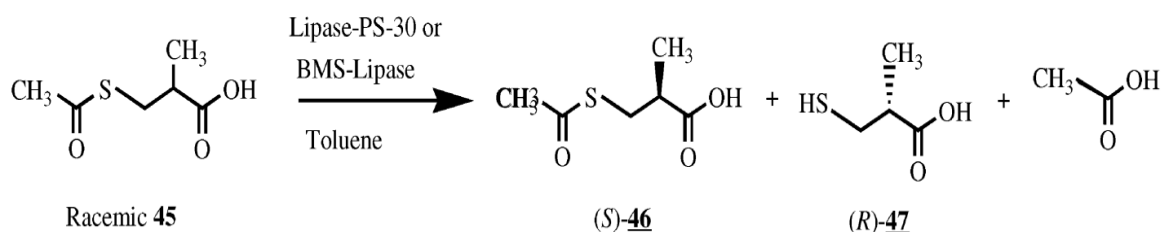
Recently, importance has been given to the use of Lobucavir as a antiviral agent for the treatment of herpes virus and hepatitis B<sup>151</sup>. Regioselective amino acylation is difficult to achieve by chemical

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procedures, however, it appeared to be suitable with an enzymatic approach. Enzymatic processes were developed for aminoacylation of either hydroxyl group of lobucavir<sup>152</sup>. Captopril is designated chemically as 1-[(2*S*)-3-mercapto-2-methylpropionyl]-l-proline. It is used as an antihypertensive agent through suppression of the renin–angiotensin–aldosterone system. Captopril prevents the conversion of angiotensin I to angiotensin II by inhibition of ACE.

The action of captopril as an inhibitor of ACE depends on the configuration of the mercapto alkanoyl moiety; the compound with the *S*-configuration is about 100 times more active than its corresponding *R*-enantiomer<sup>153</sup>. The required 3-mercapto-(2*S*)-methylpropionic acid moiety has been prepared from the microbially derived chiral 3-hydroxy-(2*R*)-methylpropionic acid, which is obtained by the hydroxylation of isobutyric acid<sup>154</sup>. The synthesis of the chiral side-chain of captopril by the lipase-catalyzed enantioselective hydrolysis of the thioester bond of racemic 3-acetylthio-2-methyl propanoic acid **45** to yield (*S*)-**46** has been demonstrated<sup>155</sup>. Lipase PS-30 from *P. cepacia* in an organic solvent catalyzed the hydrolysis of the thioester bond of undesired enantiomer of racemic 3-acetylthio-2-methyl propanoic acid **45** to yield the desired (*S*)-**46**, (*R*)-3-mercapto-2-methylpropanoic acid **47** and acetic acid.

The reaction yield >24% (theoretical maximum is 50%) and ee>95% were obtained for (*S*)-**46** using each lipase (**Figure 2.11**).



**Figure 2.11.** Synthesis of chiral side-chain of captopril by the lipase-catalyzed enantioselective hydrolysis of the thioester bond of racemic 3-acetylthio-2-methyl propanoic acid **45** to yield (*S*)-**46**

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Another ACE inhibitor is Ceranopril which requires chiral intermediate carbobenzoxy(Cbz)-l-oxyllysine.

Hanson et al developed a biotransformation process to prepare the Cbz-l-oxyllysine. *N*- $\epsilon$ -Carbobenzoxy(Cbz)-l-lysine was first converted to the corresponding keto acid by oxidative deamination using cells of *Providencia alcalifaciens* SC 9036 which contained l-amino acid oxidase and catalase. The keto acid was subsequently converted using l-2-hydroxy-isocaproate dehydrogenase from *Lactobacillus confusus*, and NADH. The reaction yield of this process is 95% with 98.5% of ee<sup>152</sup>.

Many microbial enzymes have been used in the reduction of a single keto group of  $\beta$ -keto or  $\alpha$ -keto compounds<sup>156,157</sup>. The enantioselective reduction of the diketone ethyl-3,5-dioxo-6-(benzyloxy) hexanoate to the diol ethyl-(3*R*,5*S*)-dihydroxy-6-(benzyloxy) hexanoate has been demonstrated<sup>158</sup>. This compound is a key intermediate in the synthesis of [4-[4*a*,6 $\beta$ (*E*)]-6-[4,4-bis(4-fluorophenyl)-3-(1-methyl-1*H*-tetrazol-5-yl)-1,3-butadienyl]-tetrahydro-4-hydroxy-2*H*-pyren-2-one, a potential new anticholesterol drug which acts by inhibition of HMG CoA reductase<sup>159</sup>. Among various microbial cultures evaluated for the enantioselective reduction of diketone ethyl-3,5-dioxo-6-(benzyloxy) hexanoate, glycerol-grown cell suspensions of *Acinetobacter calcoaceticus* SC 13876 were shown to give a reaction yield of 85% and ee of 97%.

$\beta$ 3-Adrenergic receptors are found on the cell surface of both white and brown adipocytes and are responsible for lipolysis, thermogenesis, and the relaxation of the intestinal smooth muscle. Consequently, several research groups are engaged in developing selective  $\beta$ 3 agonists for the treatment of gastrointestinal disorders, type II diabetes, and obesity<sup>160,161</sup>.

An important chiral intermediate for the synthesis of several potential anti-Alzheimer's drugs is the (S)-2-pentanol that inhibit beta-amyloid peptide release and/or its synthesis. Patel have studied the enzymatic resolution of racemic 2-pentanol by lipase B from *Candida*

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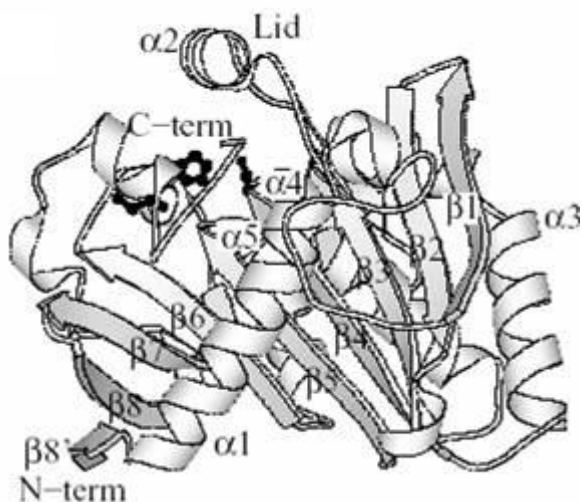
antarctica<sup>162</sup>. These lipases were screened for the enantioselective acetylation of racemic 2-pentanol in hexane using vinyl acetate as an acyl donor. *C. antarctica* lipase B efficiently catalyzed this reaction giving yields of 49% and 99% of ee for (*S*)-2-pentanol.

## 2.6 LIPASE FROM RHIZOMUCOR MIEHEI

The *Rhizomucor miehei* is a globular protein which, as demonstrated by crystallographic studies<sup>163</sup>, is composed of 2289 carbon atoms with hydrogen, of which 230 contain oxygen bound with water. This type of lipase belongs to the family of  $\alpha / \beta$  hydrolase.

Few and well done works, published in the 90's years, report on the determination of the 3D-structure of this globular and complex protein<sup>163-165</sup>.

They demonstrated that Lipase from *Rhizomucor miehei* possesses  $\alpha$ -helix and  $\beta$ -sheet secondary structures (**Figure 2.12**).



**Figure 2.12.** Tertiary structure of the *Rhizomucor miehei* Lipase<sup>166</sup>

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The RML contains eight principal sheets conformation  $\beta$  and an additional lap shorter than the other (short strand  $\beta$  8'). With the exception of the last round (8'), all others are sequential and connected together by both loops hairpin-shaped conformation  $\beta$  (technically called  $\beta$ -hairpin loops) that were carried out by dextrorotation involving reasons  $\beta$  -  $\alpha$  -  $\beta$ . Every single polypeptide chain so shaped consists of 269 amino acid residues.

The tertiary structure of RML is very asymmetrical. There is a  $\alpha$ -helical N-terminal folded in a perpendicular position with respect to the  $\beta$ -sheet. The propeller position is stabilized by a short chain N-terminal pentapeptide that forms an extra lap conformation  $\beta$  (8') linked via a hydrogen bridge to the C-terminal of the polypeptide chains of the eight principal laps (major strands). Finally, the fold of the polypeptide chains in the RML is stabilized by three bridges between these sulphide residues and their positions (Cys = Cystidine):

Cys40-Cys43

Cys29-Cys268

Cys235-Cys244

Only the disulfide bond 235-244 is internal and not accessible to the molecule; the other two can be accessible to solvents.

The structure shown in **Figure 2.12** as well as being the tertiary structure of the enzyme in question, is also its "closed form". In fact, there is a further  $\alpha$ -helix that covers the catalytic center when the enzyme is in its closed form. RML in the active site is thus located in a shallow groove covered by this  $\alpha$ -helix called "lid" when the enzyme is in its closed conformation. This  $\alpha$ -helix comprises residues 85 to 91. The arrangement of these elements and their composition is the basis of the mechanism of



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activation of the RML<sup>167</sup>. The area of the lid hinge is represented by the amino acid Arginine (Arg), which in addition to being a positively charged amino acid has also a basic behavior (see Appendix A). The electrostatic interactions between the lid and the substrate can influence and stabilize the opening of the lid, and thus the enzyme activity. The concept that involves electrostatic interactions between one or more residues of the hinge lid and another molecule is the subject of relatively recent studies<sup>76,168-170</sup>.

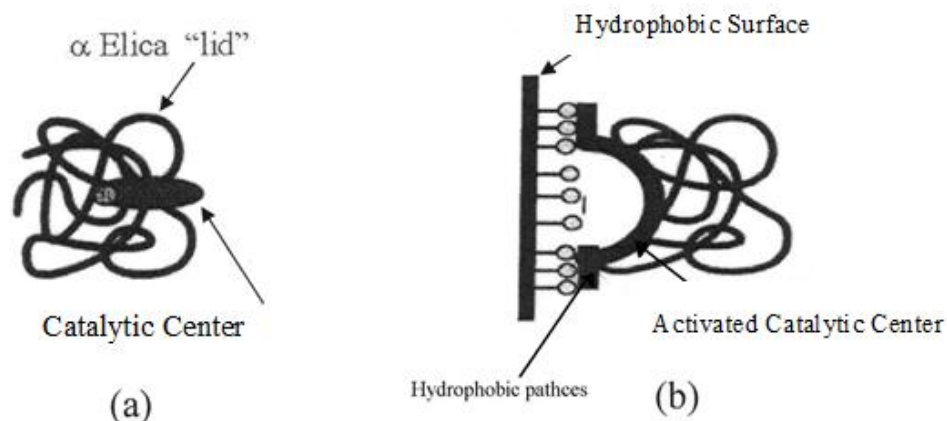
In particular, for the family of the *Rhizomucor miehei* lipase, the key residues responsible for possible electrostatic interactions are the Arg80 (Arg = Arginine) and Asp91 (Asp = Aspartic Acid), which are not linked via the hydrogen bridge to any other residue of the molecule of the enzyme.

The catalytic triad is formed by Ser 144, Asp 203 and His 257 (His = Histidina). RML forms a central  $\beta$ -sheet which contains the Ser 144 at the end of one part of the sheet. This active site is covered by the small  $\alpha$ -helix in the upper left part of the picture. This helix includes the residues 84 to 92. The three serine residues (Ser 82-84) and the four residues (92-95) placed at the ends of the helix form a hinge, so that the helical part (Leu 85-Asp 91) can move as a rigid body in the course of the activation process. The Trp 88 of that helix is labeled in **Figure 2.13**.



**Figure 2.13.** "Ribbon-model" of the main chain of the RML<sup>171</sup>

In particular, the hydrogen bridge between the carboxyl group of aspartic acid of the catalytic triad and the Histidine is very stable and involves both oxygens<sup>172</sup>. The interactions, on the other hand, that involve the lid with the rest of the body of the protein are only of a hydrophobic type. These interactions lead to the closed conformation of the enzyme in its native form (**Figure 2.14 (a)**). At the interface between the lipids and the water, there is the activation of the enzyme (**Figure 2.14 (b)**) caused by the opening of the lid, and the exposure of the heart and the hydrophobic catalytic lipophilic element towards fats decomposes, with which the enzyme comes into contact through absorption and covers the active site of the lipase.



**Figura 2.14.** (a) Native form of RML (closed form) – (b) Active form of RML (open form) <sup>166</sup>

Recent studies have allowed us to understand the composition of the residues of the zone involved during the phase of activation of the lipases<sup>167</sup>. These residues, defined "hydrophobic pieces" (or hydrophobic paths), represent the amino acids that are directly involved in hydrophobic interactions with the substrate (or with a hydrophobic surface).

Finally, most of the proteins contain water molecules within them and are strongly linked to the peptide chains by polar forces. These water molecules are part of the structure of the enzyme and an exchange of some of these molecules can lead to changes in the secondary structure of the elements where such molecules are linked causing, therefore, intermediate conformations of the enzyme itself.

Therefore, the charge distribution on the enzyme surface, influenced by the composition of the outer lid, the position of the water molecules and the presence of hydrophobic forces are the three main causes of conformational change of the enzyme and can, therefore, influence both its adsorption on the surface of a support as its final activity.

Finally, the extent of any electrostatic interactions between the lid and the surface of the enzyme adsorption can even stabilize the open form, and thus activate, the enzyme itself.

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## 2.7 RHIZOMUCOR MIEHEI LIPASE AS THE CATALYST IN THE RESOLUTION OF A CHIRAL COMPOUND.

In modern literature, many papers<sup>173-176</sup> and books<sup>177-180</sup> have been devoted to the use of enzymes in organic synthesis.

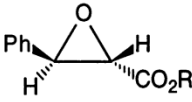
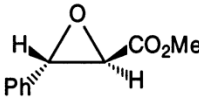
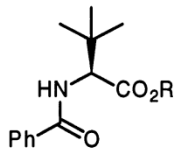
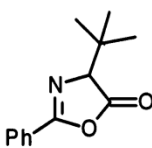
On focusing this topic on lipases, depending on the medium employed, it is evident that these enzymes are able to catalyze both the hydrolysis of esters and acyl-transfer reactions, such as esterifications, transesterifications, interesterifications (ester and acid), and to transfer acyl groups from esters to other nucleophiles such as amines, thiols or hydroperoxides. In this paragraph, we would like to focus on the possibility of using the lipase-catalyzed acyl transfer methodology as a method for the kinetic resolution of enantiomers. In this sense, obtaining homochiral compounds is one of the most exciting fields of present day research into lipase catalysis, because these optically pure compounds are very important as building blocks in Asymmetric Synthesis<sup>181-185</sup> or in the pharmaceutical industry due to the fact that the different activity of both enantiomers is a real problem, because there are more than 500 drugs currently marketed as racemic mixtures, with negligible information available about the properties of the individual stereoisomers.

Lipase catalyzed enantiomer differentiating hydrolysis of acylated racemic alcohols has only been used to a minor extent employing *Rhizomucor miehei* lipase as a catalyst. Some examples are described in the literature and lead to obtaining good enantioselectivities with *Rh. miehei* lipase: thus, the enzymatic deacylation of *meso*-(1*R*, 2*R*, 3*S*, 4*S*)-1, 2, 3, 4-tetracetoxy-5-cyclohexane and *rac*-1, 2, 3, 4-tetracetoxy-5-cyclohexane with *n*-butanol was reported using Lipozyme<sup>186,187</sup>, the regioselectively deacylated products being useful as starting materials for the synthesis of different biologically-active compounds.

Some examples of *Rhizomucor miehei* lipase-catalyzed resolution of chiral acids via enantioselective esterification or transesterification are shown in **Table 2.4**.

The kinetic resolution of methyl *trans*-*b*-phenyl glycidate<sup>188</sup> makes it possible to obtain the (2*S*, 3*R*) compound with better yields and enantioselectivities than other enzymes tested (lipases of *Pseudomonas* sp., *Candida cylindracea*, pancreatic porcine lipase or *Humicola lanuginosa*) (**number 1** in **Table 2.4**). Other interesting uses of *Rhizomucor miehei* lipase are the opening of lactone rings: for instance, this lets us indicate the alcoholysis of 2-phenyl-4-*tert*-butyloxazoline-5-(**4H**)-one in order to obtain *N*-benzoyl-L-*tert*leucine butyl ester, where hydrolysis leads to the synthesis of L-(*S*)-*tert*leucine, a non proteinogenic chiral  $\alpha$ -amino acid used as a chiral auxiliary or as a component of potentially therapeutic pseudopeptides<sup>189</sup> (**number 2** in **Table 2.4**).

**Table 2.4** *Rhizomucor miehei* lipase catalyzed enantioselective esterification and transesterification of racemic acids

Entry	Product	Remaining substrate	Enzyme	Ref.
1	 <i>n</i> -butanol or <i>i</i> -butanol 33-51% yield, 46-77% ee (E=20-114)		Lipase M-AP-10 from Amano	188
2	 <i>n</i> -butanol, ethanol or methanol, 30-67% yield, 39-99% ee E=3->100		<i>Lipozyme</i> from Novo- Nordisk	189

Nevertheless, the most widely used substrates for resolution are 2-arylpropionic acids, an important group of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs). Among the racemic drugs, 2-arylpropionic acids

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(APA's, the "profen" family) constitute an important group of nonsteroidal anti-inflammatory drugs (NSAIDs), which are widely used as racemic mixtures to control the symptoms of arthritis and related connective tissue diseases<sup>190</sup>. However, it is well documented that only the S-(+) enantiomer is pharmacologically active, while only a certain portion of the R-(-) enantiomer could be transformed into the S-(+) isomer by *in vivo*<sup>191</sup> metabolic inversion.

A systematic study of the enantioselective resolution of ibuprofen by commercial *Rhizomucor miehei* lipase (Lipozyme IM 20: lipase from *Rhizomucor miehei* immobilized on Duolite A568 ) has been carried out using 10 mg Lipozyme per millilitre of reaction mixture; isooctane as solvent (10 mL) and butanol as esterifying agent<sup>192</sup>. The main variables controlling the process (temperature, ibuprofen concentration, ratio butanol:ibuprofen) have been studied using an orthogonal full factorial experimental design, in which the selected objective function was enantioselectivity. This strategy has been resulted in a polynomial function that describes the process. By optimizing this function, optimal conditions for carrying out the esterification of racemic ibuprofen have been determined: temperature = 40°C.; concentration of ibuprofen equal to 50 mM; ratio butanol:ibuprofen equal to 1.9. The optimal ibuprofen concentration was 50 mM, which was the maximum value tested. When experiments with higher values of ibuprofen concentration were carried out, the enantioselectivity maintained values over 100, but the conversion did not reach values over 25% in any case. So, these optimal conditions (40°C, 50 mM and 1.9) have been chosen for the esterification of ibuprofen using Lipozyme as biocatalyst. Under these conditions, enantiomeric excess and total conversion values were 93.8% and 49.9%, respectively, after 112 h of reaction. These conditions have been considered in the design of a continuous reactor to scale up the process. A packed bed reactor is the most appropriate reactor. The stability of the system (up to 100 h) and the

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possibility of reutilization of the enzyme (up to four times) lead to consider this reactor as a suitable configuration for scale up of the process.

A study of the enzymatic activity of immobilized lipase from *Rhizomucor miehei* (Lipozyme IM: Lipase of *Rhizomucor miehei*, immobilized on a macroporous ion-exchange resin) in the enantioselective esterification of 2-arylpropionic acids has been carried out by Lopez-Belmonte *et al.*<sup>193</sup> The main variables controlling process (temperature, type of organic solvent, and type of alcohol moieties) have been studied. The optimal condition for carrying out the esterification of racemic ibuprofen have been determined: 300 mg of Lipozyme IM, Ibuprofen : 1-butanol molar ratio =1:1; Temperature = 37°C and cyclohexane as a solvent. Lipozyme IM shows S-(+) enantioselectivity in all case and under optimum condition the enantiomeric excess of remaining acid and the yield in ester values were 98 and 64 % respectively, after 48 hours of reaction in cyclohexane<sup>193</sup>.

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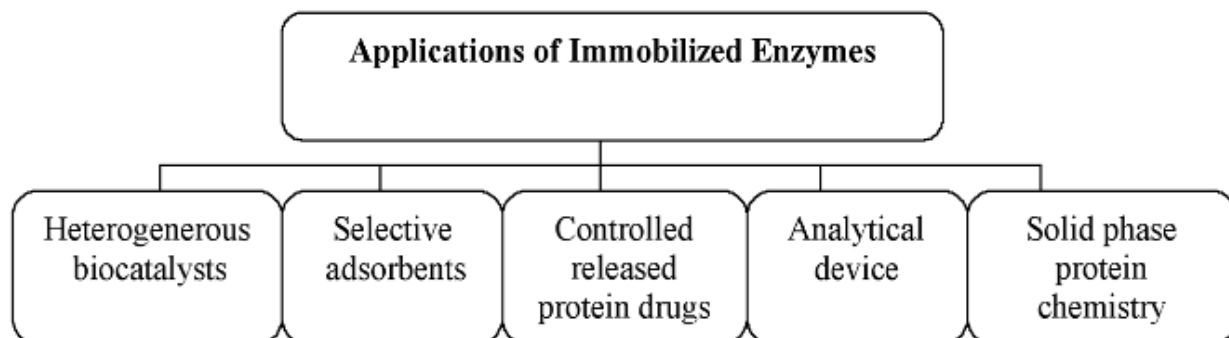
# **CHAPTER III**

## ***Immobilized Lipase***

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### 3.1 HISTORICAL BACKGROUND

Since the second half of the last century numerous efforts have been devoted to the development of immobilized enzymes for a variety of applications<sup>1</sup> (**Figure 3.1**)



**Figure 3.1** Range of application of immobilized enzymes.

Initially, immobilization techniques were mainly used to prepare adsorbents for isolation of proteins by immunologists, via adsorption on simple inorganic carriers such as glass<sup>2</sup>, alumina<sup>3</sup> or hydrophobic compound-coated glass<sup>4</sup>. Along with these prototypes a few irreversible immobilized enzymes prepared by covalent attachment were also reported in the literature<sup>5</sup>. Although in 1950s the method of enzyme immobilization was still dominated by physical methods, i.e. non-specific physical adsorption of enzymes or proteins on solid carriers, for example  $\alpha$ -amylase adsorbed on activated carbon, bentonite or clay<sup>6</sup>, AMP(Adenosine monophosphate) deaminase on silica<sup>7</sup>, the method of adsorption was gradually switched from simple physical adsorption to specific ionic adsorption, for instance, catalase on the ionic resin DEAE-cellulose<sup>8,9</sup>, lipase and catalase on styrenepolyaminostyrene (Amberlite XE-97)<sup>10</sup>. Along with physical methods of enzyme immobilization, however, other

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important methods of enzyme immobilization, for example covalent immobilization, were further investigated<sup>10-13</sup>.

Apart from the physical adsorption and covalent immobilization, it was demonstrated for the first time by Dickey that some enzymes entrapped in the sol-gel inorganic matrix formed by silicic acid derived glasses retained reasonable biological activity<sup>7</sup>. Subsequently attention was focused on no-covalent enzyme immobilization, i.e. adsorption and entrapment<sup>14</sup>. Enzyme entrapment techniques were also further extended by the use of synthetic polymeric gels<sup>15</sup> of natural polymer derivatives, such as nitrocellulose, starch, silicon elastomers, for the sol-gel process<sup>16,17</sup>.

Other techniques of enzyme immobilization, for example adsorptive cross-linking of enzymes on films and membranes, or beads for the formation of enzyme envelopes, were also developed<sup>18</sup>. The enzymes studied changed from a few classic enzymes to a broad range of enzymes such as galactosidase, urease<sup>19</sup>, chymotrypsin<sup>20</sup>, lactate dehydrogenase<sup>21</sup>, catalase, peroxidase<sup>22</sup>,  $\alpha$ -amylase<sup>23</sup>, etc, which were expected to have great potential application in chemical, pharmaceutical and medical industrial sectors. At the same time, there was increasing interest in the physical and chemical nature of the carriers (their hydrophilic or hydrophobic nature and their charges), the binding chemistry strongly dictated the catalytic characteristics of the enzyme, such as its activity and stability<sup>23-30</sup>.

### **3.2 ADVANTAGE OF IMMOBILIZED ENZYME.**

Immobilization makes possible to overcome the problems related to the use of enzymes in their native form, thereby creating numerous advantages. First of all, the immobilized technique allows to use enzyme in more operating cycles, both in batch and in continuous flow, recovering the heterogeneous catalyst at the end of the reaction. In addition, the immobilization technique forces the enzyme into a well defined position inside the environment of the reaction, so the medium of the reaction does

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not limit the catalytic activity of the enzyme. Sometimes the immobilization generates on "activated" enzyme that result much more active with respect to its native and free form. This aspect is often observed from same Lipases<sup>31</sup>. Moreover, there are other advantages related to enzymes, such as the increase of selectivity, the possibility to work in mild reaction conditions (room temperature and neutral pH). These aspects facilitate the use of enzymes in processes on an industrial scale, by reducing or eliminating the recovery operations and also decreasing the cost of the process through the possibility of reusing the enzyme and increasing the productivity of the process.

To evaluate critically the performance of immobilized enzyme, the following parameters have to be considered. *Catalytic activity* is usually important to achieve a high substrate conversion, so that the enzyme is used as efficiently as possible. This parameter is influenced to a large extent by the method of immobilization. *Product final yield* is an important parameter for practical applications. that is not much often influenced by the immobilization procedure, and it is therefore not so relevant for the evaluation of immobilized preparations in most cases. *Enzyme Operation stability* is often of crucial importance, especially for large-scale applications. Good stability of enzyme is required to achieve high productivity.

### **3.3 CHOICE OF THE SUPPORT**

The characteristics of the matrix are of paramount importance in determining the performance of the immobilized enzyme system. Ideal support properties include physical resistance to compression, inertness and biocompatibility toward enzymes; resistance to microbial attack and availability at low cost<sup>29,30</sup>. According to their chemical composition,

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supports can be classified as inorganic and organic (**Table 3.1**), and the organic supports can be subdivided into natural and synthetic polymers<sup>32</sup>.

**Table 3.1.** Classification of enzyme supports<sup>32</sup>

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<b>Organic</b>
Natural polymers
<ul style="list-style-type: none"><li>• Polysaccharides :cellulose, dextrans, agar, agrose , chitin, alginate</li><li>• Proteins: collagen, albumin</li><li>• Carbon</li></ul>
Synthetic polimers
<ul style="list-style-type: none"><li>• Polystyrene</li><li>• Other polymers: polycrilate polymethacrylate, polyacrilamide, polyamides, vinyl, and allyl-polymers</li></ul>
<b>Inorganic</b>
Natural minerals: bentonite, silica
Mesoporous material; zeolite
Processed material: glass (nonporous and controlled pore), metals, controlled pore.

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The physical characteristics of the matrices are very important for the performance of the immobilized systems and determine the type of reactor used under technical conditions. In particular, pore structure and particle size determine the total surface area available to enzyme and thus critically affect the capacity for binding of enzymes. Porous supports are generally preferred because high specific surface area allows a higher enzyme loading and the immobilized enzyme receives greater protection from the environment. Porous supports should have a controlled pore distribution in order to optimize capacity and flow properties. In spite of the many advantages of inorganic carriers (high stability against physical, chemical and microbial degradation), most of the industrial applications are performed with organic matrices. The hydrophilic character is one of the

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most important factors determining the level of activity of an immobilized enzyme<sup>33</sup>. The hydrophilicity of the support gives the dispersion and contact with the enzyme. Sometimes it cannot permit any immobilization of the enzyme, because of the preferential adsorption of H<sub>2</sub>O respect to the enzyme. It is necessary a good compromise between hydrophobicity and hydrophilicity.

### **3.4 METHODS OF ENZYME IMMOBILIZATION**

More than 5000 publications, including patents, have been published on enzyme immobilization techniques<sup>34,35</sup>. Several hundred enzymes have been immobilized in different forms and approximately a dozen immobilized enzymes, for example amino acylase, many lipases, proteases, nitrilase, amylase, invertase, etc., have been increasingly used as indispensable catalysts in several industrial processes. Although the basic methods of enzyme immobilization can be categorized into a few different methods only, for example adsorption, covalent bonding, entrapment, encapsulation, and cross-linking<sup>36</sup>, hundreds of variations, based on combinations of these original methods, have been developed<sup>37</sup>.

One way of classifying the various approaches to immobilizing enzymes is in two broad categories: irreversible and reversible methods<sup>38</sup>.

#### **3.4.1 METHODS OF IRREVERSIBLE ENZYME IMMOBILIZATION**

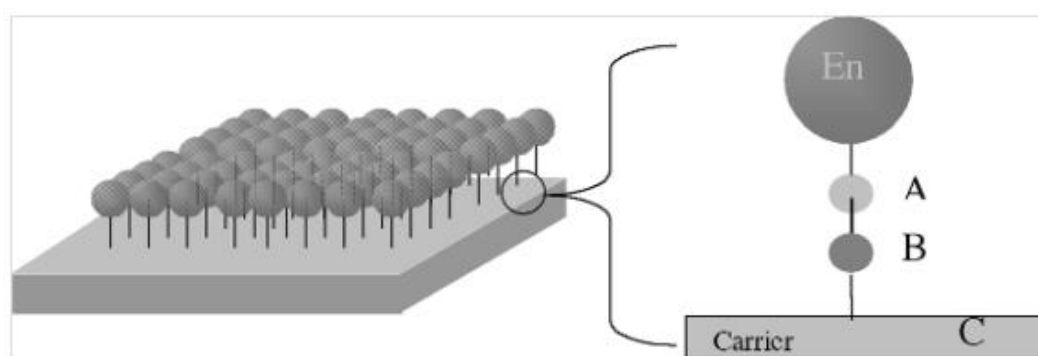
Irreversible immobilization means that once the biocatalyst is attached to the support it cannot be detached without destroying either the biological activity of the enzyme or the support. The most common procedures of irreversible enzyme immobilization are:

- covalent coupling
- entrapment or micro-encapsulation



### 3.4.1.1 COVALENT ENZYME IMMOBILIZATION

Covalent immobilization involves the formation of covalent bonds between the enzyme and the support which can be organic or inorganic, natural or synthetic. In general, covalent bonding of an enzyme to a carrier is based on the chemical reaction between the active amino acid residues (A) located on the enzyme surface and the active functionalities (B) that are attached to the carrier surface (C), or vice versa, as illustrated in **Figure 3.2**.



**Figure 3.2.** Covalent immobilization of enzyme on the carrier: (A) active amino acid residue; (B) binding functionality of the carrier; (C) carrier<sup>39</sup>.

The main advantage is the nature of the covalent immobilization bond, which makes the interaction between the enzyme and the matrix resistant to various treatments such as changes in pH, temperature, ionic strength and concentration of substrate in the reaction medium. The disadvantage is that only small amounts of enzyme can be loaded on the support, because many factors contribute to the formation of this bond. In addition, the alteration of the enzyme structure can occur as a result of the process of conjugation. The enzyme can form more than one covalent bond with the substrate, so, it is locked in a non-productive conformation. For

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example, it could find itself in a position which makes the active site inaccessible to substrates and products. The presence of high amounts of enzyme in non-catalytic conformations leads to a great decrease in the activity after the immobilization<sup>39</sup>.

The following properties are expected to affect the performance of a covalently carrier-bound immobilized enzyme:

- physical nature of the carrier (pore size, particle size, porosity, shape, etc.);
- chemical nature of the carrier ;
- the nature of the linkage or binding chemistry;
- the conformation of the enzyme after immobilization;
- enzyme orientation;
- the nature and length of the spacer between enzyme and support;
- the properties of the medium used for binding the enzymes;
- the number of bonds formed between the enzyme and the carrier;
- enzyme distribution on or within the carriers

Immobilization is sometimes carried out in the presence of substrates, substrate analogues or inhibitors, which interact reversibly with the active site, thereby preventing covalent modification in this region. In the case of lipases it might be an advantage to carry out immobilization under conditions favoring the open form after immobilization. There are several commercially available activated support materials intended for covalent enzyme immobilization. Some of them contain epoxy groups which are intended to react mainly with the amino groups of the enzyme to be immobilized. This can be done by mixing the material directly with an

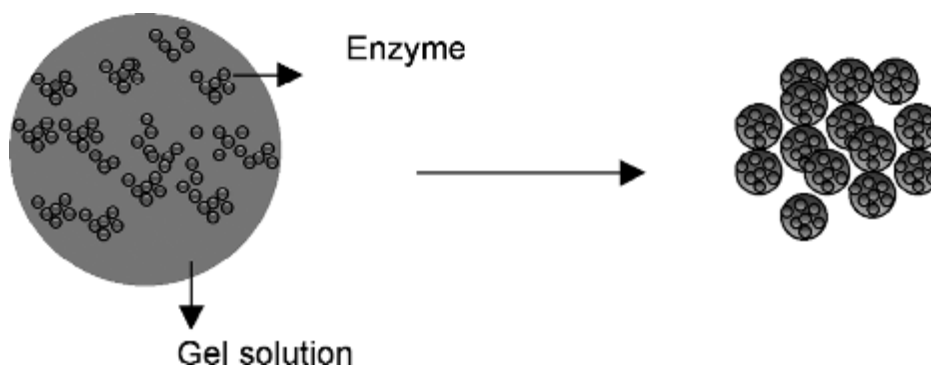
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aqueous solution of the lipase. Interesting new developments in the area of covalent lipase immobilization include immobilization in nanoparticles. For example, immobilization in a polyacrylamide nanogel was achieved by covalent acrylation of *Candida rugosa* Lipase (CRL) using N-acryloxysuccinimide as reagent, followed by the addition of acrylamide and the induction of polymerisation. The lipase-containing nanogel obtained, posses higher stability than native lipase in polar organic solvents<sup>40</sup>.

### 3.4.1.2 ENZYME ENTRAPMENT AND ENCAPSULATION

Entrapment of enzymes means that the enzyme molecules or enzyme preparations are confined in a matrix formed by dispersing the catalytic component (a soluble/insoluble enzyme preparation) in a fluid medium followed by formation of a insoluble matrix with enzymes confined by chemical or physical methods ( **Figure 3.3**).



**Scheme 3.3.** Entrapment of biocatalysts<sup>39</sup>

The entrapment technique is one of the simplest methods for immobilization of enzymes and whole cell-based enzymes. By this method more than one enzyme can be immobilized simultaneously<sup>41</sup>.

The precursor used for the preparation of the matrix is different. In most cases of polymerization entrapment, unsaturated monomers and co-monomers are used as cross-linkers and the polymerization can be

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irradiation-initiated<sup>42</sup> or photo or chemically initiated. Many enzymes have been entrapped in various gel matrices, for example lipase from *Candida rugosa* in alginate or chitosan<sup>42,43</sup>, whole cells with PGA (penicillin G acylase) activity in gelatin<sup>44</sup> or polyacrylamide, glucose oxidase (GOx) entrapped in Poly(2-hydroxyethyl methacrylate)-hydrogel microspheres (**PHEMA**-hydrogel), microbial whole cell in cross-linked ENTP-2000 (synthesized from poly(propylene glycol)-2000, hydroxyethylacrylate, and isophorone diisocyanate)<sup>45</sup>.

The geometric properties of the entrapped enzymes can be easily adapted in various forms such as beads, film, fibre, etc., depending on the application and the method of entrapment. Although it was found that entrapment can lead to serious diffusion limitation, reducing the apparent activity of the enzyme,<sup>46</sup> there are also many examples of enzymes entrapped in the matrix with good, comparable or better activity retention than other enzyme immobilized by different methods, such as the covalent<sup>46-48</sup>. One of the entrapment method, in fact, is usually very mild compared with covalent enzyme immobilization. Thus, this method might be extremely useful for the enzymes which can be easily deactivated by covalent enzyme immobilization.

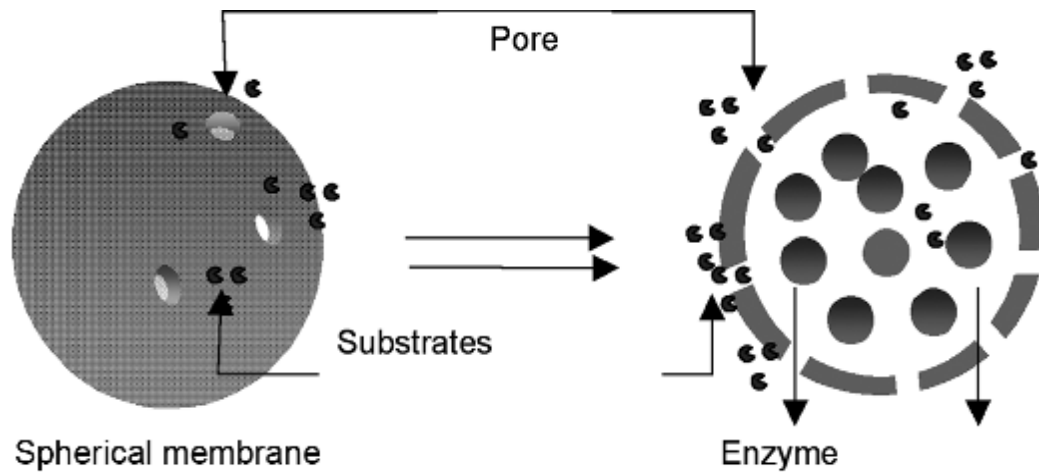
Encapsulation of an enzyme is the formation of a membrane-like physical barrier around an enzyme preparation. There are many methods of preparation of encapsulated enzymes: in-situ encapsulation, encapsulation-cross-linking, immobilization and encapsulation, and post-loading encapsulation.

A particular encapsulation in the micro-encapsulation. Micro-encapsulation of enzymes refers to the process by which an enzyme preparation such as dissolved enzymes, or lyophilized enzymes, or whole-cell catalysts are enclosed physically or chemically within spherical semi-permeable polymer membranes with diameters in 1-100  $\mu$  m range.

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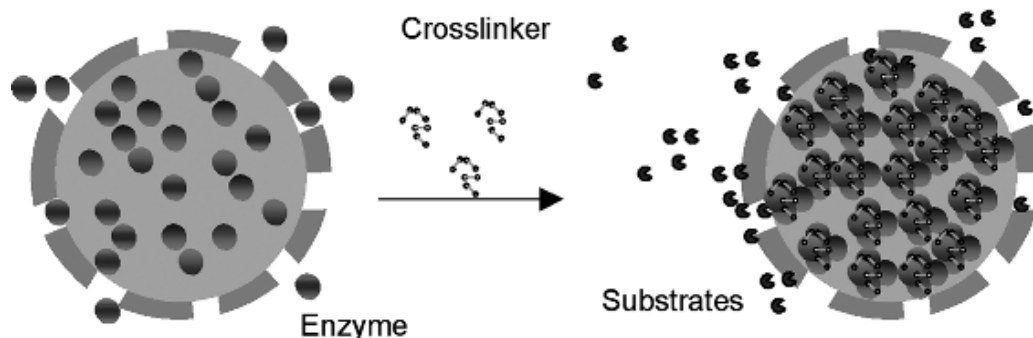
The enzyme molecules are physically confined in the interior by the membrane formed around the drops of enzyme solution, the substrates or products are able to diffuse freely across the membrane, depending on the pore size of the membranes (**Figure 3.4**). The membrane functions as a physical barrier to the enzyme molecules only.



**Schema 3.4.** Micro-encapsulation of enzymes in microcapsules<sup>39</sup>

### 3.4.1.3 CROSS-LINK IMMOBILIZATION

This technique involves the formation of a covalent bond between various molecules of the same enzyme to form a three-dimensional network with cross-bridges that stabilize the structure (**Figure 3.5**). Due to the problems of diffusion of the substrate within the lightweight aggregates that are formed the enzymatic activity is reduced. Moreover, the gelatinous nature of these aggregates makes it difficult to use them in industrial reactors. In the case of covalent immobilization, it is in most cases the amino groups of the enzyme that participate in bond formation and glutaraldehyde is the most common cross-linking reagent<sup>49</sup>.



**Scheme 3.5.** Cross-link immobilization<sup>39</sup>

### 3.4.2 METHODS OF REVERSIBLE IMMOBILIZATION

The use of reversible methods for enzyme immobilization is highly attractive, mostly for economic reasons because when the enzymatic activity decrease, the support can be regenerated and re-loaded with fresh enzyme. The reversible immobilization of enzymes is particularly important for immobilizing labile enzymes and for applications in bioanalytical systems.

The most common procedures of reversible enzyme immobilization are based on adsorption techniques.

Adsorption is perhaps the simplest method of enzyme immobilization because the technique involves the simple mixing of the substrate with the enzyme. The forces of adsorption are various (Van der Waals forces, ionic interactions and hydrogen bonds) and are relatively weak. There are intrinsic advantages in adsorptive enzyme immobilization methods:

- reversibility, that permits the reuse of the carriers;
- simplicity, which enables enzyme immobilization under mild conditions;

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- possible high retention of activity because there is no chemical modification, in contrast with covalent enzyme immobilization<sup>50</sup>.

The bond between the enzyme and the surface of the substrate is generated by a combination of hydrophobic interactions and electrostatic attraction forces between these molecules. The choice of the support which must ensure the adsorption of the enzyme appears to be important. Moreover, the support must be chosen in order to minimize the loss of enzyme during use which may be due to the weakness of the bond and the possibility of separation of the enzyme caused by changes in pH and ionic strength.

**3.4.2.1 Adsorption on/in organic supports:** Porous, inorganic supports were already in use in the early development of biocatalysis in organic media. If the interaction between the support and the enzyme is not strong enough for efficient adsorption, a mixture of aqueous enzyme solution and support can simply be dried to leave the enzyme deposited on the support surface. As long as the enzyme is not soluble in the reaction media used, it will remain on the support surface.

Hydroxylapatite and alumina were used in lipase-catalysed conversions of triacylglycerols<sup>51</sup> and different kinds of celite have been used as supports for a wide variety of enzymes, including many lipases<sup>52</sup>. Gitlesen *et al.* have been studied the adsorption of different lipases by EP-100 polypropylene powder from crude and pure lipase preparations<sup>53</sup>. Also membranes of chitosan has been utilized as carriers for immobilization of lipases. Chitosan is an attractive natural biopolymer from renewable resources with the presence of reactive amino and hydroxyl functional groups in its structure. Due to the its good biocompatibility it can be used in magnetic-field assisted enzyme. application of chitosan nanoparticles to immobilize enzymes strongly increases stability of immobilized enzymes

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and their easy separability from the reaction mixture at the end of the biochemical process<sup>54-58</sup>.

Several porous organic polymers have been used as supports for lipase immobilization under appropriate conditions (pH, ionic strength, and temperature). One frequently used form of *Candida antarctica* lipase B (CALB) is Novozym 435, which contains the acrylate-based polymer Lewatit VP OC 1600 as support. A porous styrene–divinylbenzene polymer has been evaluated as a CALB support for the esterification of acetic acid with 1-butanol in hexane<sup>59</sup>. The most widely used polymers are Sepabeads<sup>60</sup>, polypropene (Accurel MP-1000)<sup>61</sup>, alginate<sup>62</sup>. Simple and effective method of lipase immobilization on organic polymers have been reported by Basri *et al.*. Lipase from *Candida rugosa* has been adsorbed on to organic polymer beads at 40°C and at pH of adsorption between 6 and 7. Lipase immobilized in this manner produced high catalytic recoveries<sup>64</sup>. Microbial lipase from *Candida rugosa* has been immobilised, also, by physical adsorption onto styrene–divinylbenzene (STYDVB) copolymer. Better results were found when the coupling procedure was performed in the presence of heptane. Operational stability tests indicated that a small enzyme deactivation occurs after 12 consecutive batches of 24 h each<sup>64</sup>.

Immobilization of lipase onto porous Accurel EP400 powder, for the enzymic hydrolysis of sunflower oil is described by Murray *et al.*<sup>65</sup> Satisfactory immobilization of lipase onto EP400 particles was attained only at high enzyme loadings. However, under these conditions, rates of hydrolysis and equilibrium conversions obtained using lipase immobilized onto EP400 particles were comparable to those achieved in free enzyme systems. In addition, once bound to the EP400 support, the enzyme displayed good recycle potential<sup>65</sup>.



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### 3.4.2.2 Adsorption on mesoporous silica and zeolites

Immobilization of enzyme on mesoporous silica has attracted special interest because of the possibility of tailoring these materials with respect to pore sizes<sup>66-68</sup>. The surface properties of mesoporous silica can be tailored using various silanes as building blocks or deriving agents. For lipases, it is of special interest to use silanes containing hydrophobic groups<sup>69</sup>.

A series of mesoporous silica materials containing different functional groups has been prepared. Controlled pore glass derivatized with various organo-silanes was used as a support for *Rhizomucor miehei* lipase catalysing the esterification of oleic acid with 1-octanol, and the highest catalytic activity was observed with the most hydrophobic material, containing octadecyl groups<sup>70</sup>. A mesoporous organo-silicas led to the highest activity of *Thermomyces lanuginosus* Lipase in a transesterification reaction between 1-butanol and vinyl propionate in hexane<sup>71</sup>. Compared to pure silica, the introduction of methylene groups increased the activity of adsorbed *Rhizopus oryzae* Lipase, catalysing a transesterification reaction between (S)-glycidol and vinyl butyrate in isooctane<sup>72</sup>. Octyl groups were found to provide a good microenvironment for *Candida antarctica lipase B* on a mesoporous silica in the acylation of ethanolamine with lauric acid in acetonitrile<sup>73</sup>. Activation of a mesoporous silica having high surface area with octyltriethoxysilane has proven successful to cover the hydrophilic surface of silica with a hydrophobic layer of octyl groups. This has enabled to accomplish our objective: To achieve an efficient lipase immobilization in a monolayer, avoiding the formation of enzyme aggregates. Lipase from *Candida antarctica B* was easily adsorbed onto this matrix by hydrophobic interactions between both macromolecules<sup>74</sup>.

Of great interest is the use of zeolites as a support for the immobilization of enzymes. Macario *et al.*<sup>75</sup> have studied the catalytic performance in biodiesel production of different kind of zeolites catalysts. The catalysts tested have been: strong acid catalysts (USY, BEA, FAU-X),

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weak acid catalysts (MCM-41 and ITQ-6 with Si/Al =  $\infty$ ), acid–base catalysts (K-MCM-41, K-ITQ-6), potassium silicate (K<sub>2</sub>SiO<sub>3</sub>) and hydroxide (KOH). The enzyme used as biocatalyst has been the *Rhizomucor miehei* Lipase. This enzyme has been immobilized in/on zeolite and related materials by adsorption. Among inorganic solid catalysts tested, the highest triglycerides conversion and biodiesel yield were achieved by K-ITQ-6 catalysts, after 48 h of reaction at 180 °C<sup>75</sup>. Serralha *et al.* have studied the immobilized by adsorption of *recombinant cutinase from Fusarium solani pisi* on several zeolites and its activity towards the alcoholysis reaction of butyl acetate with hexanol, in isooctane<sup>76</sup>. In a work of Galarneau *et al.*<sup>77-79</sup>, two lipases from *Mucor miehei* have been immobilized by adsorption in MCM-41 materials featuring different hydrophilic/hydrophobic surfaces and by encapsulation either in hydrophobic silica sol-gel or in Sponge Mesoporous Silicas (SMS), a new procedure based on the addition of a mixture of lecithin and amines to a sol-gel synthesis to provide pore-size control. The resulting biocatalysts have been evaluated for various ester hydrolysis reactions and compared with commercially available immobilized lipases in silica sol-gel (Sol-gel AK-Fluka) and in ion-exchange resin (Lipozyme-Fluka). An adequate hydrophobic/hydrophilic balance of the support, such as supported-micelle, provides the best route to enhance lipase activity. The SMS encapsulation procedure enables the highest activity for the lipases<sup>77-79</sup>.

In order to create pores of the desired size and shape in the mesoporous silica, template molecules, such as surfactants, are used in the preparation procedure. For example, Takahashi *et al.*, reported the immobilization of horseradish peroxidase and subtilisin in FSM-16, prepared with a cationic surfactant. Enzyme immobilization in FSM-16 show the best stability and catalytic activity in a organic solvent when the average pore size just exceeded the molecular diameter of enzyme<sup>80</sup>. A new mesoporous micelle-templated silica (MTS) route for enzyme encapsulation

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is presented by *Mureseanu et al*<sup>81</sup>. The pore structure is given by a new association of lecithin and dodecylamine as cosurfactant. The mixed-micelles give after the addition of tetraethyl orthosilicate a well-ordered mesoporous material with a spongelike rigid structure stable after calcination at 550 °C, with size of the pores lies between 30 and 40 Å. The activity of this heterogeneous catalyst was tested in the hydrolysis of the ethylthiostearate. These new biocatalysts were very active, more than hydrophobic sol-gel materials and commercially available sol-gel encapsulated lipase<sup>81</sup>.

Normally, these are later removed by extraction with an organic solvent and/or by calcination at high temperature. However, it has been shown that it can sometimes be advantageous to leave some of the template in the structure<sup>83</sup>. An example is the application of hierarchical ZSM-5 zeolites, combining micropores and intracrystalline mesopores, as carriers for lipase enzyme, compared with purely microporous ZSM-5 and mesoporous MCM-41 have been studied by *Mitchell et al.*<sup>82</sup> Strategies to improve enzyme immobilization by modification of the support porosity and surface properties have been also reported. Modification of the mesopore walls prior to enzyme immobilization is essential to attain an active and recyclable biocatalyst. Enzymes immobilized on purely inorganic supports exhibit rapid loss of activity attributed to enzyme leaching. Despite the high mesopore surface area of surface-functionalized MCM-41, the mono-dimensionality of the mesopores results in restricted accessibility and a reduced enzyme uptake. In comparison, the interconnected mesopores of the hierarchical zeolites remain accessible after surface functionalization showing good adsorption properties. So, lipase immobilized on thiol-functionalized mesoporous ZSM-5 was found to be the most efficient biocatalyst<sup>82</sup>.

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### **3.4.2.3 Protein-coated microcrystals.**

One way to obtain highly active enzyme preparations in organic media is to prepare protein- (enzyme-) coated microcrystals (PCMCs)<sup>83</sup>. In this method, an aqueous enzyme solution is mixed with a concentrated solution of an excipient, which is normally an inorganic salt, but can be a sugar or amino acid. The mixed solution is slowly added to a water-miscible organic solvent to induce crystallization of the excipient and precipitation of the enzyme on the surface of the crystals. The size of the crystals is normally in the range 0.1–5 mm. This small size and the fact that the enzyme is located primarily on the surface allow efficient mass transfer of substrates and products.

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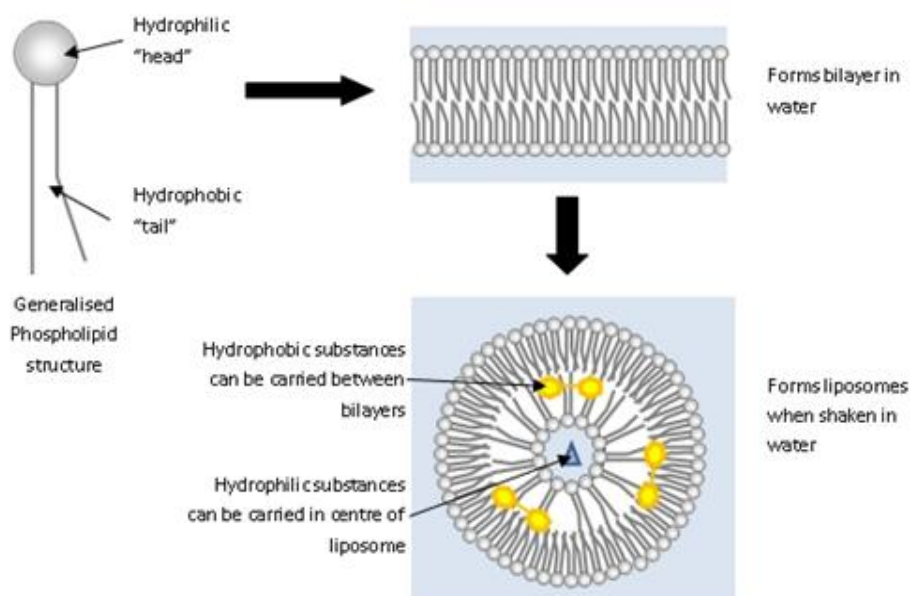
## **CHAPTER IV**

*Introduction of the experimental  
work of the thesis*

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As discussed in the previous chapter, immobilization is a technique that allows us to overcome main problems associated to the use of free enzymes. During the enzyme immobilization procedure, the main purpose is that one to obtain an activated and stable enzyme form for this reason, we have studied in this work the possibility to immobilize a particular enzyme, Lipase from *Rhizomucor miehei*, in a hybrid matrix containing a Liposomal phase, as organic shell, and silica, as inorganic protection. Due to the high ability of enzyme to catalyze racemic solution with specific selectivity, we have choose to test immobilized enzyme activity in a biotransformation enantioselective esterification of racemic ibuprofen ester. The choice toward liposome as organic protection of enzyme is due to their high versatility in biology, biochemistry and medicine.

Liposomes are small vesicles with a diameter of 50-500 nanometers. Their structure is characterized by the presence of one or more external phospholipid bilayers, delimiting the inside of a heart in which the hydrophilic material is located in the aqueous phase (**Figure 4.1**).



**Figure 4.1.** Structure of liposome<sup>1</sup>

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Liposomes were discovered by A. Bangham<sup>2</sup> about 40 years ago and since then they have become very versatile tools in biology, biochemistry and medicine. It has been shown that phospholipids spontaneously form closed structures when they are hydrated in aqueous solutions.

The liposome are classified on the basis of:

➤ **Structural parameters**

1. ***Unilamellar vesicles:***
  - Small unilamellar vesicles (SUV): size range from 20-40 nm.
  - Medium unilamellar vesicles (MUV): size range from 40-80 nm
  - Large unilamellar vesicles (LUV): size range from 100-1000 nm.
2. ***Oligolamellar vesicles:*** They are made up to 2-10 bilayers of lipids surrounding a large internal volume.
3. ***Multilamellar vesicles:*** they have several bilayers and then compartmentalize the aqueous volume in a infinite numbers of ways.

➤ **Methods of liposome preparation**

REV: Single or oligolamellar vesicles made by Reversed phase Evaporation methods.

MLV-REV: Multilamellar vesicles made by Reversed phase Evaporation methods.

SPLV: Stable Plurilamellar Vesicles

FATMLV: Frozen and Thawed MLV

VET: vesicles prepared by extrusion technique

DRV: Dehydration- rehydration methods.

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Due to their resemblance to natural membranes and their non-toxic nature, liposomes provide an excellent system for the encapsulation of active molecules. For example, Chemo therapy drugs, like Doxil, are encapsulated in liposomes to enhance their efficacy and reduce side effects when used to treat a wide range of cancer ailments, including breast cancer<sup>3</sup>. These drug molecules are more easily transported through biological elements.

Generally, polar compounds are encapsulated in the liposome aqueous interior, while non-polar solutes are incorporated in the lipid bilayer. Similarly, significant results have been achieved in the last two decades in the area of gene delivery and cellular/tissue imaging using amphiphilic lipid mixtures, both in vivo and in-vitro. Liposomes have also found wide applications in biosensors and flow based bio-analytical assays, due to their relatively large internal volume (marker encapsulation) and the ability to modify their outer-membrane with various bio-recognition elements<sup>4</sup>.

There are many advantages in using liposomes as support to encapsulate the active molecules:

- Liposomes are biocompatible, completely biodegradable, not toxic and not immunogenic;
- Liposome are suitable for delivery of hydrophobic, amphipathic and hydrophilic molecules;
- They protect the encapsulated molecules from the external environment;
- They reduce exposure of sensitive tissue to toxic molecules.

However, the liposomes, as all the organic systems used for encapsulation, reveal limitations related to their thermal and chemical stability. Their stability can be increased by creation of outer of inorganic silica, creating nanoparticles structure.

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## 4.1 AIM OF THE THESIS WORK

In this thesis has been described the synthesis of heterogeneous biocatalyst formed by hybrid-nanospheres containing an inorganic silica shell, in which there is an organic phase where biocatalyst is confined (Lipase + liposome).

The porous structure of the shell allows the entry of the substrate which will be converted by the enzyme as well as the leakage of the products, but could also promote the release of the enzyme, causing its high loss. In order to focus on this aspect, we prepare hybrid-nanospheres with and without surfactant, that should act as diameter reducing of the external porous silica matrix.

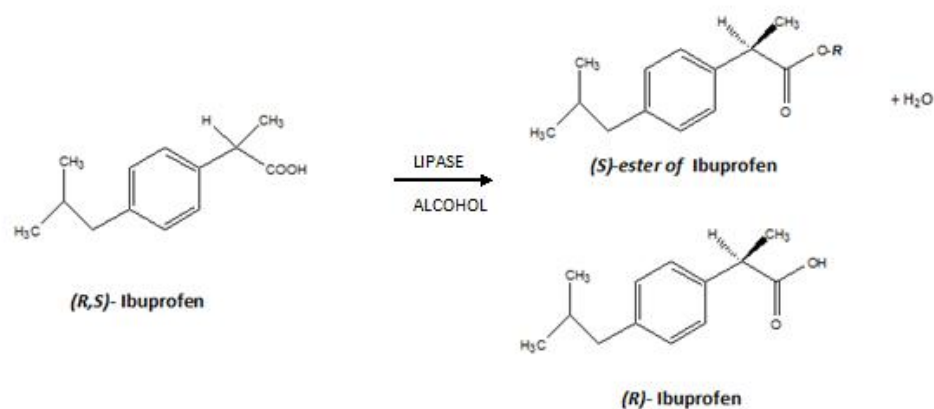
The catalyst has been characterized by usual and advanced techniques for porous solid, in order to understand the role of liposome and silica shell in the catalyst performance.

The influence of several experimental factors in the synthesis of immobilized lipase from *Rhizomucor miehei* in liposomal phase, has been studied:

- *Silica/liposomal weight ratio*
- *mixing time between the Liposome and the Lipase solution.*

The enzyme immobilization optimized procedure has allowed to obtain perfect hybrid-nanospheres in which Lipase is confined into liposomal phase.

Afterwards, the optimized heterogeneous catalyst has been used in enantioselective esterification of Ibuprofen (**Figure 4.2**).



**Figure 4.2.** Enantioselective esterification of (R,S)-ibuprofen with alcohol using lipase of *Rhizomucor miehei* (RML).

In recent years Lipases have been widely used for the production of enantiomerically pure compounds, resolving racemic alcohols and organic acids<sup>5</sup>. There is an increasing trend toward the use of optically pure enantiomers for drugs because they are more specific targets and have fewer side-effects than racemic mixtures. Among 1800 drugs currently available, about half are of a chiral mixture. Racemic drugs have usually the desired therapeutic activity mainly in only one of the enantiomers. In this sense, 2-aryl propionic acids (profens), that make up an important group of non-steroidal anti-inflammatory drugs (NSAIDs) used in the treatment of arthritis and other related diseases<sup>6,7</sup>, show mainly the pharmacological activity by their corresponding (S)-enantiomer<sup>8</sup>. In fact, it has been reported that (S)-enantiomer of ibuprofen is 100-fold more active than its antipode in the *in vitro* synthesis of prostaglandin<sup>9</sup>. Therefore, important efforts are being made to synthesize pure enantiomers of 2-aryl-propionic acids, since pharmacological studies have indicated that gastrointestinal problems are among the most frequent side effects associated with the consumption of profens<sup>10</sup>. Ibuprofen is a racemic carboxylic acid, showing in each enantiomer a physiologically different behavior, being the (S)-enantiomer

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the form that exhibits an anti-inflammatory property<sup>11</sup>. In recent years, lipases have been used for the chiral resolution of (R,S)-ibuprofen through mainly direct enantioselective esterification in organic media<sup>12-14</sup> or enantioselective hydrolysis of its chemically synthesized racemic ester<sup>15-17</sup>.

Among the high number of lipases described in the literature, only the enzymes belonging to a few species have been demonstrated to have adequate stability and biosynthetic capabilities to allow routine use in organic reactions and hence their applications as industrially relevant enzymes<sup>18,19</sup>. Among lipase, only *Candida cylindracea* and immobilized *Rhizomucor miehei* lipases (Lipozyme, Novo Industry Denmark) were able to catalyze the esterification reaction. In the literature there are few studies regarding the uses of the commercial *Rhizomucor miehei* lipase (Lipozyme IM) in enantioselective reactions<sup>20,21</sup>. Lipozyme IM is a commercial lipase, supplied by Novo Nordisk Industry, in which enzyme is immobilized on a macroporous ion exchange resin.

On the contrary, there are no reports about the immobilization of *Rhizomucor miehei* lipase in other supports and its use in this type of reaction.

The influence of several catalytic parameters on the activity of hybrid nanospheres, such as:

- type of the solvent (polar iso-octane and apolar dimethylformamide);;
- nature of alcohol (1-methanol, 1-propanol and 1-butanol);
- reaction temperature (27°, 37°, 50° and 80°C).

have been investigated.

The reusability and storage stability of the catalyst were also determined for a potential industrial application. Two important parameters in enzymatic catalysis, have been evaluated: turnover number (**TON**) and turnover number of frequency (**TOF**).



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#### 4.1.1 IBUPROFEN: GENERAL INFORMATION

It is known that ibuprofen have anti-inflammatory properties by Dr. Stewart Adams at the Boots Company in Nottingham (UK). Since then, ibuprofen has evolved and has become one of the most widely-used analgesic–antipyretic–anti-inflammatory drugs. It is available in nearly all countries in the world as both a prescription and over-the-counter (OTC) sale drug for the treatment of a wide variety of painful and inflammatory conditions, although its principal approved application is the treatment of mild-moderate pain, including muscular-skeletal condition, headache/migraine, fever, and accidental injuries.

As an over-the-counter sale remedy it is registered for sale in 82 countries worldwide. It probably ranks after aspirin and paracetamol in non-prescription OTC use for the relief of symptoms of acute pain, inflammation and fever, although the patterns of use of these analgesics vary considerably from country to country worldwide.

It is well documented that their pharmacological activity rests mainly on the (*S*)-enantiomer<sup>22</sup>. For example, (*S*)- ibuprofen is 100-fold more active than its antipode<sup>23</sup>. Therefore, considerable efforts are made to synthesize pure enantiomers of ibuprofen. Previous pharmacological studies of the acidic NSAIDs indicated that gastrointestinal (GI) side effects constitute the most frequent of all the adverse reactions. These range in both severity and frequency from relatively mild to the more serious states of GI ulceration and hemorrhage<sup>24</sup>. Therefore, developing their bioreversible derivatives to decrease the toxicity induced by the acid moiety of the profens is most necessary.

The prodrug is a pharmacologically inactive derivative of a parent drug that requires spontaneous or enzymatic transformation within the body to release the active drug. Thus, the prodrug temporarily masking the acid moiety of the profen is a promising means for reducing or abolishing the GI toxicity due to the locally direct contact effect<sup>25</sup>. When it is designed to be

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administered orally, good aqueous solubility is essential to give an acceptable bioavailability.

Esterification of the profens is an effective way to prepare the prodrug, since the organism is rich in enzymes capable of hydrolyzing the resultant ester<sup>26</sup>. With a careful selection of the alcohol moiety, the resultant ester prodrug can often provide bioactivity comparable to that of the parent drug<sup>24,25,27</sup>. For example, Tammara *et al.* indicated that morpholinoalkyl esters of indomethacin and naproxen represent potentially useful derivatives to decrease GI side effects without altering the pharmacological profile of the parent compounds. However, at least two synthesis steps are required in obtaining the (*S*)-ester prodrug from the racemate, which include an optical resolution of the racemate to give the (*S*)-enantiomer by using chemical or biochemical methods and then the chemical synthesis of the desired product<sup>28-30</sup>.

In conclusion, it is evident that to perform an efficient way to produce active enantiomer from racemic product is suitable for medical application.

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# **CHAPTER V**

## ***Materials and Methods***

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In this chapter, the materials and methodologies used to prepare and testing heterogeneous biocatalysts are reported.

## 5.1 MATERIALS

- ▶ **Preparation of organic nanospheres.** For the preparation of organic parts of nanospheres, we used L- $\alpha$ -phosphatidylcholine as lecithin liposome precursor, purchased from Sigma Aldrich, while PALATASE 20000L, was the lipase enzyme. This lipase is produced by Novo Nordisk (Denmark). This enzyme is a purified 1,3-specific lipase (EC 3.1.1.3) from *Rhizomucor miehei* (RML).
- ▶ **Preparation of silica porous shell.** For the preparation of silica porous shell, we used: Tetraethyl orthosilicate (99%), as silica source; Hexadecylamine (98 %) as a template; sodium fluoride (99%) as mineralizing agent. All of these products were purchased from Sigma Aldrich.
- ▶ **Catalytic Tests:** For the reaction tests, different alcohol were tested: methanol (99.9%), 1-Propanol (99.9%), 1-butanol (99.9%). The solvent used were: Isooctane (99.9%) and Dimethylformamide (99%). Racemic Ibuprofen (98 %) and the pure enantiomers of ibuprofen (*R* and *S*), were purchased from Sigma-Aldrich.

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## **5.2 DETAILS ON USED ENZYME: *RHIZOMUCOR MIEHEI* LIPASE**

The lipase used in the work is Palatase 20000L produced by Novo Nordisk (Denmark) and commercialized by Sigma-Aldrich. The Palatase is a 1,3 specific lipase (EC 3.1.1.3) produced by a fungus called *Rhizomucor miehei*, for fermentation by a microorganism, genetically modified, *Aspergillus oryzae*. Its molecular weight is equal to 29536 g/mol, while the calculated spherical diameter is  $41\text{Å}^1$ . The Palatase 20000L exhibits its maximum activity at a pH comprised between 6-8.5 with the greatest value equal to 7.5. The optimum temperature of reaction is 40°C.

The highest activity of this enzyme is ensured only if stored at 5 ° C and for a period not exceeding 6 months. In fact, long periods of storage reduce the activity of the enzyme, so that if we want to achieve the same catalytic performance of the enzyme when cool, a greater amount must be used. In commercial solutions, polysaccharides, polyols or sugars can also be present. These components can also be added to retain water to reduce the possibility of enzyme denaturation. In fact, the enzyme in the presence of water can assume different conformations and may be denatured over time.

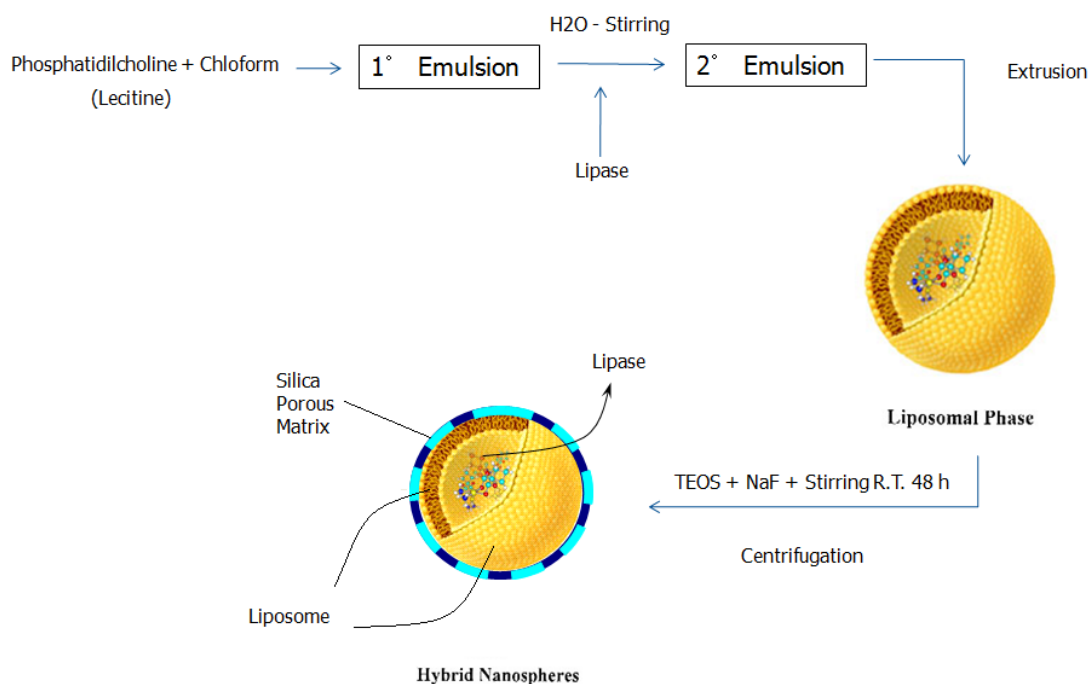
Furthermore, the same enzyme during the storage period can produce pigments such as phenols. Studies carried out in the literature about the presence or absence of these compounds, shows that there is no presence of phenols; the quantity of polyphenols appears to be equal only to 0.124 mg/ml; the content of sugar is equal to 6,12 mg/ml; the water content is equal to 48% w/w; while that of total protein is equal to 164 mg/ml<sup>2</sup>.

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### 5.3 METHODS OF LIPASE IMMOBILIZATION: General procedure

The methodology used to prepare the hybrid nanosphere has been the following: an amount of lecithin has been mixed with chloroform (10mL) in order to dissolve and create an homogeneous solution. The solution has been vigorously stirred to obtain an emulsion which was yellow in color. Thereby, this solution has been mixed with 40 mL of 0.2 M phosphate buffer pH=7 containing 7.1 mL of RML (commercial PALATASE). The suspension has been maintained at 40°C in continuous stirring for a defined time. After this period the liposomal/enzyme solution has been extruded twice using cellulose filters with a pore diameter equal to 0.20  $\mu\text{m}$ , in order to obtain homogeneous nanospheres. Subsequently an external inorganic shell has been formed around the liposome/enzyme nanospheres. For this, an amount of tetraethyl orthosilicate has been added to the liposomal/enzyme solution. The suspension formed was stirred at room temperature for 24 hours and subsequently an amount of NaF (7.1 mg) has been incorporated to initialize the condensation of silane groups. The stirring has been continued for 48 h at room temperature. To separate the supernatant from the medium containing the enzyme immobilized nanospheres, the mixture has been centrifuged at 10000 rpm for 30 min. The recovered solid has been washed with distilled water, dried at 30 °C overnight and stored at 0 °C to preserve the functionality of the enzyme until its use. The supernatant and the initial solution has been assayed for protein content (see §5.5). The difference between protein loaded and that remaining unbound indicates the quantity of protein bound inside the nanosphere. The immobilization of lipase inside the nanosphere is schematically represented in **Figure 5.1**.





**Figure 5.1.** Preparation procedure of hybrids nanospheres without surfactant.

The summery regarding the condition of all sample is reported in **Table 5.1.**

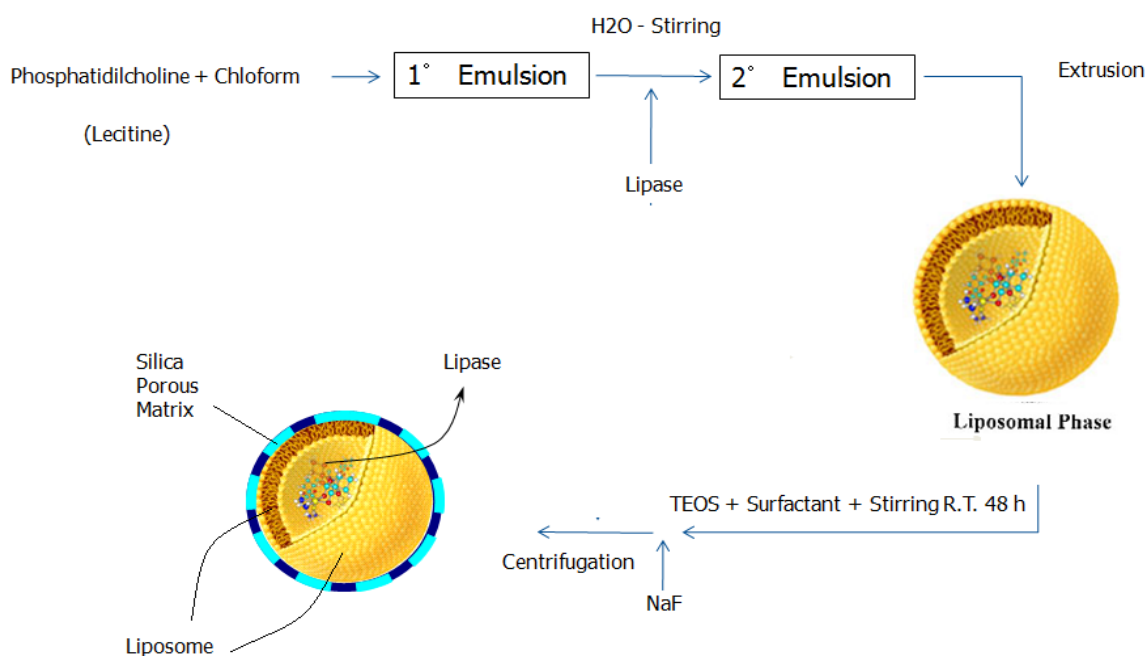
**Tabella 5.1** Summery condition of all sample prepared

<b>Sample</b>	<b>Lecithin Amount [gr]</b>	<b>Silica Amount (TEOS) [gr]</b>	<b>SiO<sub>2</sub>/Liposome Weight ratio</b>
<b>RL1</b>	0.44	0.88	2
<b>RL2</b>	0.44	0.44	1
<b>RL3</b>	0.88	0.44	0.5

Each of these samples have been synthesized according to the above procedure and varying the mixing time of Liposome/Lipase solution ranging from 1 and 12 hours.

All samples have been characterized in order to check the best procedure to obtain perfect hybrid nanosphere where lipase is in the core of the organic phase. The optimized procedure has been used to prepare hybrid

nanosphere with different silica shell. In particular, the silica cover has been prepared using hexadecylamine as surfactant. To prepare the different silica shell, amount of hexadecylamine (TEOS/hexadecylamine molar ratio equal to 4) has been dissolved in a 40 ml of ethanol. This solution has been added drop-wise to the Lipase/Liposomal solution with vigorous stirring at room temperature for 2 hours. Subsequently, 7.1 mg of NaF was incorporated to initialize the condensation of silane groups. The resulting mixture was allowed to react at room temperature for 48 hours following which the solid product was obtained by filtration and air dried at room temperature (**Figure 5.2**).



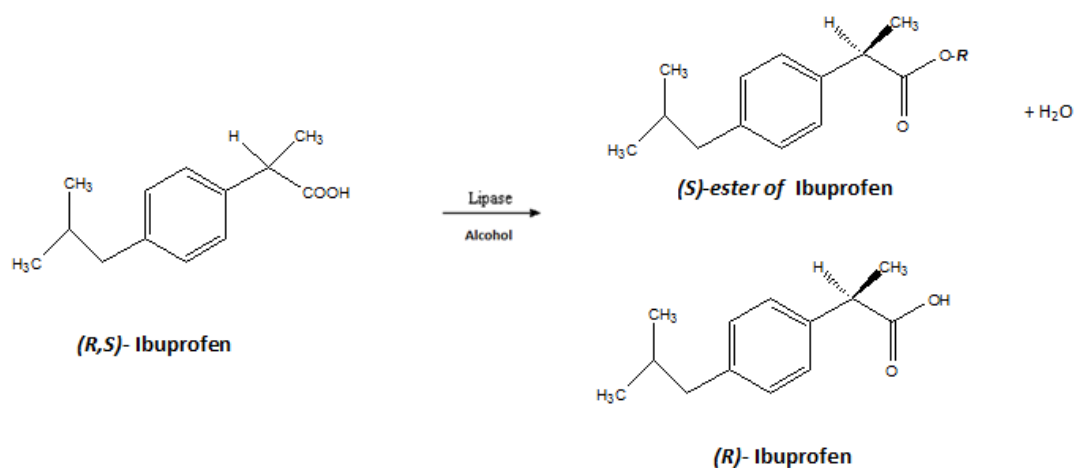
**Figure 5.2.** Preparation procedure of hybrids nanospheres with surfactant.

Before the use in the reaction, all prepared catalysts have been activated: first have been washed with 100 ml of solvent (isooctane), then with 900 ml of distilled water, and finally dried at 30 °C overnight. After 24 hours the sample is ready to be use in the reaction.

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## 5.4 ESTERIFICATION REACTION OF (R,S)-IBUPROFEN USING IMMOBILIZED LIPASE.

The biotransformation reactions of racemic ibuprofen (**Figure 5.3**) has been conducted in 20 mL conical flasks. The standard reaction mixture has been composed of (R,S)-ibuprofen (66mM), alcohol (66mM), and solvent (10mL), without the addition of water<sup>3</sup>. The reaction has been started by adding prepared heterogeneous biocatalyst (7%wt of Lipase with respect to ibuprofen) to the solution and carried out in a flask by orbital magnetic stirring at 135 rpm at different temperature.



**Figure 5.3.** Esterification of (R,S)-ibuprofen with 1-propanol using lipase with (S)-stereopreference

The quantity of Lipase utilized in reaction (7%wt of Lipase with respect to ibuprofen: 10 mg of Lipase and 990 mg of support) has been chosen as a minimum quantity from which to carry out the reaction.

Aliquots of 50  $\mu$ L of the solution have been withdrawn at different times and diluted in 50  $\mu$ L of solvent in order to following the time profile of the reaction. In particular, the amount of ester have been monitored by gas chromatography technique. Different variables were studied in this process:

- 
- type of the solvent (apolar iso-octane and polar dimethylformamide);
  - nature of the alcohol (1-methanol, 1-propanol and 1-butanol);
  - reaction temperature (27°, 37°, 50° and 80°C).

After 72 hour of reaction the reaction the mixture was filtered and analyzed by chiral gas chromatography to determine enantiomeric excess. The final time of reaction (72 hours) has been chosen considering some work reported in the literature<sup>4,5</sup>, in which has been used a commercial lipase resulting from the *Rhizomucor miehei* and immobilized on a macroporous ion-exchange resin (Lipozyme IM).

Enantiomeric excess (ee) has been calculated according to Eqs. (1)

$$(1) \quad ee \% = \frac{R-S}{S+R} * 100 \quad \text{For } R > S$$

R is the peak area for the R(-) enantiomer of ibuprofen and S is the peak area for the S(+) enantiomer of ibuprofen, obtained by chiral gas chromatography .

The solid recovered has been washed several time with 100 ml of solvent (isooctane), then with 900 ml of distilled water, and finally dried at 30 °C overnight , for 24 hours, until the next reuse. Stability of catalyst has been studied. We have been evaluated two important parameters in enzymatic catalysis such as turnover number (**TON**) and Turnover number of frequency (**TOF**):

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## 5.5 PROTEIN ESTIMATION

The protein estimation has been done by using UV-VIS absorption method at 280/235 nm<sup>6</sup>, where  $A_{235}$  and  $A_{280}$  are the absorbance values at the 235 or 280 nm, respectively. The NanoDropND-1000 full-spectrum (220-750 nm) spectrophotometer, that measures 1 $\mu$ l samples without the use of cuvettes or the need for dilution, has been used. The protein exhibits a maximum value of absorbance at 280 nm due to the presence of tyrosine and tryptophan; while the absorbance at 235 nm is due to the presence of a peptide bond<sup>6</sup>.

The difference in absorbance between 235 and 280 nm, due primarily to the peptide bond, can be used to calculate protein concentration using the equation:

$$\text{Protein Concentration} \left( \frac{\text{mg}}{\text{ml}} \right) = (A_{235} - A_{280}) / 2,51$$

where the factor 2,51 is the difference between the average measured extinction coefficient at 235 and 280 nm.

Previously proposed methods for the calculation of protein concentration were designed to correct interference by nucleic acids<sup>6</sup>.

The quantity of protein immobilized into the nanospheres was determined by a mass balance between the initial solution and the final solution:

$$W_{IM} = (C_0 * V_0) - (C_f * V_f)$$

Where :

$W_{IM}$  = mg of immobilized enzyme;

$C_0$  = enzyme concentration in initial solution [mg/ml];

$C_f$  = enzyme concentration in final solution [mg/ml];

$V_0$  = volume of initial solution;

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$V_f$  = volume of final solution.

## 5.6 CATALYST CHARACTERIZATION

### 5.6.1 THERMOGRAVIMETRIC ANALYSIS.

TGA is commonly used to determine selected characteristics of materials that exhibit either mass loss or gain due to decomposition. Common applications of this analysis are: materials characterization through analysis of characteristic decomposition patterns; studies of degradation mechanisms and reaction kinetics; determination of organic content and inorganic content in a sample, which may be useful for corroborating predicted material structures or simply used as a chemical analysis.

**Base principles of thermogravimetric analysis.** *Thermogravimetric analysis* is a method of thermal analysis in which changes in physical and chemical properties of materials are measured as a function of increasing temperature (with constant heating rate), or as a function of time (with constant temperature and/or constant mass loss)<sup>7</sup>. Thermogravimetric analysis (TGA) is an analytical technique used to determine a material's thermal stability and its fraction of volatile components by monitoring the weight change that occurs as a specimen is heated. The measurement is normally carried out in air or in an inert atmosphere, such as Helium or Argon, and the weight is recorded as a function of increasing temperature in addition to weight changes; some instruments also record the temperature difference between the specimen and one or more reference pans (differential thermal analysis, or DTA) or the heat flow into the specimen pan compared to that of the reference pan (differential scanning calorimetry, or DSC). The latter can be used to monitor the energy released or absorbed via chemical reactions during the heating process.

The instrumentation used for the thermogravimetry is composed of four parts:

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- 
- thermal scale;
  - furnace;
  - system of purge gas which ensures an environment which is inert or reactive;
  - computer for controlling the instrument.

The usual scales available for these types of analyses operate in a range between 5-20 mg, although, for specific requirements, there are scales that operate up to 100mg .

Thermogravimetric analysis can not only determine the weight loss associated with the content of water present and the organic phase present in the material, but also the structural change of the crystal phase. The latter is an exothermic process in which weight loss does not occur.

***Instrument and conditions used.*** The measurements have been carried out with Metler Toledo TGA/SDTA 851E, in a temperature range between 20 and 800 ° C (at this temperature all the organic matter present in the material is removed), with a heating rate of 10°C/min and in a synthetic air stream with a flow of 50mL/min.

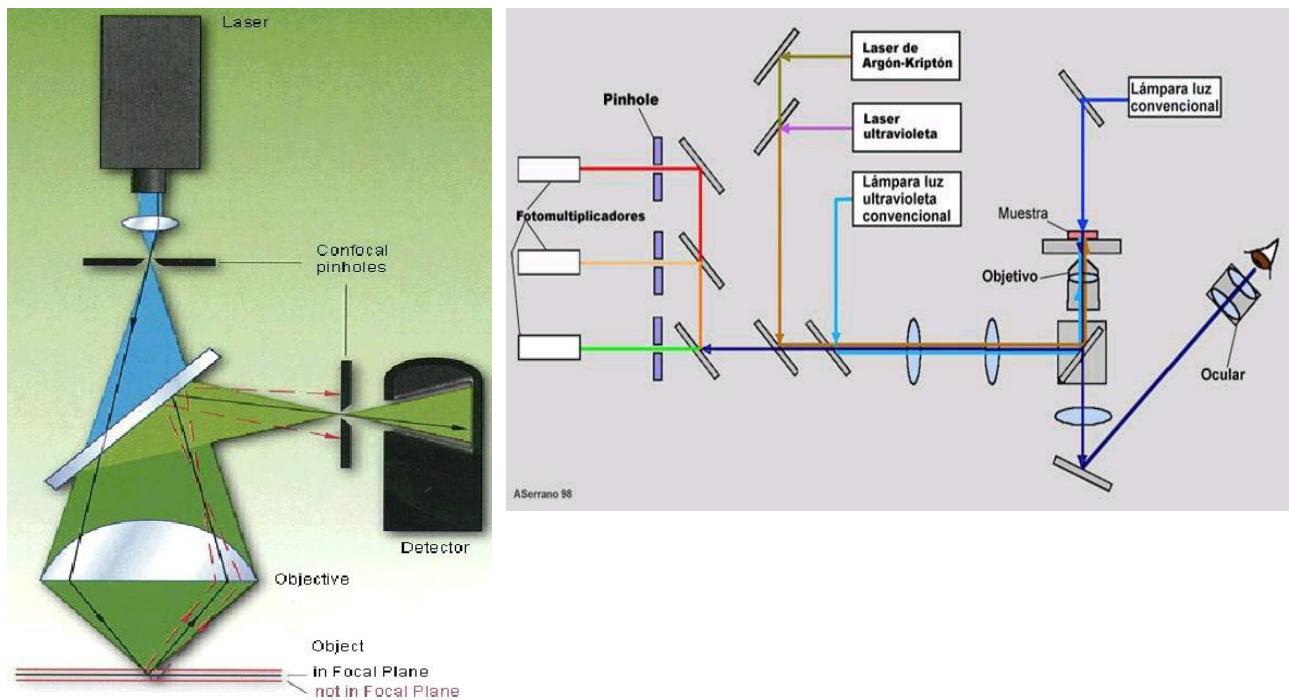
## **5.6.2 FLUORESCENCE CONFOCAL MICROSCOPY**

The fluorescence confocal microscopy has been used, in this work, to clarify the exact position of the enzyme inside the nanospheres.

***Base principles of fluorescence confocal microscopy.*** Confocal microscopy has been pioneered by Marvin Minsky in 1955 while he has been a Junior Fellow at Harvard University<sup>8</sup>. A confocal microscope creates sharp images of a specimen that would otherwise appear blurred when viewed with a conventional microscope. This is achieved by excluding most of the light from the specimen that is not from the microscope's focal

plane. The image has less haze and better contrast than that of a conventional microscope and represents a thin cross-section of the specimen. Thus, besides allowing better observation of fine details, it is possible to build three-dimensional (3D) reconstructions of a volume of the specimen by assembling a series of thin slices taken along the vertical axis.

In the figure below it is possible to observe the principal component of a confocal microscope (**Figure 5.4**).



**Figure 5.4** Component of fluorescent confocal microscope

The laser provides the intense blue excitation light. The light reflects off a dichroic mirror, which directs it to an assembly of vertically and horizontally scanning mirrors. These motor-driven mirrors scan the laser across the specimen. The dye in the specimen is excited by the laser light and fluoresces. The fluorescent (green) light is de-scanned by the same mirrors that are used to scan the excitation light (blue) from the laser and then passes through the dichroic mirror. Thereafter, it is focused onto the pinhole. The light that makes it through the pinhole is measured by a detector such as a photomultiplier tube<sup>9</sup>. The ability of a confocal



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microscope to create sharp optical sections makes it possible to build 3D renditions of the specimen.

***Instrument and conditions used.*** In this work we use LEICA TSC-SL fluorescence confocal microscopy to clarify with greater accuracy the exact position of the enzyme inside the nanospheres. During the nanospheres synthesis process, lipase RML was mixed together with a fluorescent compound for 2 hours. The fluorescent compound used is a fluorescein isothiocyanate (99%) purchased from Sigma Aldrich. For the analysis, a small amount of sample, crushed finely, has been placed over the microscope slide (is a thin flat piece of glass, typically 75 by 26 mm and about 1 mm thick, used to hold objects for examination under a microscope) and a drop of water has been injected. A cover glass, a smaller and thinner sheet of glass, has been placed over the specimen. The sample is ready to be analyzed.

### **5.6.3 N<sub>2</sub> ADSORPTION/DESORPTION**

This technique has been applied, for all samples synthesized, in order to obtain information about specific surface area, pore size and pore volume of the nanospheres produced.

***Base principles of N<sub>2</sub> adsorption/desorption technique.*** The physical adsorption of a gas involves interaction between the solid phase (or adsorbent) and inert gas (or adsorbate), due to the effect of Wan der Waals forces. It is reversible, involves small heat adsorption, and it is an exothermic process. By physical adsorption it is possible to determine various properties such as:

- the shape of the pores, which can be determined by the crystal structure;
- the specific surface area which is measured on the surface area available per mass unit;

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- specific porosity, which represents the volume of the available empty spaces, internal to the particles, per unit mass of solid.

The amount of gas adsorbed by a solid sample at a given temperature is a function of the equilibrium pressure of the system. The relationship between the mass of gas and the relative pressure is represented by a defined adsorption isotherm curve. This isotherm allows us to evaluate, by varying the relative pressure, the exact amount needed to fill the pores before reaching the saturation pressure (i.e. all the material is covered by a layer of liquid adsorbed and is always equal to 1).

Depending on the porosity of the material, there are different curves of adsorption. To determine the exact isotherm of adsorption / desorption, the material is subjected to degassing, under vacuum, to remove adsorbed molecules on the surface of the pores, and to acquire thus the real weight of the sample. Through equipment that measures volumetric and gravimetric results, it is possible to evaluate the amount of gas adsorbed on the solid at different values of relative pressure and then build the isotherm. Through the interpretation of the adsorption- desorption isotherms it is possible to obtain information about specific surface area, pore size and pore volume.

Determination of the specific surface area is carried out following the Brunauer–Emmett–Teller (BET) theory. The concept of the theory is an extension of the Langmuir theory, which is a theory for monolayer molecular adsorption, to multilayer adsorption with the following hypotheses: (a) gas molecules physically adsorb on a solid in layers in an infinitive way; (b) there is no interaction between each adsorption layer; and (c) the Langmuir theory can be applied to each layer. The resulting BET equation (1) is:

$$\frac{1}{v\left[\left(\frac{p_0}{p}\right)-1\right]} = \frac{c-1}{v_m c} \left(\frac{p}{p_0}\right) + \frac{1}{v_m c} \quad (1)$$

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where  $p$  and  $p_0$  are the equilibrium and the saturation pressure of adsorbates at the temperature of adsorption,  $v$  is the adsorbed gas quantity (for example, in volume units), and  $v_m$  is the monolayer adsorbed gas quantity,  $c$  is the BET constant (2):

$$c = \exp\left(\frac{E_1 - E_L}{RT}\right) \quad (2)$$

where  $E_1$  is the heat of adsorption for the first layer, and  $E_L$  is that for the second and higher layers and is equal to the heat of liquefaction.

Equation (1) is an adsorption isotherm and can be plotted as a straight line with  $\frac{1}{v\left[\left(\frac{p_0}{p}\right) - 1\right]}$  on the y-axis and  $\left(\frac{p}{p_0}\right)$  on the x-axis according to experimental results. This plot is called a BET plot. The linear relationship of this equation is maintained only in the range of  $0.05 < \left(\frac{p}{p_0}\right) < 0.35$ .

The BET method is widely used in surface science for the calculation of surface areas of solids by physical adsorption of gas molecules. The total surface area  $S_{total}$  and the specific surface area  $S_{BET}$  are given by:

$$S_{total} = \frac{(v_m N s)}{V} \quad (3)$$

$$S_{BET} = \frac{S_{total}}{a} \quad (4)$$

where  $v_m$  is in units of volume which are also the units of the molar volume of the adsorbate gas,  $N$  is Avogadro's number,  $s$  the adsorption cross section of the adsorbing species,  $V$  the molar volume of the adsorbate gas, and  $a$  the mass of the solid sample or adsorbent.

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According to A.S.T.M (American Section of the International Association for Testing Materials), it is possible to distinguish three types of pore in relation to their size:

- Micropore : size less than that 20 Å;
- Mesopore: size between 20 and 600 Å;
- Macropore: size greater than 600 Å.

***Instrument and conditions used.*** Nitrogen adsorption isotherms were measured at -196 °C with a Micromeritics ASAP 2010 volumetric adsorption analyser. Before analyses, all samples have been calcinated at 600°C under vacuum condition for 8 hours, and before the measurements, the samples were outgassed for 12 h at 100 °C. The BET specific surface area was calculated from the nitrogen adsorption data in the relative pressure range from 0.04 to 0.2. The total pore volume was obtained from the amount of N<sub>2</sub> adsorbed at a relative pressure of about 0.99. External surface area and micropore volume were estimated using the t-plot method in the t range from 3.5 to 5. The pore diameter and the pore size distribution were calculated using the Barret-Joyner-Halenda (BJH) method on the adsorption branch of the nitrogen isotherms.

#### **5.6.4 TRANSMISSION ELECTRON MICROSCOPY (TEM)**

This analysis will be conducted to observe the morphology and size of the hybrid nanospheres produced.

***Base principles of TEM technique.*** *Transmission electron microscopy (TEM)* is a microscopy technique in which a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through. TEMs are capable of imaging at a significantly higher resolution than light microscopes owing to the small de Broglie wavelength of electrons. This enables the instrument's user to examine fine detail even as small as a single column of atoms, which is thousands of times smaller

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than the smallest resolvable object in a light microscope. TEM forms a major analysis method in a range of scientific fields, in both physical and biological sciences. TEMs find application in cancer research, virology, material science as well as pollution, nanotechnology, and semiconductor research.

The first TEM was built by Max Knoll and Ernst Ruska in 1931, with this group developing the first TEM with a resolution greater than that of light in 1933 and the first commercial TEM in 1939.

A TEM is composed of several components, which include a vacuum system in which the electrons travel, an electron emission source for generation of the electron stream, a series of electromagnetic lenses, as well as electrostatic plates. The latter two allow the operator to guide and manipulate the beam as required. What is also required is a device to allow the insertion into, motion within, and removal of specimens from the beam path. Imaging devices are subsequently used to create an image from the electrons that exit the system.

To increase the mean free path of the electron gas interaction, a standard TEM is evacuated at low pressures, typically in the order of  $10^{-4}$  Pa. The need for this is twofold: first the allowance for the voltage difference between the cathode and the ground without generating an arc, and secondly to reduce the collision frequency of electrons with gas atoms to negligible levels. TEM components such as specimen holders and film cartridges must be routinely inserted or replaced requiring a system with the ability to re-evacuate on a regular basis. As such, TEMs are equipped with multiple pumping systems and airlocks and are not permanently vacuum sealed. Sections of the TEM may be isolated by the use of pressure-limiting apertures, to allow for different vacuum levels in specific areas, such as a higher vacuum of  $10^{-4}$  to  $10^{-7}$  Pa or higher in the electron gun in high-resolution or field-emission TEMs. High-voltage TEMs require ultra-high vacuums with a range of  $10^{-7}$  to  $10^{-9}$  Pa to prevent generation of an

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electrical arc, particularly at the TEM cathode<sup>10</sup>. As is the case for higher voltage TEMs, a third vacuum system may operate, with the gun isolated from the main chamber either by use of gate valves or by the use of a differential pumping aperture. The differential pumping aperture is a small hole that prevents diffusion of gas molecules into the higher vacuum gun area faster than they can be pumped out. For these very low pressures either an ion pump or a getter material is used.

Poor vacuum in a TEM can cause several problems, from deposition of gas inside the TEM onto the specimen as it is being viewed through a process known as electron beam induced deposition, or in more severe cases, damage to the cathode from an electrical discharge<sup>10</sup>. Vacuum problems due to specimen sublimation are limited by the use of a cold trap to adsorb sublimated gases in the vicinity of the specimen<sup>11</sup>.

In a TEM, the electrons make up the beam, through a section where the vacuum has been created previously, and then pass completely through the sample. This must have an extremely reduced thickness, between 50 and 500 nm. The resolving power is approximately 0.2 nm, which is about 500,000 times greater than that of the human eye. This type of microscope is provided with complex systems using the modification of electrical and magnetic fields which are able to drive the electrons through magnetic "lenses" necessary to widen considerably the electron beam already passed through the sample to ensure that the image is not magnified. So this type of instrument, given its high resolving power, makes it possible to obtain a certain size: the morphology of samples of a much smaller size compared to those that can be analyzed with the scanning electron microscope, and seems to be suitable for the samples analyzed in this work that are apparently of a size approximately equal to 2 nm.

***Instrument and conditions used.*** The transmission electron micrographs which are presented in this work have been obtained with *JEOL 1200*, operating at 120 KeV. The samples were prepared by

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suspension of the solid in water and, subsequently, a drop of the upper part of the suspension is deposited on a mesh of copper (300 mesh) covered with a thin perforated carbon layer (honey carbon film).

### **5.6.5 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)**

Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical chemistry technique used for determining the content and purity of a sample as well as its molecular structure.

**Base principles of Nuclear Magnetic Resonance.** Nuclear magnetic resonance spectroscopy, most commonly known as NMR spectroscopy, is a research technique that exploits the magnetic properties of certain atomic nuclei. It determines the physical and chemical properties of atoms or the molecules in which they are contained. It relies on the phenomenon of nuclear magnetic resonance and can provide detailed information about the structure, dynamics, reaction state, and chemical environment of molecules. The intramolecular magnetic field around an atom in a molecule changes the resonance frequency, thus giving access to details of the electronic structure of a molecule.

Most frequently, NMR spectroscopy is used by chemists and biochemists to investigate the properties of organic molecules, although it is applicable to any kind of sample that contains nuclei possessing spin.

Suitable samples range from small compounds analyzed with 1-dimensional proton or carbon-13 NMR spectroscopy to large proteins or nucleic acids using 3 or 4-dimensional techniques. The impact of NMR spectroscopy on the sciences has been substantial because of the range of information and the diversity of samples, including solutions and solids. Recently, two-dimensional NMR for solids has started to be used. Thanks to this it is possible to obtain information through the connectivity between

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the atoms. So, it is possible to analyze the connectivity Si-O-Si of zeolites ( $^{29}\text{Si}$ ).

***Instrument and conditions used.*** Solid state MAS-NMR spectra were recorded at room temperature under magic angle spinning (MAS) in a Bruker AV-400 spectrometer. The single pulse  $^{29}\text{Si}$  spectra were acquired at 79.5 MHz with a 7 mm Bruker BL-7 probe using pulses of 3.5  $\mu\text{s}$  corresponding to a flip angle of  $3/4 \pi$  radians, and a recycle delay of 240 s. Pulses of 0.5  $\mu\text{s}$  to flip the magnetization  $\pi/20$  rad, and a recycle delay of 2 s were used. The  $^{13}\text{C}$  spectra were recorded with a 7 mm Bruker BL-7 probe and at a sample spinning rate of 5 kHz.  $^{13}\text{C}$  and  $^{29}\text{Si}$  were referred to adamantane and tetramethylsilane, respectively.  $^{13}\text{C}$  NMR is an application of NMR that has been used to know the carbon atoms in organic molecules, while  $^{29}\text{Si}$ -NMR, allowed to identify quantitatively and qualitatively how this is linked to the other silicon atoms or OH groups present.

### **5.6.6 POWERED X-RAY DIFFRACTION (XRD)**

XRD technique has been used to quantify the various components of a solid sample, and also to obtain information on the crystalline structure and crystallite size.

***Base principles of XRD diffraction technique:*** *X-ray crystallography* is a tool used for identifying the atomic and molecular structure of a crystal, in which the crystalline atoms cause a beam of incident X-rays to diffract into many specific directions. By measuring the angles and intensities of these diffracted beams, a crystallographer can produce a three-dimensional picture of the density of electrons within the crystal. From this electron density, the mean positions of the atoms in the crystal can be determined, as well as their chemical bonds, their disorder and other information.

In its first decades of use, this method determined the size of atoms, the lengths and types of chemical bonds, and the atomic-scale differences

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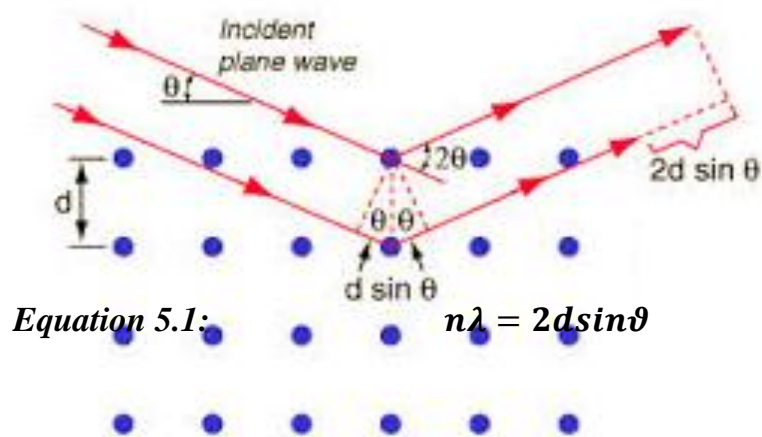
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among various materials, especially minerals and alloys. The method also revealed the structure and function of many biological molecules, including vitamins, drugs, proteins and nucleic acids such as DNA. X-ray crystallography is still the chief method for characterizing the atomic structure of new materials and in discerning materials that appear similar using other experiments. X-ray crystal structures can also account for unusual electronic or elastic properties of a material, shed light on chemical interactions and processes, or serve as the basis for designing pharmaceuticals to be used to combat diseases.

X-ray crystallography is related to several other methods for determining atomic structures. Similar diffraction patterns can be produced by scattering electrons or neutrons, which are likewise interpreted as a Fourier transformation. If single crystals of sufficient size cannot be obtained, various other X-ray methods can be applied to obtain less detailed information; such methods include fiber diffraction, powder diffraction and small-angle X-ray scattering (SAXS). If the material under investigation is only available in the form of nanocrystalline powders or suffers from poor crystallinity, the methods of electron crystallography can be applied to determine the atomic structure.

X-rays were discovered by Wilhelm Conrad Röntgen in 1895, just as the studies of crystal symmetry were being concluded. Physicists were initially uncertain of the nature of X-rays, although it was soon suspected (correctly) that they were waves of electromagnetic radiation, in other words, another form of light. After Von Laue's pioneering research, the field developed rapidly, most notably by the physicist William Lawrence Bragg. In 1912–1913, the younger Bragg developed Bragg's law (**Equation 5.1**), which explains why the cleavage faces of crystals appear to reflect X-ray beams at certain angles of incidence  $\theta$ . The variable  $d$  is the distance between atomic layers in a crystal, and the variable  $\lambda$  is the wavelength of the incident X-ray beam;  $n$  is an integer (**Figure 5.5**).

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**Figure 5.5.** Bragg's Law.

The XRD-diffraction is a technique that :

- Measures the average spacing between layers or rows of atoms
- Determines the orientation of a single crystal or grain
- Finds the crystal structure of an unknown material
- Measures the size, shape and internal stress of small of crystalline regions.

**Instrument and conditions used.** In this work, for the sample in which has been used a surfactant (hexadecylamine), powered X-ray diffraction (XRD) has been carried out with a Philips X'PERT diffractometer. Data were collected stepwise over the  $2^\circ \leq 2\theta \leq 20^\circ$  angular region, with steps of  $0.01^\circ 2\theta$ , 20-s/step accumulation time and Cu  $K\alpha$  ( $\lambda = 1.54178 \text{ \AA}$ ) radiation.

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## 5.7 PRODUCTS REACTION DETERMINATION

### 5.7.1 GAS CHROMATOGRAPHY ANALYSIS

*Base principles gas chromatography technique.* In the early 1900s, Gas chromatography (GC) was discovered by Mikhail Semenovich Tsvett as a separation technique to separate compounds. In organic chemistry, liquid-solid column chromatography is often used to separate organic compounds in solution. Among the various types of gas chromatography, gas-liquid chromatography is the method most commonly used to separate organic compounds. The combination of gas chromatography and mass spectrometry is an invaluable tool in the identification of molecules. In a GC analysis, a known volume of gaseous or liquid analyte is injected into the "entrance" (head) of the column, usually using a micro-syringe (or, solid phase micro-extraction fibers, or a gas source switching system). As the carrier gas sweeps the analyte molecules through the column, this motion is inhibited by the adsorption of the analyte molecules either onto the column walls or onto packing materials in the column. The rate at which the molecules progress along the column depends on the strength of adsorption, which in turn depends on the type of molecule and on the stationary phase materials. Since each type of molecule has a different rate of progression, the various components of the analyte mixture are separated as they progress along the column and reach the end of the column at different times (retention time). A detector is used to monitor the outlet stream from the column; thus, the time at which each component reaches the outlet and the amount of that component can be determined. Generally, substances are identified (qualitatively) by the order in which they emerge (elute) from the column and by the retention time of the analyte in the column.

A typical gas chromatograph consists of an injection port, a column, carrier gas flow control equipment, ovens and heaters for maintaining temperatures of the injection port and the column, an integrator chart recorder and a detector.

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**Injection.** A sample port is necessary for introducing the sample at the head of the column. Modern injection techniques often employ the use of heated sample ports through which the sample can be injected and vaporized in a near simultaneous fashion. A calibrated micro-syringe is used to deliver a sample volume in the range of a few microliters through a rubber septum and into the vaporization chamber. Most separations require only a small fraction of the initial sample volume and a sample splitter is used to direct excess sample to waste. Commercial gas chromatographs often allow for both split and split-less injections when alternating between packed columns and capillary columns. The vaporization chamber is typically heated to 50 °C above the lowest boiling point of the sample and subsequently mixed with the carrier gas to transport the sample into the column.

**Carrier Gas:** The carrier gas plays an important role, and varies in the GC used. Carrier gas must be dry, free of oxygen and have a chemically inert mobile-phase employed in gas chromatography. Helium is most commonly used because it is safer than but comparable to hydrogen in efficiency, has a larger range of flow rates and is compatible with many detectors. Nitrogen, argon, and hydrogen are also used depending upon the desired performance and the detector being used. Both hydrogen and helium, which are commonly used on most traditional detectors such as flame Ionization(FID), thermal conductivity (TCD) and Electron capture (ECD), provide a shorter analysis time and lower elution temperatures of the sample due to higher flow rates and low molecular weight. For instance, hydrogen or helium as the carrier gas gives the highest sensitivity with TCD because the difference in thermal conductivity between the organic vapor and hydrogen/helium is greater than other carrier gases. Other detectors such as mass spectroscopy, use nitrogen or argon which offer

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much better advantages than hydrogen or helium due to their higher molecular weights, which improve vacuum pump efficiency.

***Detector.*** The detector is the device located at the end of the column which provides a quantitative measurement of the components of the mixture as they elute in combination with the carrier gas. In theory, any property of the gaseous mixture that is different from the carrier gas can be used as a detection method. These detection properties fall into two categories: bulk properties and specific properties. Bulk properties, which are also known as general properties, are properties that both the carrier gas and the analyte possess but to different degrees. Specific properties, such as detectors that measure nitrogen-phosphorous content, have limited applications but compensate for this by their increased sensitivity.

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**Tabella 5.2 Type of GC Deterctor**

Type of Detector	Applicable sample	Detection Limit
Mass Spectrometer (MS)	Tunable for any sample	0.25 to 100 pg
Flame Ionization (FI)	Hydrocarbons	1pg/s
Thermal Conductivity (TCD)	Universal	500 pg/ml
Electron-Capture (ECD)	Halogenated hydrocarbons	5 fg/s
Atomic Emission (AED)	Element- Selective	1 pg
Chemiluminescence (CS)	Oxidizing Reagent	Dark current of PMT
Photoionization (PID)	Vapor and gaseous compound	0.002 to 0.02 $\mu$ /L

Each detector has two main parts. The first part of the detector is the sensor which is placed as close to the column exit as possible in order to optimize detection. The second is the electronic equipment used to digitize the analog signal so that a computer may analyze the acquired chromatogram. An ideal GC detector is distinguished by several characteristics. The first requirement is adequate sensitivity to provide a high resolution signal for all components in the mixture. The sensitivities of the detectors are in the range of 10<sup>-8</sup> to 10<sup>-15</sup> g of solute per second. There are different GC detectors:

**Column Oven:** The thermostatic oven serves to control the temperature of the column within a few tenths of a degree to carry out precise work. The oven can be operated in two manners: isothermal programming or temperature programming. In isothermal programming, the temperature of the column is held constant throughout the entire separation. The optimum column temperature for an isothermal operation is about the middle point of the boiling range of the sample. In the temperature programming method, the column temperature is either increased continuously or in steps as the separation progresses. This

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method is well suited to separating a mixture with a broad boiling point range.

**Column:** There are two general types of column: packed and capillary (also known as open tubular). Packed columns contain a finely divided, inert, solid support material (commonly based on diatomaceous earth) coated with a liquid stationary phase. Most packed columns are 1.5 - 10m in length and have an internal diameter of 2 - 4mm. Open tubular columns, which are also known as capillary columns, come in two basic forms. The first is a wall-coated open tubular (WCOT) column and the second type is a support-coated open tubular (SCOT) column. WCOT columns are capillary tubes that have a thin layer of the stationary phase coated along the column walls. In SCOT columns, the column walls are first coated with a thin layer (about 30 micrometers thick) of adsorbent solid, such as diatomaceous earth, a material which consists of single-celled, sea-plant skeletons. The adsorbent solid is then treated with the liquid stationary phase. While SCOT columns are capable of holding a greater volume of stationary phase than a WCOT column due to its greater sample capacity, WCOT columns still have greater column efficiency.

In addition to the equipment described above, a gas chromatograph can also be equipped with **Mass Spectrometry Detectors.** Mass Spectrometer (MS) detectors are the most powerful of all gas chromatography detectors. In a GC/MS system, the mass spectrometer scans the masses continuously throughout the separation. When the sample exits the chromatography column, it is passed through a transfer line into the inlet of the mass spectrometer. The sample is then ionized and fragmented, typically by an electron-impact ion source. During this process, the sample is bombarded by energetic electrons which ionize the molecule by causing them to lose an electron due to electrostatic repulsion. Further bombardment causes the ions to fragment. The ions are then passed into a

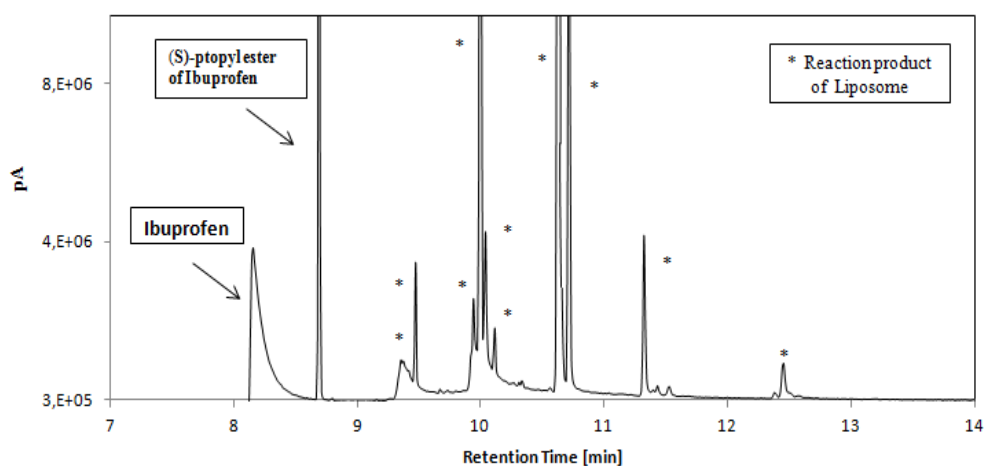
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mass analyzer where the ions are sorted according to their  $m/z$  value, or mass-to-charge ratio. Most ions are only singly charged.

The Chromatogram will point out the retention times and the mass spectrometer will use the peaks to determine what kind of molecules exist in the mixture.

***Instrument and condition used.*** For mass spectroscopy, has been used an Agilent 6890N equipped with a column HP5MS (5%-Phenyl-95% methylpolysiloxane; 30m x 250  $\mu\text{m}$  x 0.25  $\mu\text{m}$ ). By GC/MS analysis it is possible to Know retention times and the mass spectrometer will use the peaks to determine what kind of molecules exist in the mixture. An example is given in **Figure 5.6**



**Figure 5.6** Mass spectrometry chromatogram of esterification of racemic ibuprofen after 72h of reaction (66mM ibuprofen; 66 mM 1-propanol; 10 ml of isooctane; T=37°C)

On the contrary, gas chromatography was performed using a Agilent 7890A gas chromatograph equipped with a flame ionization detector (FID) and a BP5MS column (SGE Analytic Science; low polarity phase; 5% phenyl 95% Polysilphenylene-siloxane; 30m x 250  $\mu\text{m}$  x 0.25  $\mu\text{m}$ ). The injector temperature was 280°C and that of the detector 300°C ; the oven temperature was maintained at 50°C. The carrier gas was nitrogen with a flow rate of 25 mL/min. The temperature program of the column is:

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- 2 min at 50°C;
- 30°C/min until 280°C

The ester yield were calculated by the following formula:

$$\text{ester yield \%} = \left[ \frac{(A_E/PM_E)}{\left(\frac{A_E}{PM_E}\right) + \left(\frac{A_{ib}}{PM_{ib}}\right)} \right] * 100$$

Where the  $A_E$  and  $PM_E$  are the peak area and molecular weight of the (S)-ester of ibuprofen (desired product) respectively, while  $A_{ib}$  and  $PM_{ib}$  are respectively, the peak area and molecular weight of ibuprofen.

$A$  indicated the peak area of the component, corrected by corresponding response factor. In order to calculate the corrected area of each reactant and product present in the reaction mixture, the standards of following compound has been analyzed by GC and the correspondent response factor has been calculated. The quantitative analysis to measure formed ester and remaining acid has been carried out using internal standardization method.

**Ester yield [%]**, is given as a percentage of (S)-ibuprofen esterified after the reaction time calculated by gas chromatography analysis.

Considering that the object of this work is to study the catalytic performance of the biocatalyst, have been evaluated two important parameters in enzymatic catalysis: turnover number (TON) and turnover number of frequency (TOF). These parameters have been respectively calculated in accordance with the equations (2) and (3).

$$TON = \left( \frac{\%Ester\ yield}{100} \right) * \left( \frac{mol\ iburpofen}{mol\ enzyme} \right) \quad [/] \quad (2)$$

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**Ester yield** [%] is given as a percentage of (S)-ibuprofen esterified after the reaction time calculated by gas chromatography analysis.

$$TOF = \frac{TON}{Time} \quad [h^{-1}] \quad (3)$$

TON and TOF numbers have been calculated in correspondence with a reaction time equal to 1 hours, for better and more accurate comparison and to minimize the inhibition or competitive effect of the product.

### 5.7.2 CHIRAL GAS CHROMATOGRAPHY ANALYSIS

Chiral GC has seen a dramatic explosion of applications in many fields of study including natural products, asymmetric synthesis, biological studies, environmental contaminants, agriculture, food, flavor, and fragrance with the evaluation of essential oils.

The chromatographic instrumentation necessary for the satisfactory separation of a mixture of stereoisomers is essentially the same as that used in general GC analyses, with the exception that the columns and/or packing need to be specially prepared to provide chiral selectivity. The chromatographic components comprise a number of gas supplies and controls which provide the carrier gas for the column and any other gases that may be necessary, e.g., hydrogen, and air or oxygen for a flame ionization detector (FID). These gases pass through a set of flow controllers and thence, either to the sampling device, or to the appropriate detectors. The injection device can take various forms, depending on the type of column that is to be used. The sample passes directly from the injection device directly into the column which is situated in an oven. The oven temperature is thermostatically controlled, and is fitted with a temperature programmer so that its temperature can be changed linearly at chosen rates, over selected temperature limits during the chromatographic development.

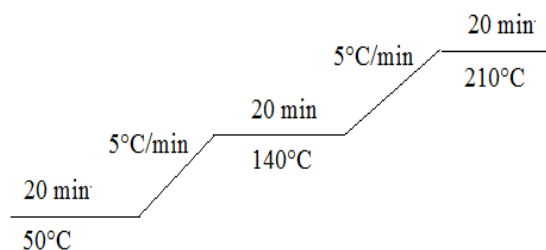
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The column mobile phase (carrier gas) then passes to the detector which is situated in its own thermostatic oven and the concentration of solute in the exiting carrier gas is continuously monitored. A range of different detectors is usually available and their output is acquired and processed by the dedicated computer system. The modern gas chromatograph includes a computer which acquires the data, processes it using standard software, and then reports the results on an appropriate printer. The data from the detector is first digitized, then acquired by the computer and stored on disk. At the end of the run, the data is retrieved, processed, the report constructed and then printed out.

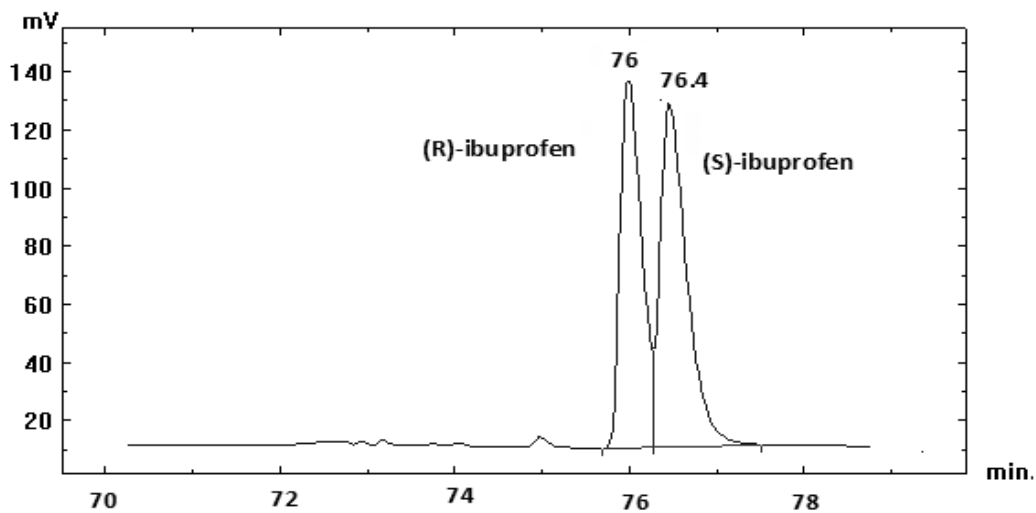
**Instrument and condition used.** Chiral analysis of both enantiomers of ibuprofen were conducted by chiral gas chromatography Agilent 8000series using a BETADEx™ 120 column (35% phenyl 65% dimethylsiloxane; 30m x 0.25 mm x 0.25µm). The temperature program of column is:

- 20 minutes at 50°C
- 5°C/min until 140°C
- 20 minutes at 140°C
- 5°C/min until 210°C
- 20 minutes at 210°C



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The retention time is 76.4 min for (S) -ibuprofen and 76 for (R)-  
ibuprofen (**Figure 5.7**)



**Figure 5.7** Chromatogram of (R,S)-ibuprofen by chiral gas chromatography.

Enantiomeric excess (ee) has been calculated according to Equation.

$$ee \% = \frac{R-S}{S+R} * 100 \quad \text{For } R > S$$

*R* is the peak area for the R(-) enantiomer of ibuprofen and *S* is the peak area for the S(+) enantiomer of ibuprofen.

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# **CHAPTER VI**

## ***Results and Discussions***

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## 6.1 CHARACTERIZATION CATALYSTS RESULTS

The preparation of this type of hybrid nanospheres was carried out through two consecutive steps: (a) synthesis of liposome with encapsulated enzyme (lipase) by means of emulsion methodology from lecithin in chloroform/water system, and (b) formation of porous silica shell around the liposomal phase. The process of preparation of the samples has been described in Chapter V, paragraph 5.3.

This first part of work focused on the influence of various parameters in the preparation of hybrid nanospheres:

- silica/liposome weight ratio
- mixing times of Liposome/Lipase solution

These parameters were analyzed and optimized in order to obtain biocatalysts with a homogeneous organic and inorganic final conformation maintaining a high loading of contained enzymes.

In **Table 6.1** and **Figure 6.1 (a, b)** the tendency of enzyme immobilization quantity towards Liposome/Lipase solution mixing time (from 1 to 12 hours), for each Liposome/Silica ratio tested, is reported.

**Table 6.1 Immobilization results at different mixing time of Liposome/Lipase solution, obtained by UV-VIS methods 235/280 nm\*.**

Mixing Time [h]	Sample Code	Lecithin Amount [g]	Silica Amount (TEOS) [g]	SiO <sub>2</sub> /Liposome Weight ratio	mg of Immob. Enzyme	$\frac{(mg\ Immob.\ Enzyme)}{g\ SiO_2}$
1h	RL1	0.44	0.88	2	2.7	3.1
	RL2	0.44	0.44	1	2	4.5
	RL3	0.88	0.44	0.5	2.5	5.7
2h	RL1	0.44	0.88	2	4.5	5.1
	RL2	0.44	0.44	1	3.1	7.1
	RL3	0.88	0.44	0.5	3.9	8.7
4h	RL1	0.44	0.88	2	1.7	1.9
	RL2	0.44	0.44	1	2.1	4.8
	RL3	0.88	0.44	0.5	2.3	5.2
6h	RL1	0.44	0.88	2	1.6	1.8
	RL2	0.44	0.44	1	1.2	2.7
	RL3	0.88	0.44	0.5	1.5	3.4
12h	RL1	0.44	0.88	2	1.5	1.7
	RL2	0.44	0.44	1	1	2.3
	RL3	0.88	0.44	0.5	1.2	2.7

*The values shown in the figure are the average of the values obtained after three tests.*



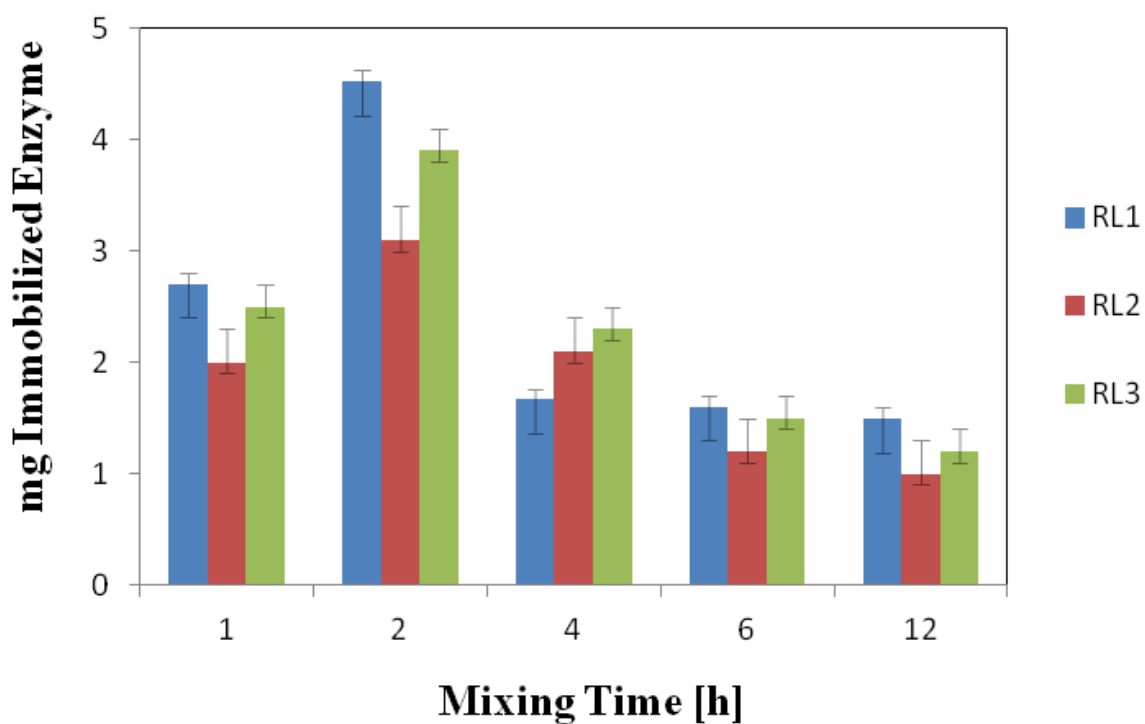


Figure 6.1(a) mg of immobilized enzyme vs Lipase/Liposome mixing time.

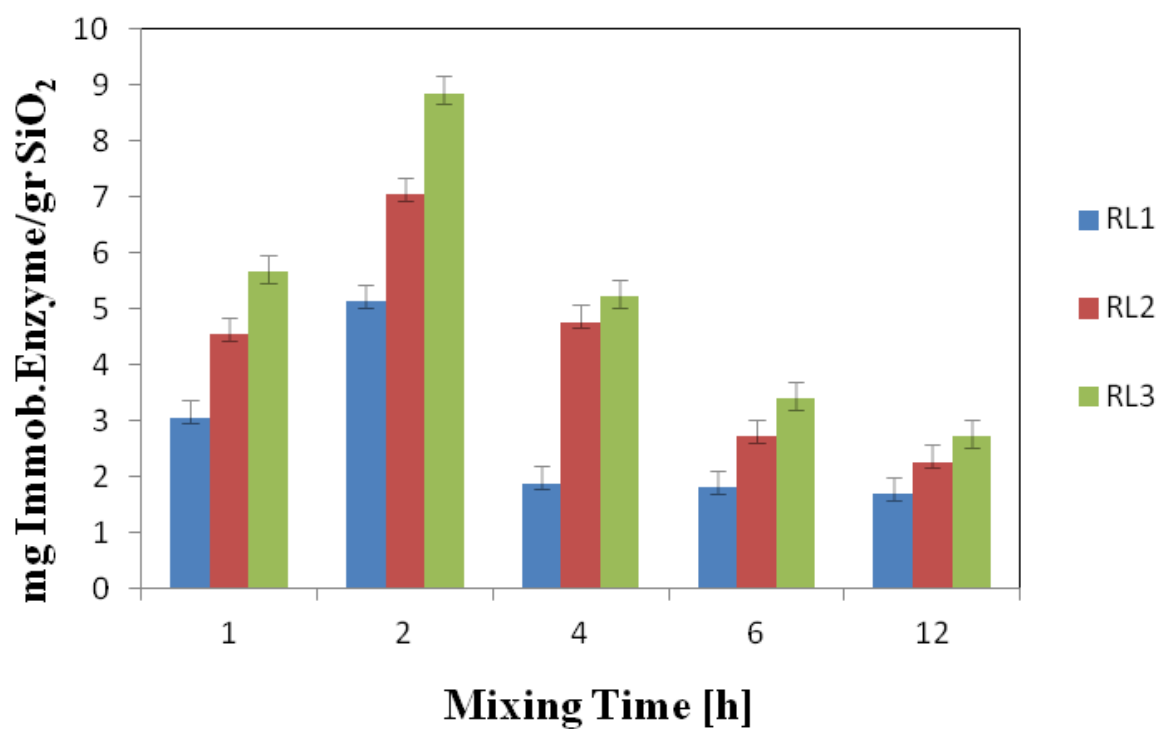


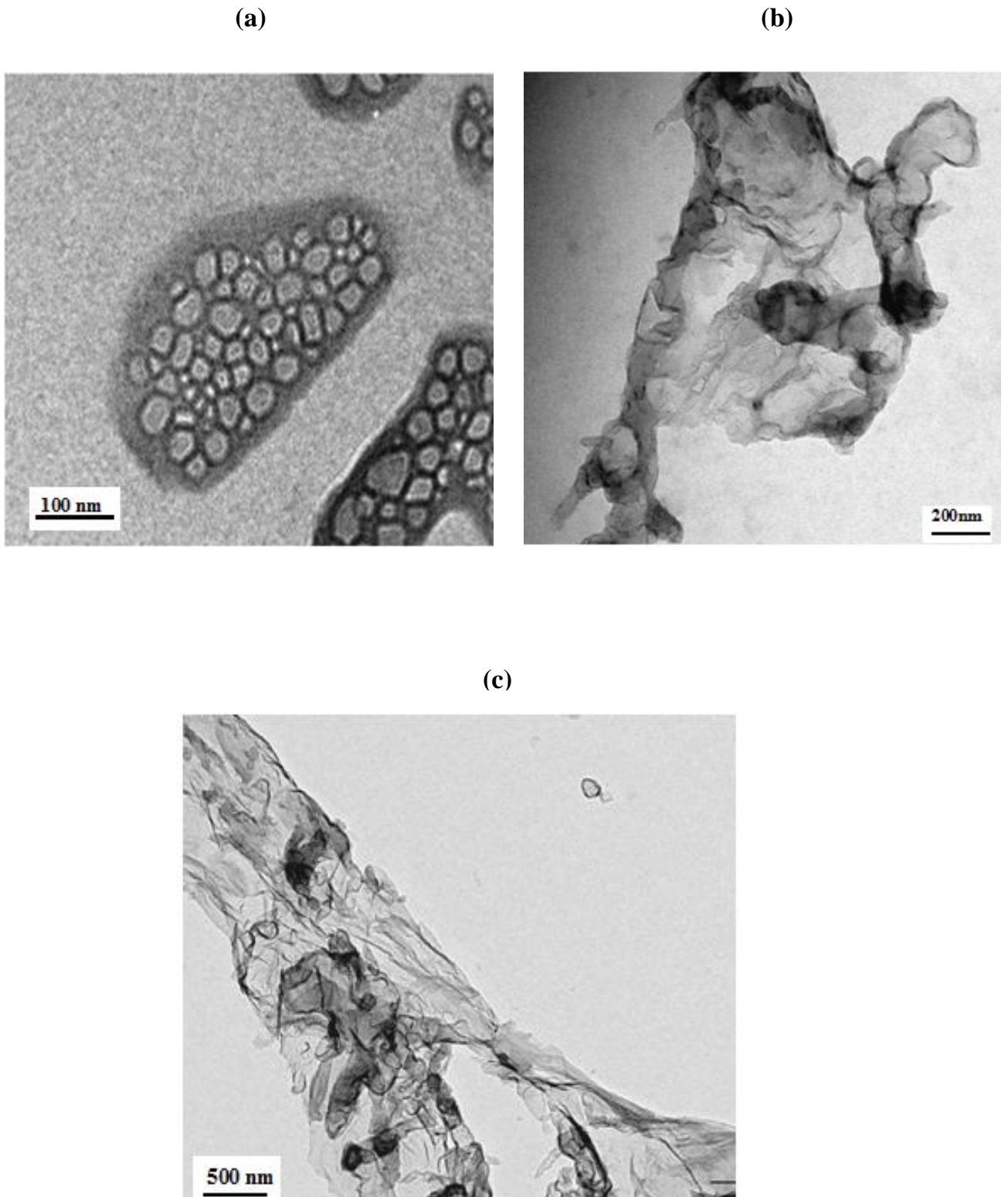
Figure 6.1 (b) mg of enzyme immobilized/grams of SiO<sub>2</sub> Lipase/Liposome mixing time.

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From **Figure 6.1 (a)** (and more detailed by **Table 6.1**) it is immediately evident that the mixing time between Liposome and Lipase strongly affects the amount of immobilized enzyme. Particularly, after only 2 h, for each Silica/ Liposome ratio tested, the highest Lipase immobilized amount is obtained. Increasing the mixing time, the amount of retained enzyme decrease, most probably due to the damage/break of Liposomal shell. Moreover, at same Liposome/Lipase mixing time, the specific amount of immobilized enzyme (mg Immob. Enzyme/ gr SiO<sub>2</sub>) strongly increases at the decreasing of SiO<sub>2</sub> amount used, as expected.

By TEM analysis results, reported in **Figure 6.2 (a-b-c)**, it is possible to notice, for representative samples prepared with an amount of silica equal (RL2) or lower (RL3) than Liposome, two aspects:

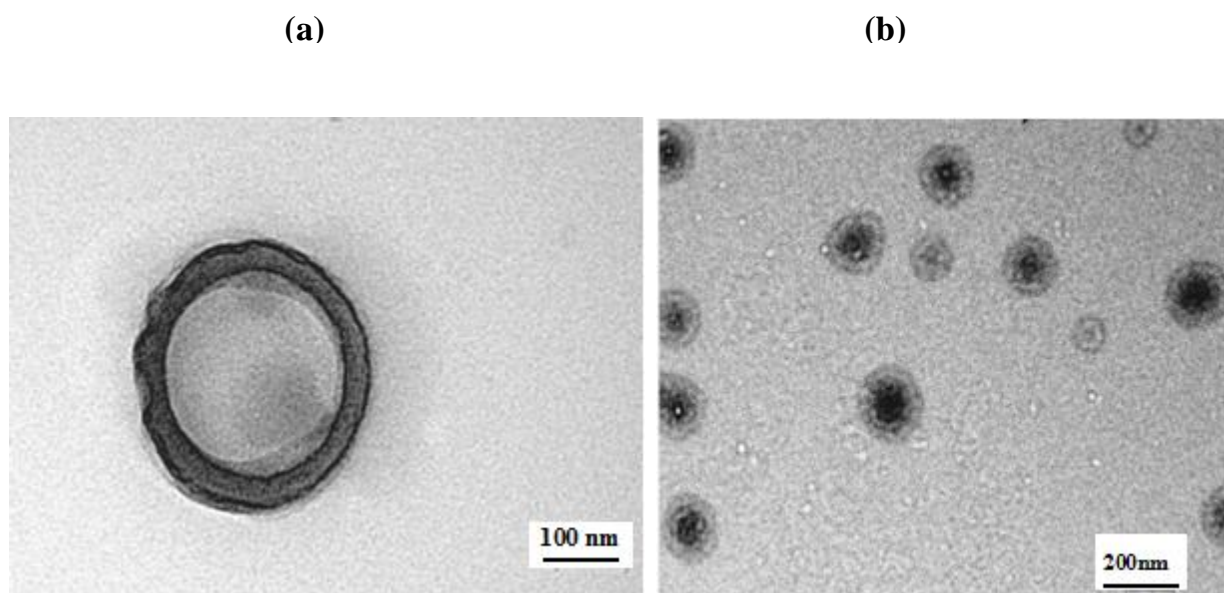
- the equal or lower amount of SiO<sub>2</sub> with respect Liposome (RL2 and RL3 samples) does not permits the complete single liposome sphere coverage by the silica shell (**Figure 6.2a**), but more spheres are included in an unique silica shell;
- increasing the time mixture between Lipase and Liposome, the damage/break and the opening of liposomal shells are evident (**Figure 6.2b** and **6.2c**, respectively for RL2 and RL3 samples). Both aspects, also affect the amount of encapsulated enzyme.



**Figure 6.2** TEM images of: RL2 sample at 2 h (a) and 12 h (b) of mixing time between Lipase and Liposome and (c) TEM image of RL3 at 12 h of mixing time between Lipase and Liposome

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On the contrary, perfect coverage of Liposomal cells by the silica shell can be observed for sample RL1 (where the amount of silica is double respect to the amount of Liposome), both for low (2 hours) and high (12 hours) mixing time between Liposome and Lipase (**Figure 6.3 a and b**). Considering the enzyme immobilization results reported in **Table 6.1** and **Figure 6.1**, the most spheres obtained after 12 hours of mixing time between Lipase and Liposome (**Figure 6.2b**) are probably without enzyme, because only few liposome cells remain unaltered after this long mixing time.



**Figure 6.3** TEM images of RL1 sample at 2 h **(a)** and 12 h **(b)** of mixing time between Lipase and Liposome

In conclusion, matching the results obtained by Uv-Vis method (for enzyme immobilization amount) reported in **Table 6.1** and by TEM analysis (for catalyst morphology), it is possible to say that the optimal hybrid nanospheres are those obtained by a double amount of silica with respect to Liposome quantity and after only 2 hour of mixing time between Liposome and Lipase. This optimized time permits to obtain the spherical

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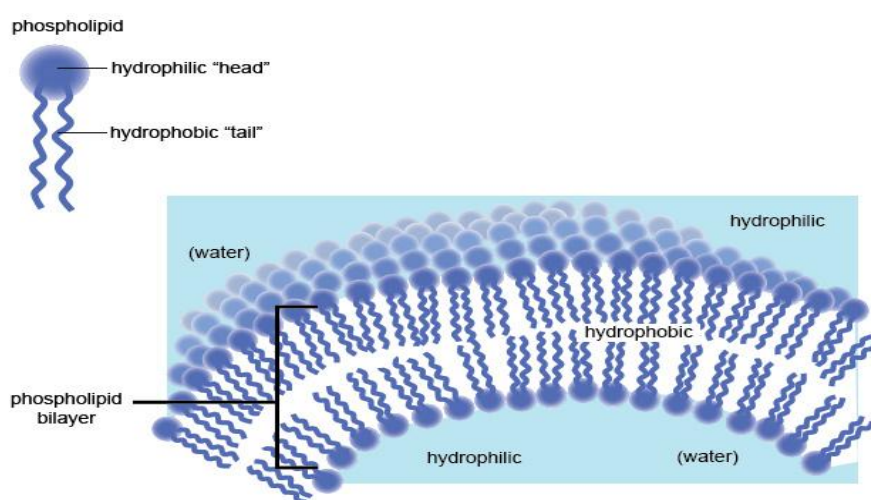
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morphology of Liposomal cells, subsequently covered by adequate amount of silica (**Figure 6.3-RL1 sample**).

Finally, the nanospheres dimensions of RL1 sample are rather homogeneous and closed to 200 nm. Therefore, sample RL1 was selected as reference catalyst and, then, was analyzed with more details.

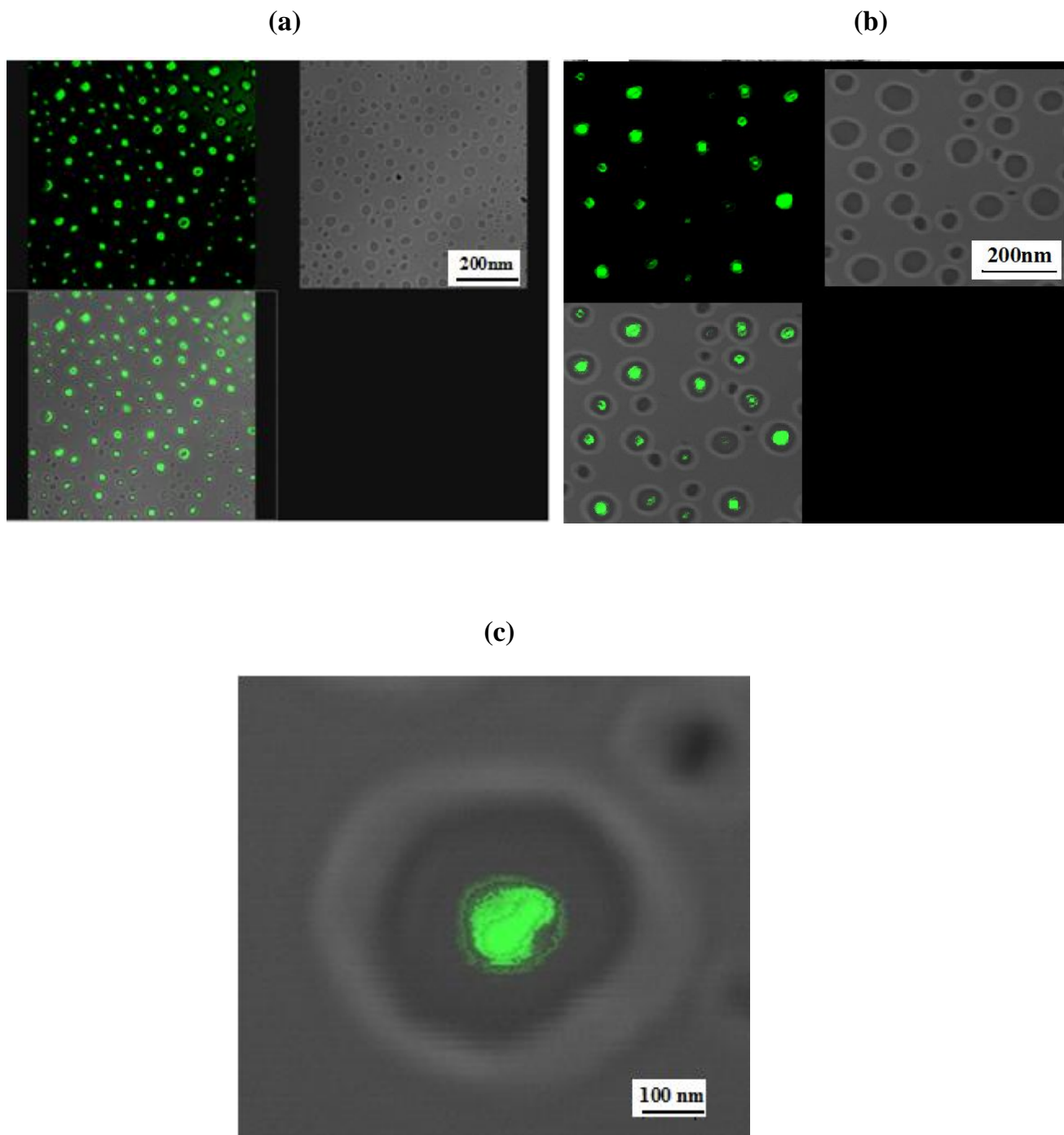
### 6.1.1 Detailed characterization results of selected catalyst

Taking account that both sides of the Liposome are hydrophilic (**Figure 6.4**), it could happen that the enzyme cannot be placed only inside the Liposome, but could be also in contact with the silica shell being located in the outer part of the liposomal phase.



**Figure 6.4** Liposome structure

To clarify more accurately the exact position of the enzyme inside the optimized nanospheres of sample RL1, we used *fluorescence confocal microscopy*. The images obtained by fluorescence confocal microscopic analysis (**Figures 6.5 a-b-c**) confirms, without doubts that, the enzyme is distributed inside the internal liposomal phase.



**Figure 6.5 (a-b-c).** Fluorescence Confocal Microscopy images of sample RL1 (different resolution).

Moreover, the organic Liposome + Lipase phase has been protected by inorganic matrix. In the **Figure 6.6**, a more clear representation of the hybrid-nanospheres structure is reported.

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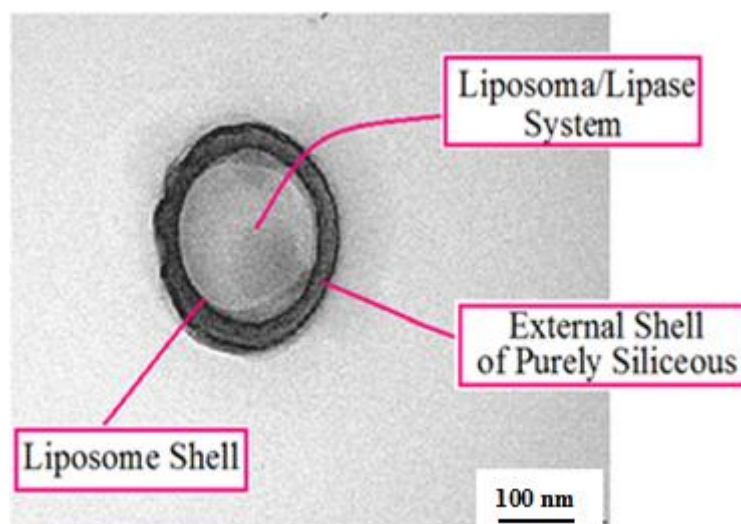


Figure 6.6 TEM image of RL1 sample

The optimized procedure ( $\text{SiO}_2/\text{Liposome}$  ratio equal to 2, and the optimum Lipase/Liposome solution mixing time equal to 2 hours) has been used to prepare different hybrid nanospheres, with silica shell prepared by surfactant. In particular, the silica cover has been prepared using hexadecylamine as surfactant.

In **Table 6.2** the synthesis conditions of nanospheres prepared with surfactant are reported.

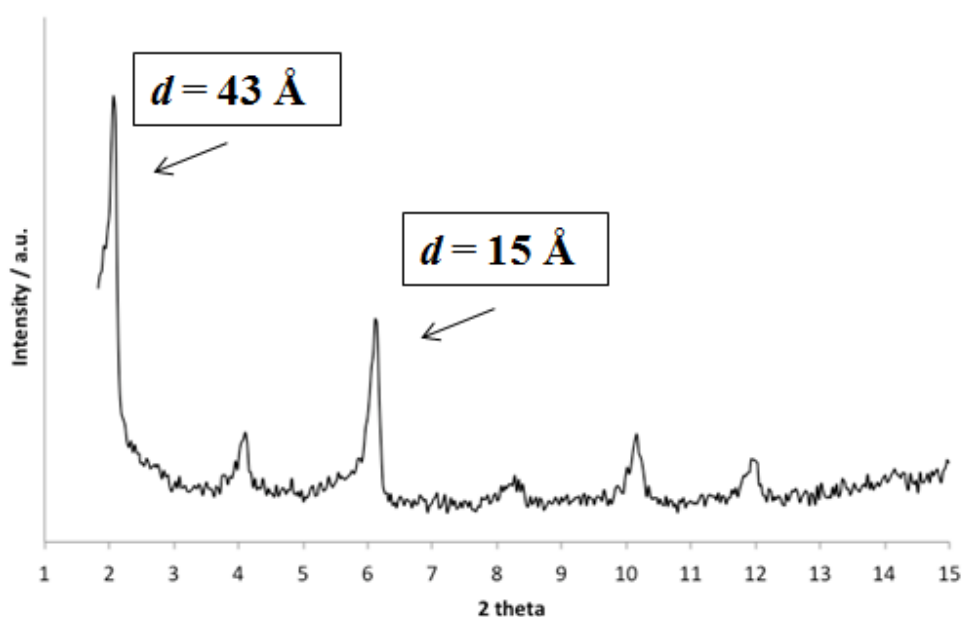
Table 6.2 Enzyme immobilization results at optimized conditions of hybrid nanospheres prepared with surfactant (sample RLH1)

Sample	Lecithin Amount [gr]	Silica Amount (TEOS) [gr]	$\text{SiO}_2/\text{Lipos.}$ Weight ratio	<i>Hexadec</i> $\text{SiO}_2$ molar ratio	Lipasi/Lipos. mixing time [h]	mg of immob. Enzyme	$\frac{\text{mg Immob Enzyme}}{\text{gr SiO}_2}$
RLH1	0.44	0.88	2	0.25	2	2.2	3

The values shown in the figure are the average of the values obtained after three tests.

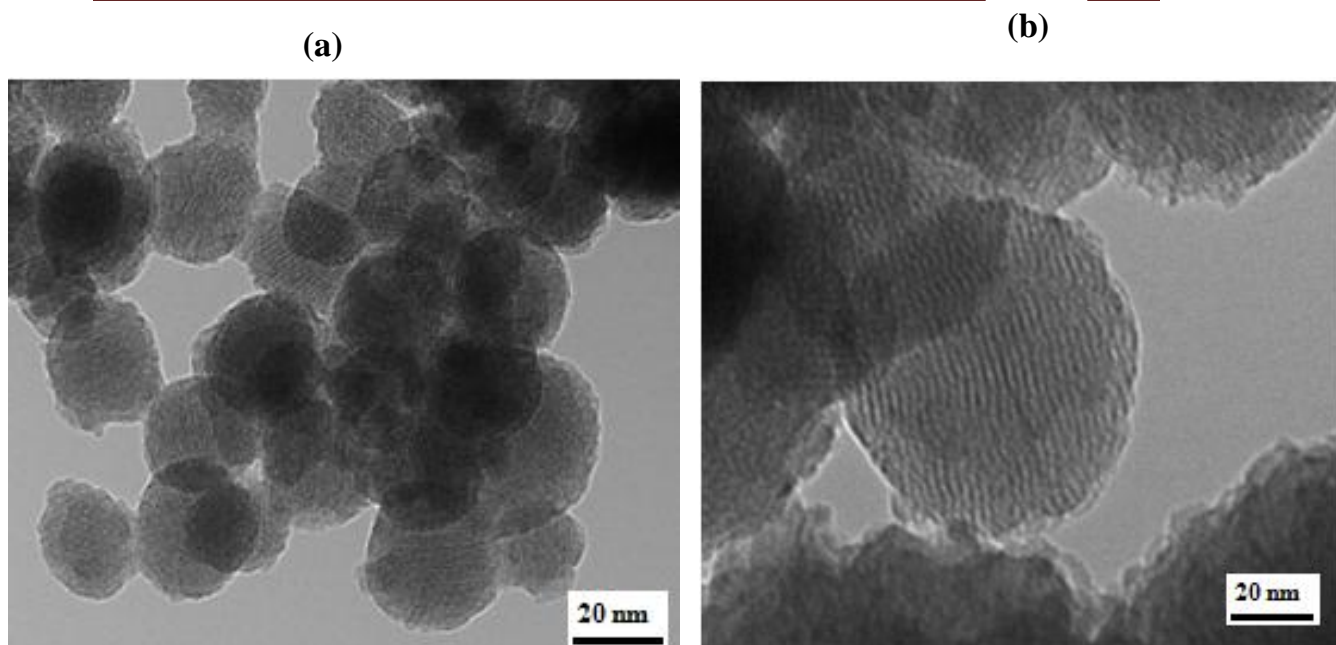
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To get more information about the external silica structure of RLH1 sample, prepared using surfactant, the XRD analysis has been carried out (**Figure 6.7**). With respect to the nanospheres synthesized without surfactant (in which the external silica shell is amorphous, as corroborate by XRD pattern – results not shown), the silica shell of RLH1 sample posses a mesoporous structure, as showed by the XRD diffraction pattern (**Figure 6.7**), where it is possible to observe two main reflection peaks. Particularly, a very sharp diffraction peak with a  $d$  value of 43 Å, which accompanied second-order reflection ( $d$  value of c.a. 15 Å), has been observed in the XRD pattern. Considering also the results of TEM analysis, **Figure 6.8 a**, is evident that the RLH1 sample shows a typical worm-hole structure and the arrays clearly visible on **Figure 6.8 b** are the same that those observed in typical hexagonal structures (MCM-41 or SBA-15). In this type of structure, the channels are always parallel to the support surface. Hence this could be in accordance to the seen parallel arrays. It is possible to conclude that the RLH1 sample is a mesoporous material.



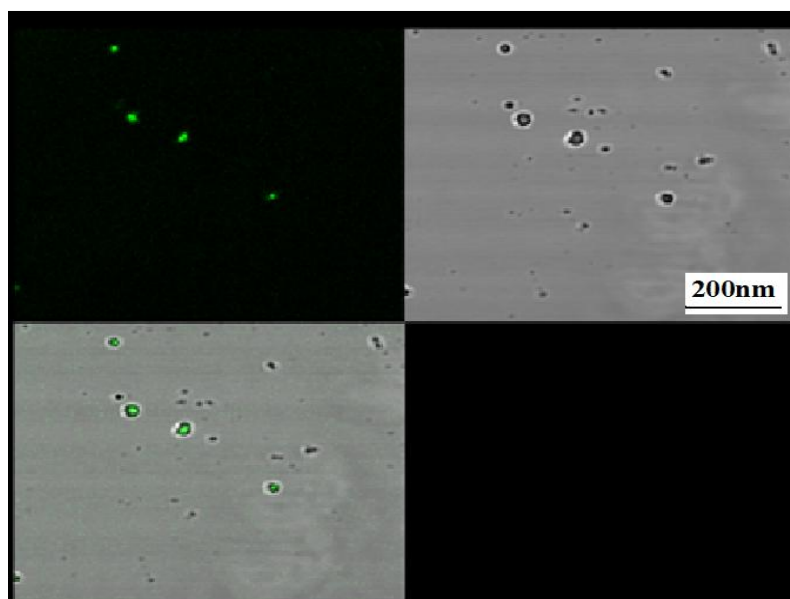
**Figure 6.7.** XRD diffraction pattern of RLH1 sample.





**Figure 6.8 (a-b).** TEM images of RLH1 sample (Nanoparticles with dimension 20÷70 nm)

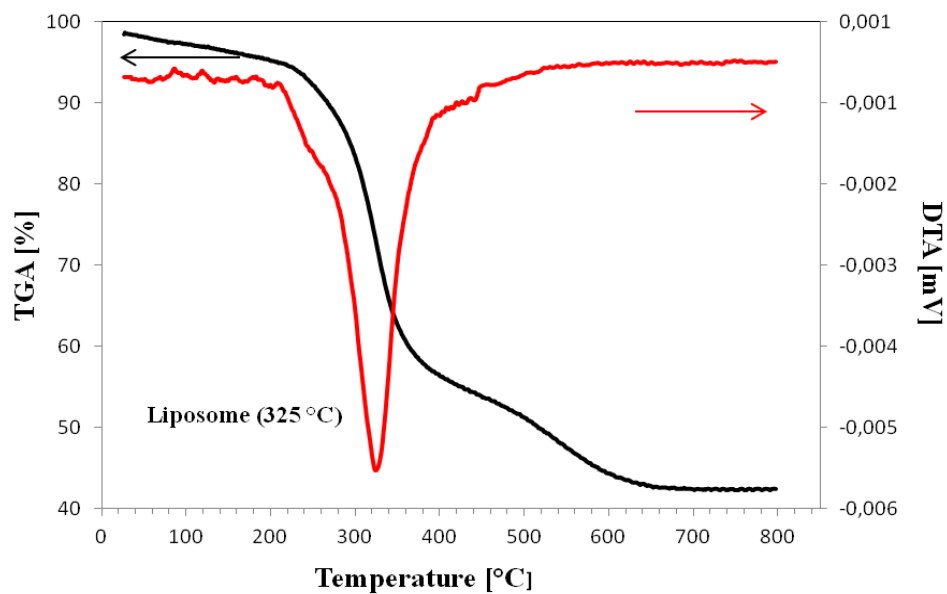
To verify if the use of surfactant does not make a change in the internal structure of nanospheres the confocal fluorescent microscopy has been also applied to the RLH1 sample. In the **Figure 6.9** it is possible to observe that the use of surfactant does not affect the final conformation of the nanospheres.



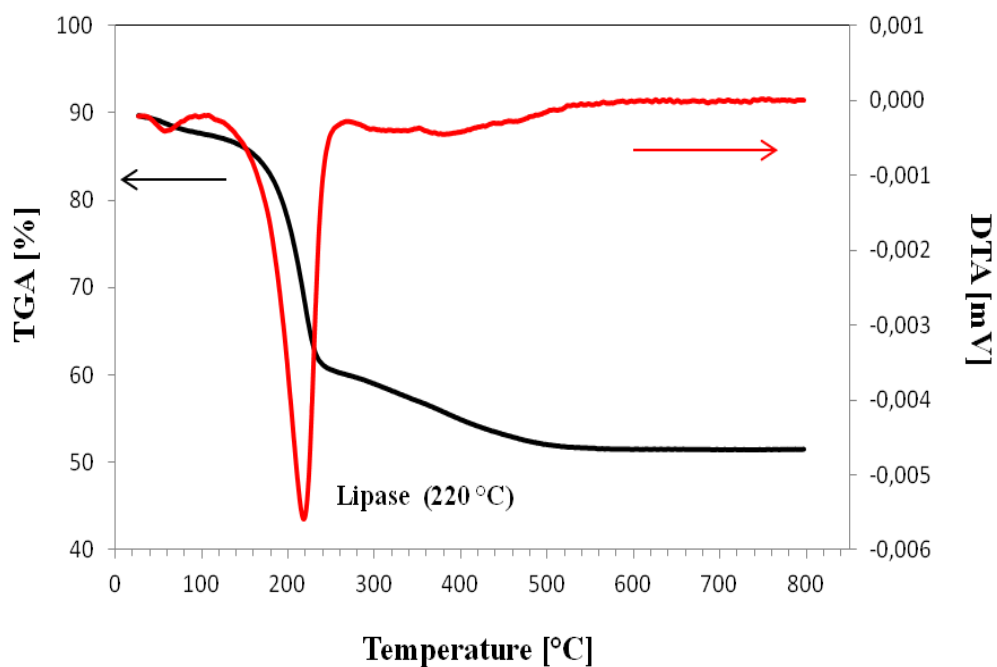
**Figure 6.9.** Fluorescence Confocal Microscopy images of sample RLH1

The incorporation of organic phase (liposome/lipase) into all synthesized hybrid nanospheres is corroborated by TGA-DTA analysis.

In order to identify the characteristic peaks of used substances, the TG-DTA analysis has been carried out on blank samples containing only liposome or lipase, separately (**Figures 6.10** and **6.11**, respectively)

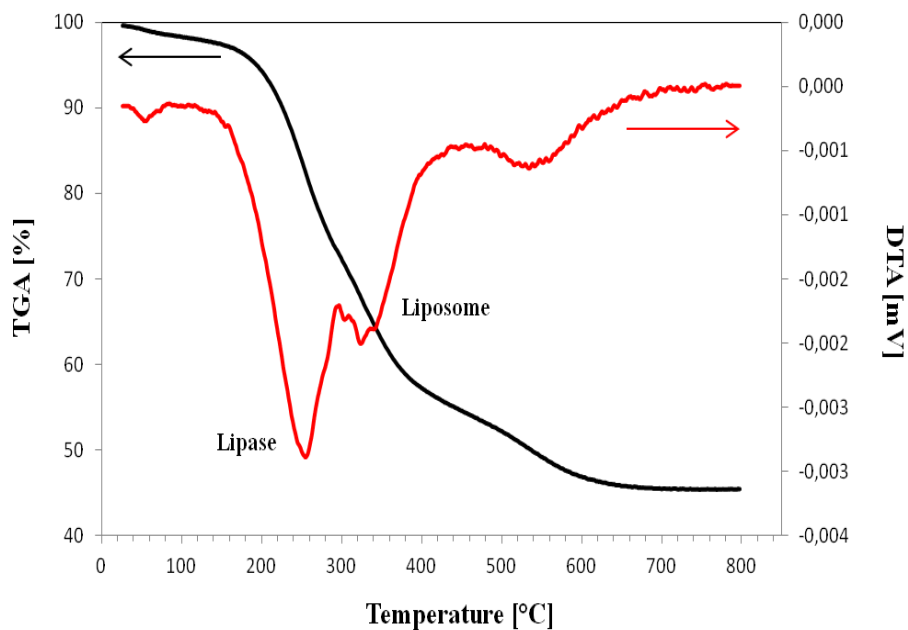


**Figure 6.10.** TGA-DTA of blank sample containing only liposome

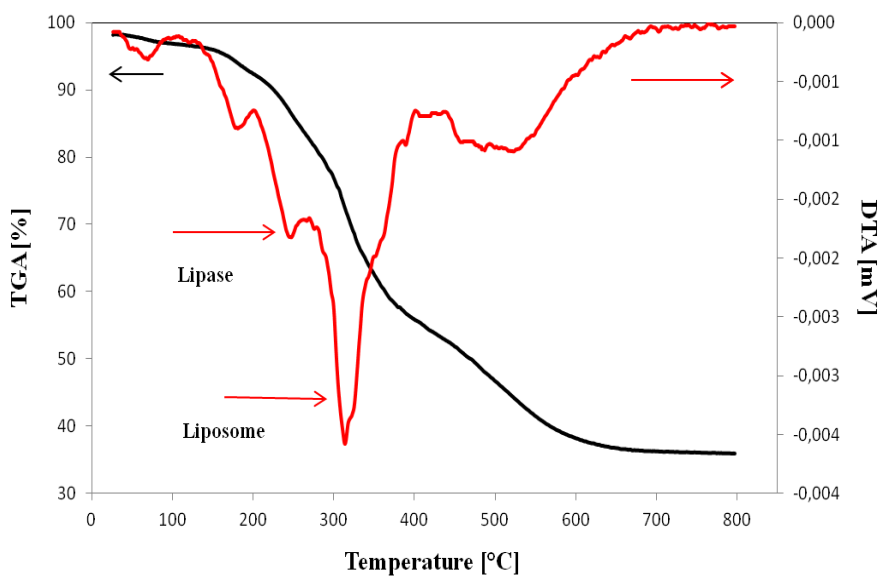


**Figure 6.11.** TGA-DTA of blank sample containing only lipase (Palatase 20000L)

The coexistence of organic phases (lipase and liposome) in the nanospheres is confirmed by the TGA-DTA analysis carried out on the samples RL1 and RLH1 ((**Figures 6.12-6.13** respectively)). In these cases, it is possible to observe the contemporary presence of the two characteristics peaks of lipase (at c.a. 250°C) and of liposome (at c.a. 340°C), for both samples.



**Figure 6.12.** TGA-DTA of Sample RL1

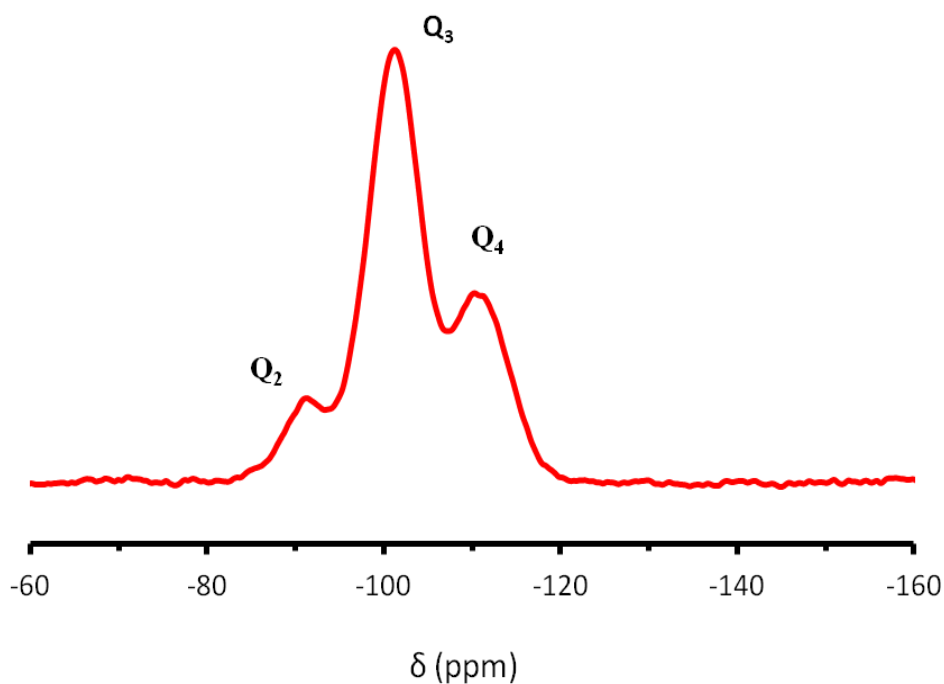


**Figure 6.13.** TGA-DTA of sample RLH1

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The Si chemical neighborhood of nanospheres shell has been investigated by  $^{29}\text{Si}$  and  $^{13}\text{C}$ -NMR analyses.

By the  $^{29}\text{Si}$ -NMR spectrum of RL1 sample (**Figure 6.14**) shows lines in the range of -90 to -120 ppm, corresponding to different Si environments in the silica shell<sup>1</sup>. The signals above -110 ppm can be assigned to Q<sup>4</sup> silicon species  $[\text{Si}(\text{OSi})_4]$ , the line between -104 and -101 ppm are attributed to Q<sup>3</sup> silanol or single hydroxyl groups  $[\text{HOSi}(\text{OSi})_3]$ , while the chemical shift at -93 ppm can be attributed to Q<sup>2</sup> species or geminal groups<sup>1</sup>. The presence of these signals in the  $^{29}\text{Si}$ -NMR spectrum of RL1 confirms the formation of silica shell.

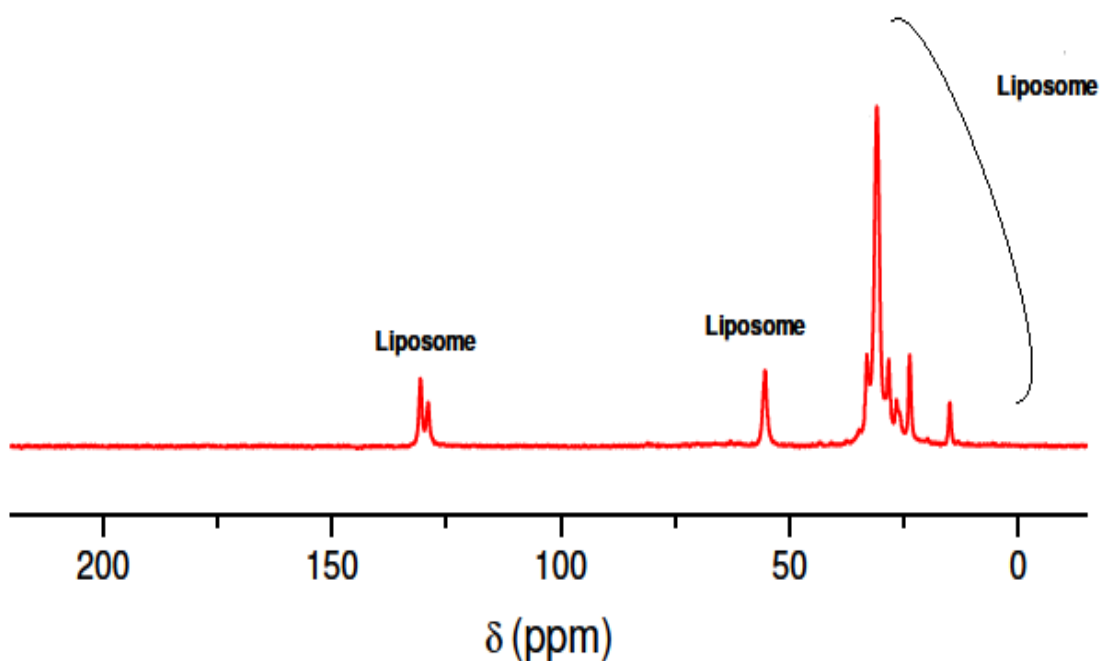


**Figure 6.14.**  $^{29}\text{Si}$  CP/MAS NMR spectrum of sample RL1

The  $^{13}\text{C}$ -NMR spectrum of RL1 sample (**Figure 6.15**) shows the typical lines assigned to the carbon atom of Liposome<sup>2</sup>. This spectrum confirms that the Liposome organic fragment remains intact during the immobilization and are effectively present into nanospheres. The

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assignment of the other carbon atoms from Lipase is not possible because the intensity of the peaks is much lower than those of the Liposome, due to the lower concentration of the enzyme with respect to those of Liposome. It should be possible also to observe the presence of probable no-hydrolyzed alkoxide groups of TEOS, but the signals relative to the carbon atoms of Liposome may hide the signals of carbon relative this groups. However, considering that a sol-gel hydrolysis in a medium fluoride has been applied, we would expect that a complete hydrolysis of TEOS occurred.



**Figure 6.15.**  $^{13}\text{C}$  CP/MAS NMR spectrum of sample RL1

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Finally, textural properties of nanospheres have been analyzed by N<sub>2</sub> adsorption/desorption technique. In the **Table 6.3** these results for RL1 and RLH1 samples are summarized. Before analyses, all samples have been calcinated at 600°C under vacuum condition for 8 hours.

**Table 6.3** Textural properties of RL1 and RLH1 silica shell

Sample	SBET [m <sup>2</sup> g <sup>-1</sup> ]	Pore Size* [Å]	Pore Volume [cm <sup>3</sup> /g]
<b>RL1</b>	430	114	1.2
<b>RLH1</b>	370	151	1.4

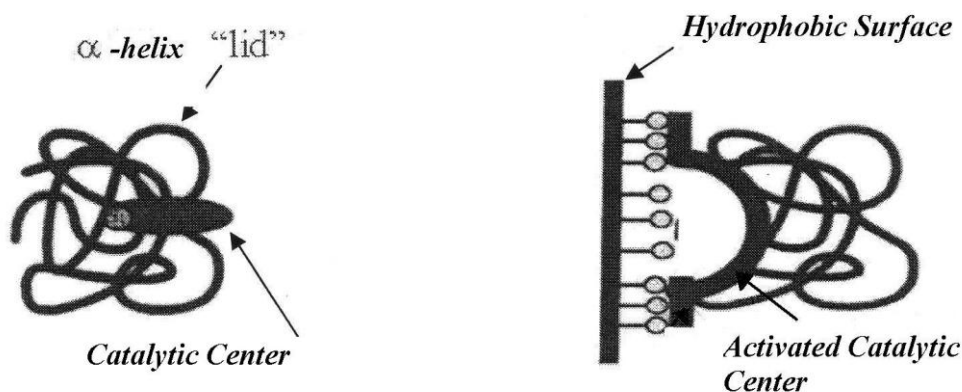
\* calculated by BJH method on adsorption branch of nitrogen isotherms

The specific surface area of both samples, high and closed to c.a 400 m<sup>2</sup>/gr, permits to obtain a preliminary knowledge of the structural properties of the materials, also observed by XRD and TEM analyses results.

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## 6.2 CATALYTIC TEST RESULTS

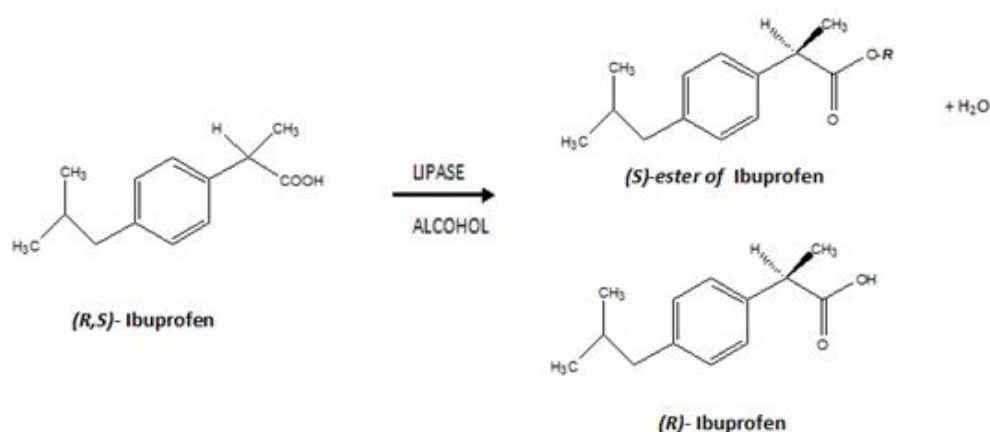
As discussed in the **Chapter II-Section 2.6**, the *Rhizomucor miehei* enzyme has a "closed conformation" in its native and free form (**Figure 6.16(a)**). In the presence of hydrophobic substrate, there is an activation of the enzyme due to the opening of the *lid* and the exposure of its catalytic center toward the substrate, that can be converted (**Figure 6.16 (b)**).



**Figure 6.16.** (a) "Closed Form" (Native Form) and (b) "Open Form" (Active Form) of *Rhizomucor miehei* Lipase.

For this reason, should be suitable that, during the immobilization procedure, the enzyme is immobilized in its active conformation. This should take place preferably without enzyme catalytic center deactivation and, therefore, the interactions between *lid* and support should involve the amino acids of the lid rather than the amino acids of the catalytic center.

The catalytic performances of optimized hybrid catalysts (RL1 and RLH1 samples) are studied in the enantioselective esterification of (R,S) - ibuprofen. The scheme of reaction is reported in **Figure 6.17**.



**Figure 6.17.** Enantioselective esterification of (R,S)-ibuprofen with alcohol using Lipase of *Rhizomucor miehei* (RML) as catalyst.

The standard reaction mixture was composed of:

- 10 ml of solvent, containing:
  - ◆ 136 mg of (R,S)-ibuprofen (66 mM);
  - ◆ 50 $\mu$ l of alcohol (66 mM);
  - ◆ ibuprofen: alcohol molar ratio =1:1
  - ◆ 7 % wt of immobilized Lipase with respect ibuprofen;

without of water addition.

The choice to work without water addition, has been the consequence of some considerations made on the basis of the information reported in the literature.

The role of water in functioning biological system operation is contradictory and can have both positive and negative effects on the rate of lipase-catalyzed reactions<sup>4</sup>. The presence of water increases active site polarity, proton conductivity and internal flexibility of the enzyme. Furthermore, the increased flexibility of the enzyme caused by water, which results in high catalytic activity, facilitates the inactivation reactions. Besides, water can lead to the formation of a diffusion barrier for



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hydrophobic substrates and cause hydrolysis which competes with the desired reaction (esterification, transesterification)<sup>4</sup>. Also, previous observations on the effects of the support material on enzymatic activity indicates that the water absorbing capacity (the "aquaphilicity") of the support material influences the catalytic activity of the immobilized enzyme to a large extent<sup>5</sup>. Several later studies have confirmed that enzyme stability generally decreases with increasing water activity<sup>6,7</sup>. Therefore, it was realized that elimination of the major part of the water from the surroundings of the enzyme can cause pronounced stabilization compared to the situation in aqueous solution.

In addition, it is known that some Lipases, such as *Rhizomucor miehei*, *Rhizopus lipases* and *Candida Antarctica Lipase B* show optimal activity at low water content (<0.02 g/g) or thermodynamic water activity  $a_w < 0.2$ <sup>8,9</sup>.

The quantity of Lipase used in the reaction is always the 7%wt with respect to ibuprofen. This is the enzyme minimum quantity easy to handle. In fact, it is important to notice that the amount of Lipase in the catalyst is very low (5.1 mg Lipase/gr SiO<sub>2</sub> for RL1 sample, see **Table 6.1**, and 3 mg Lipase/gr SiO<sub>2</sub> for RLH1 sample, see **Table 6.2** ). Finally, the quantities of alcohol (66 mM; 1:1 molar ratio with respect to ibuprofen) and solvent (10 ml) used are defined after reading of published works<sup>10-12</sup>.

At fixed time, an aliquot of 50  $\mu$ L of the solution have been withdrawn and diluted in 50  $\mu$ L of solvent, analyzed by gas chromatography technique.

Different variables were studied in this process:

- type of the solvent (apolar iso-octane and polar dimethylformamide);
- nature of the alcohol (1-methanol, 1-propanol and 1-butanol);

- 
- reaction temperature (27°, 37°, 50° and 80°C).

After 72 hour of reaction, the mixture was filtered and analyzed by chiral gas chromatography to determine enantiomeric excess. The final time of reaction (72 hours) is defined after reading of several published works<sup>11,12</sup>, in which the authors (Sanchez et al. and Lopez-Belmonte et al.) have obtained the highest performance of a commercial lipase from *Rhizomucor miehei* (Lipozyme IM) in the esterification of racemic ibuprofen after 72 hours of reaction. Sanchez et al. tested also longer reaction time (up to 112 h), but they did not register higher catalytic performance of biocatalyst.

### 6.2.1 Influence of catalyst composition

Before the use of catalyst in the enantioselective esterification of (R,S)-ibuprofen reaction, reaction test to evaluated the role of catalyst components in the reaction, have been carried out.

First of all, we analyzed the role of Liposome. A specific amount of lecithin has been mixed with chloroform in order to dissolve it and to create a homogeneous solution. The solution has been vigorously stirred for about 10 min, until to obtain an emulsion of yellow color. 50 µl of this solution has been analyzed with a Mass Spectrometry Detectors (GC-MAS), in order to determine the representative peaks of Liposome (**Figure 6.18 A**). The remaining part of the solution has been placed in a rotavapor to remove the chloroform and, thereby, has been put in a stopped conical flask with 66 mM of racemic ibuprofen, 66 mM of alcohol, 10 ml of solvent and the reaction has been carried out in a orbital magnetic stirring at 135 rpm, 37°C for 72h. At 72 hours, an aliquot of 50 µl of this solution has been withdrawn and analyzed, also, by GC-MS (**Figure 6.18 C**). At the same reaction conditions the activity of:

- Liposome/alcohol/solvent solution (**Figure 6.18 B**);
-

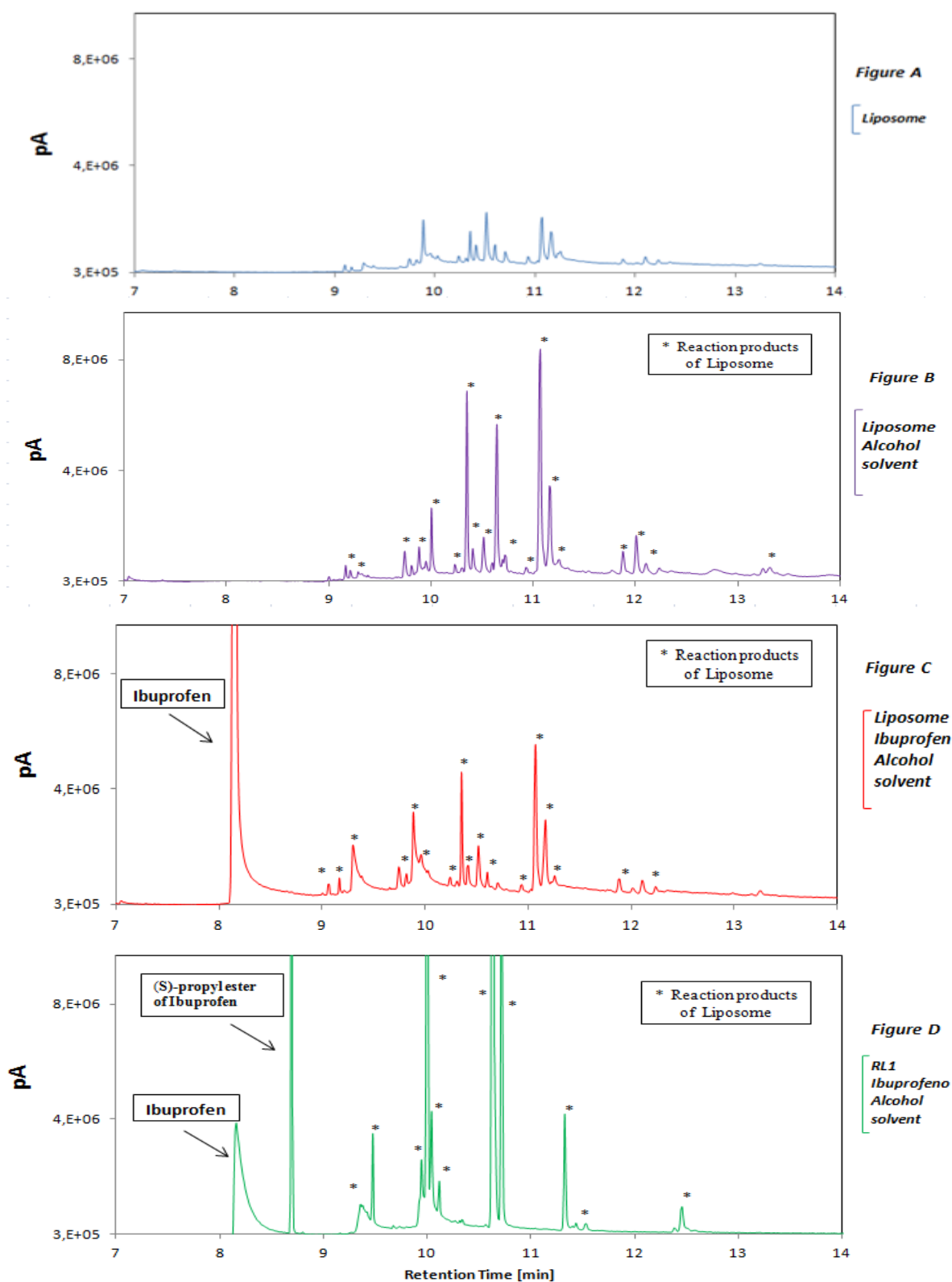
- 
- RL1/alcohol/ibuprofen/solvent solution (**Figure 6.18 D**)

have been analyzed.

In advance, the identification peaks of ibuprofen and (S)-propylester of ibuprofen have been analyzed by GC-MS. The respective retention time are: 8.20 and 8.68 min.

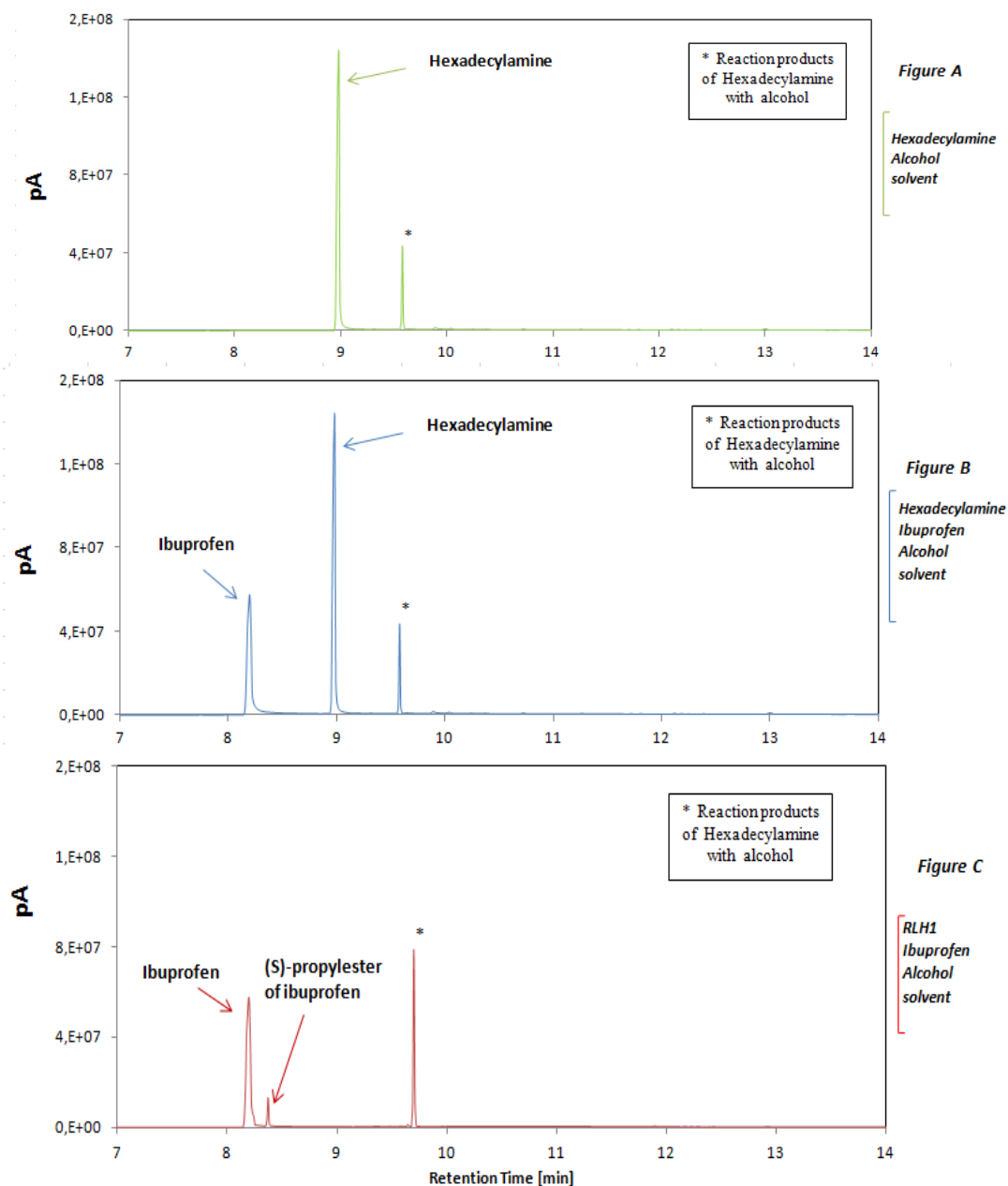
All these chromatograms are compared in order to clarify if the Liposome can reacted with the substrate (ibuprofen), thereby distorts qualitative and quantitative evaluations of the desired product (S-ester of ibuprofen) when the RL1 catalyst will be used.

The chromatograms are reported in the **Figures 6.18** where it is clear that there is no reaction between liposome and ibuprofen because in the **Figure 6.18 C** no peaks related to S-propylester of ibuprofen has been detected. Only after RL1 catalyst adding, the ester appears as reaction product.



**Figure 6.180.** GC-MS chromatograms of: a) pure Liposome; b) Liposome + alcohol + solvent after 72 h of reaction at 37°C; c) Liposome + ibuprofen+ alcohol+ solvent after 72 h of reaction at 37°C; d) RL1 catalyst+ ibuprofen + alcohol+ solvent after 72h of reaction at 37°C. For each test: 66mM of ibuprofen; 66mM of 1-propanol as a alcohol, 10 ml of isoctane as a solvent, 7 %wt of immobilized Lipase (only for test with RL1 catalyst) with respect to ibuprofen, have been used.

The same analysis has been carried out for the sample RLH1 in order to understand the role of surfactant in the reaction and thus the possible reaction between hexadecylamine and ibuprofen. The GC-MS chromatograms are reported in **Figures 6.19**: is evident that no ibuprofen conversion with surfactant occurs and the ester is formed only after RLH1 catalyst adding.



**Figure 6.19.** GC-MS chromatograms of: a) Hexadecylamine + alcohol + solvent after 72 h of reaction at 37°C; b) hexadecylamine + ibuprofen+ alcohol+ solvent after 72 h of reaction at 37°C; c) RLH1 catalyst + ibuprofen + alcohol+ solvent after 72h of reaction at 37°C. For each test: 66mM of ibuprofen; 66mM of 1-propanol, as a alcohol, 10 ml of isoactane, as a solvent, 7 %wt of immobilized Lipase (only for the test with RLH1 catalyst) with respect to ibuprofen, have been used.

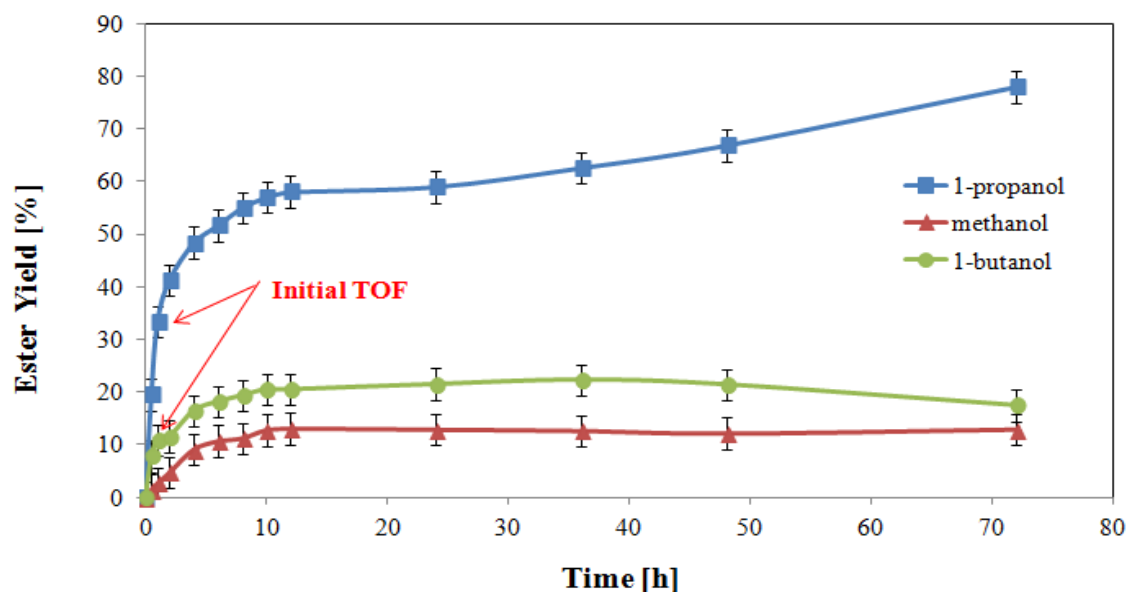
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### 6.2.2 Influence of the alcohol

Due to the hydrophobic nature of the enzyme catalytic center, for lipase recognition appears to be the most appropriate the use of hydrophobic alcohols. Furthermore, previous results show the significant importance of the alcohols geometry, in addition to their lipophilicity on the enzymatic catalytic activity.

It is well know that the lipase from *Rhizomucor miehei* shows good activity in esterification reactions of primary alcohol, which decreases with secondary alcohols, while appears to be inactive with tertiary alcohols<sup>13</sup>. For this reason, our attention has been directly focused on the study of the influence of primary alcohols with different chain lengths, i.e, methanol, 1-propanol and 1-butanol. By the data reported in **Figure 6.20** and **Table 6.4** it is possible to observe that the stereobias (S-(+)-preference) is the same for all the nucleophiles tested. However the catalyst performance is strong influenced by the length of the used alcohol. With 1-butanol and methanol, immobilized *Rhizomucor miehei* lipases shows low activity, probably due to the different substrate specificity of the lipase and/or to the different substrate solvation of the alcohol, as also suggested by previous studies<sup>14</sup>.

The highest enantiomeric excess (**ee**), ester yield and turnover numbers (**TON** and **TOF**) are obtained in the reaction where 1-propanol is used as alcohol (**Table 6.4**).



**Figure 6.20.** Influence of alcohol type in the esterification of ( $\pm$ )-ibuprofen catalyzed by RL1 catalyst. For each test: 66mM of ibuprofen; 66mM of 1-propanol as a alcohol, 10 ml of isooctane as a solvent, 7 % wt of immobilized Lipase with respect to ibuprofen, have been used.

T= 37°C, 72 h of reaction. Each data point has been calculated by gas chromatography analysis and represent the average of three experiments

**Table 6.4.** Esterification of (R,S)-ibuprofen with different alcohols and RL1 as catalyst after 72 hours of reaction. 66mM of ibuprofen; 66mM of alcohol, 10 ml of isooctane as a solvent and 7 %wt of immobilized Lipase with respect to ibuprofen, have been used. Each data point represent the average of three experiments

Alcohol	Yield in ester <sup>a</sup> [%]	ee <sup>b</sup>	TOF <sup>c</sup>	Stereopreference <sup>d</sup>
methanol	12.9	28	54	S
1-propanol	78.0	60	660	S
1-butanol	17.5	39	215	S

<sup>a</sup> Yield in ester is given as a percentage of (S)-ibuprofen esterified after the reaction time.

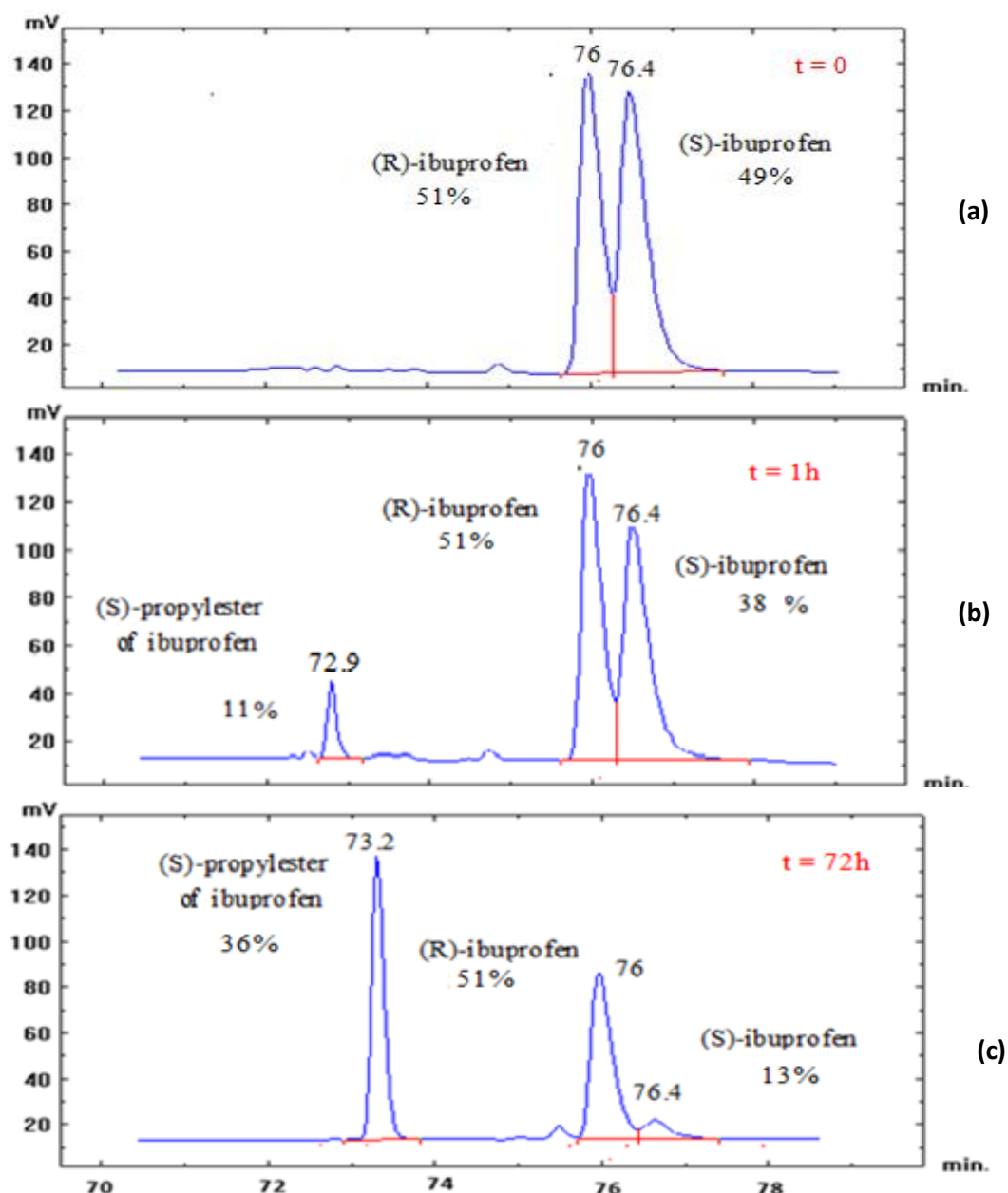
<sup>b</sup> Enantiomeric excess calculated at 72 h of reaction

<sup>c</sup> Calculated at 1 h of reaction

<sup>d</sup> Configuration of the enantiomer found in the ester

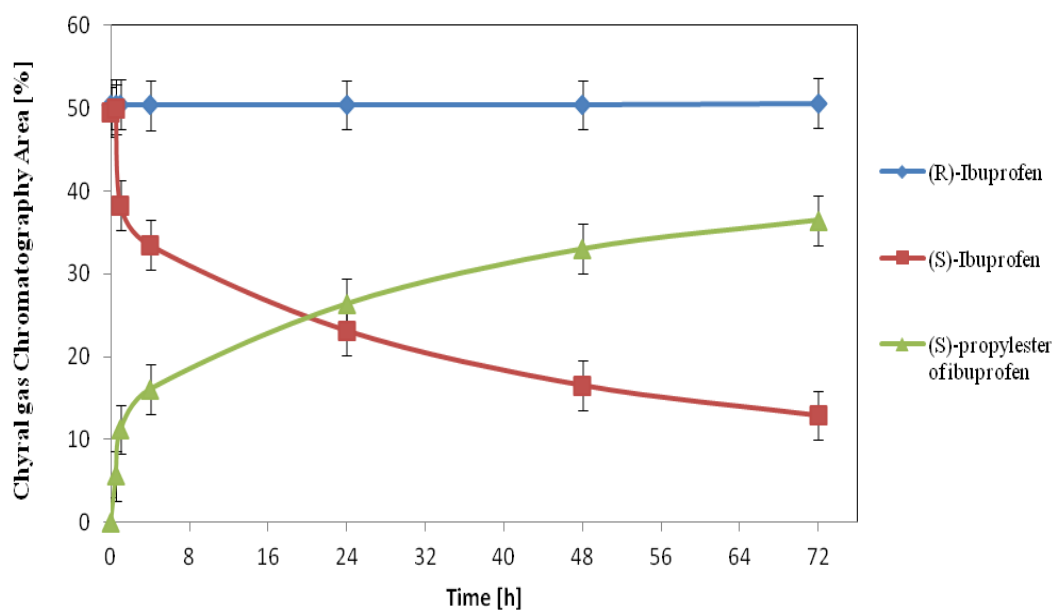
In the **Figure 6.21** the progress of reaction, monitored by chiral gas chromatography and carried out in presence of 1-propanol, is reported, while in the **Figure 6.22** the whole time profile of the reaction is reported.

All Figures clearly show that only the (S)-enantiomer of ibuprofen has been converted to the desired product, while the (R)-enantiomer is not chemically modified.



**Figure 6.21.** Chiral gas chromatograms of reaction catalyzed by RL1 catalyst. Reaction condition: 66mM of ibuprofen; 66mM of 1-propanol as a alcohol, 10 ml of isooctane, as a solvent, and 7 %wt of immobilized Lipase with respect to ibuprofen, at 37°C for: (a) t = 0; (b) t = 1h; (c) t = 72h. These dates are the average of three experiments.





**Figure 6.22.** Time profile of reaction catalyzed by RL1 catalyst reaction conditions: ibuprofen (66mM), 1-propanol (66mM), 10 ml of iso-octane, 37°C, 7%wt of immobilized Lipase with respect to ibuprofen. These dates have been evaluated by chiral gas chromatography are the average of three experiments.

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### 6.2.3 Influence of the Temperature.

The effect of temperature on the enzyme activity in the enantioselective esterification of (R,S)-ibuprofen was examined. The results are reported in **Table 6.5**. As consequence of previous results, all catalytic test are carried out in presence of 1-propanol as alcohol. At all temperature tested, *Rhizomucor miehei* Lipase shows S-(+)-enantioselectivity. We can observe that highest ester yield and turnover numbers are obtained at 37°C.

**Table 6.5.** Effect of temperature on the conversion and enantiomeric excess in the reaction catalyzed by RL1 after 72 hours of reaction. Reaction conditions: 66mM ibuprofen, 66mM 1-propanol in 10 ml of iso-octane, 7% wt of immobilized Lipase respect to ibuprofen. Each data point represent the average of three experiments

Temperature [°C]	Yield in ester <sup>a</sup> [%]	ee <sup>b</sup>	TOF <sup>c</sup>	Stereopreference <sup>d</sup>
27°C	25.8	16	393	S
37°C	78.01	60	660	S
50°C	70.6	58	297	S
80°C	43.0	9	159	S

<sup>a</sup> Yield in ester is given as a percentage of (S)-ibuprofen esterified after the reaction time.

<sup>b</sup> Enantiomeric excess calculated at 72 h of reaction

<sup>c</sup> Calculated at 1 h of reaction

Temperature lower than 37°C reduce the enzyme and substrate mobility, while at temperature higher than 37°C partial catalytic center deactivation of the enzyme occurs.

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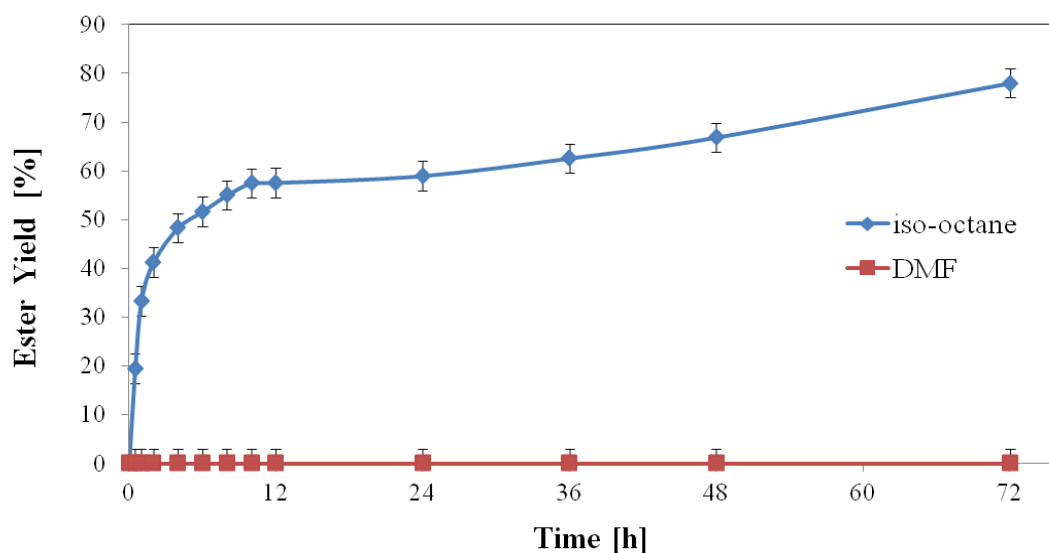
#### 6.2.4 Influence of the Solvent nature.

The enzyme activity in organic solvents depends very much on the nature of the solvent. The solvent can influence an enzymatic reaction both by direct interaction with the enzyme and by influencing the solvation of the substrates and products in the reaction medium. An example of direct interaction between solvent and enzyme is when the solvent acts as an inhibitor of the enzyme. In other cases the solvent causes conformational changes in the enzyme, thereby changing its catalytic properties and can also influence the amount of water bound to the enzyme. Enzyme stability in organic solvents depends on the direct interactions between enzyme and solvent. In several cases few solvents are able to dissolve proteins<sup>10,15</sup> due to disruption of its tertiary and sometimes also secondary structure.

For these reasons, the influence of the solvent nature on the catalytic efficiency of enzymes has been also studied.

Beyond using a-polar isooctane solvent, we have tested the activity of immobilized *Rhizomucor miehei* Lipase, in presence of polar solvent: DMR (dimethylformamide).

As clearly showed by the results reported in **Figure 6.23**, the polar DMF totally deactivates the enzyme contained into RL1 catalyst.



**Figure 6.23.** Influence of the solvent nature in the reaction catalyzed by RL1 catalyst. Reaction conditions: 66mM of ibuprofen, 66mM of 1-propanol as a alcohol, 10 ml of solvent, 7 %wt of immobilized Lipase with respect to ibuprofen, T= 37°C. Each data point has been calculated by gas chromatography analysis and represent the average of three experiments

These results are in accordance with previous studies<sup>11</sup>, where the negative effect of polar solvents on Lipase has been observed. Generally, this polar solvents remove the necessary water for maintaining the native and active conformation of the enzyme, while hydrophobic solvents allow to obtain higher enzymes activity due to their less tendencies to strip essential water in the micro-environment of enzyme.

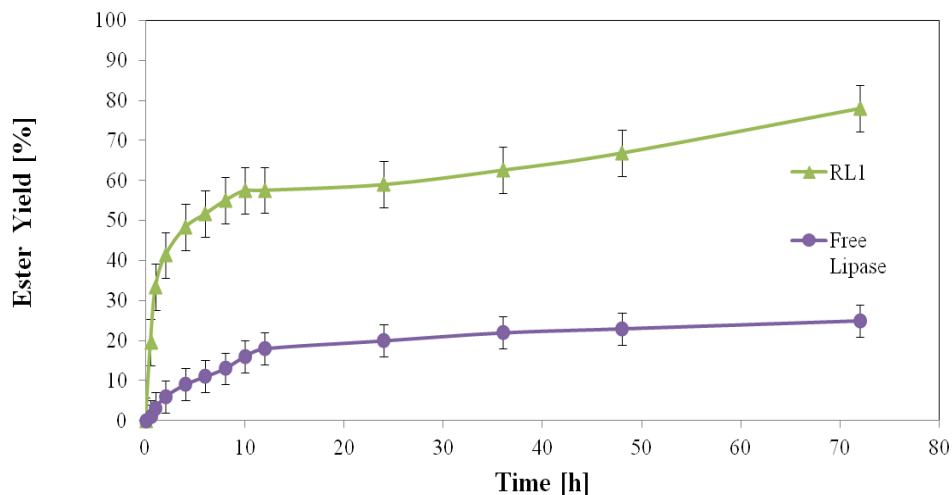
In conclusion the results reported in **Figure 6.23**, demonstrate that the protection of the enzyme in hybrid nanospheres does not prevent the enzyme deactivation by polar solvent.

### 6.2.5 Comparison with Free Lipase.

After selection of best reaction conditions, below summarized:

- temperature 37°C;
- 1-propanol as alcohol;
- iso-octane as a solvent;

a comparison between free and immobilized enzyme performances, has been carried out. The results of this comparisons are reported in **Figure 6.24** and **Table 6.6**



**Figure 6.24.** Comparison between catalytic performance of RL1 and Free Lipase. Reaction conditions: 66mM of ibuprofen, 66mM of 1-propanol as a alcohol, 10 ml of isooctane as a solvent. T= 37 °C , 72 h of reaction. The quantity of Immobilized Lipase used in the reaction is 7%wt with respect to ibuprofen. The same quantity of Free Lipase is used. The Each data point has been calculated by gas chromatography analysis and represent the average of three experiments

**Table 6.6. Catalytic results of free and immobilized lipase (RL1).**

SAMPLE	Yield in ester [%] <sup>a</sup>			ee <sup>b</sup> [%]			TON			TOF		
	1 h	24 h	72 h	1 h	24 h	72 h	1 h	24 h	72 h	1 h	24 h	72 h
Free Lipase	2	20	25	8	32	41	40	397	492	40	17	7
RL1	33	58	78	14	37	60	660	1142	1547	660	48	22

<sup>a</sup> Yield in ester is given as a percentage of (S)-ibuprofen esterified after the reaction time.

<sup>b</sup> Enantiomeric excess

First of all, the reaction parameters analyzed (ee, ester yield and turnover numbers), increase on reaction time, for both catalysts. But all parameters, at the same detected reaction time, are higher for immobilized

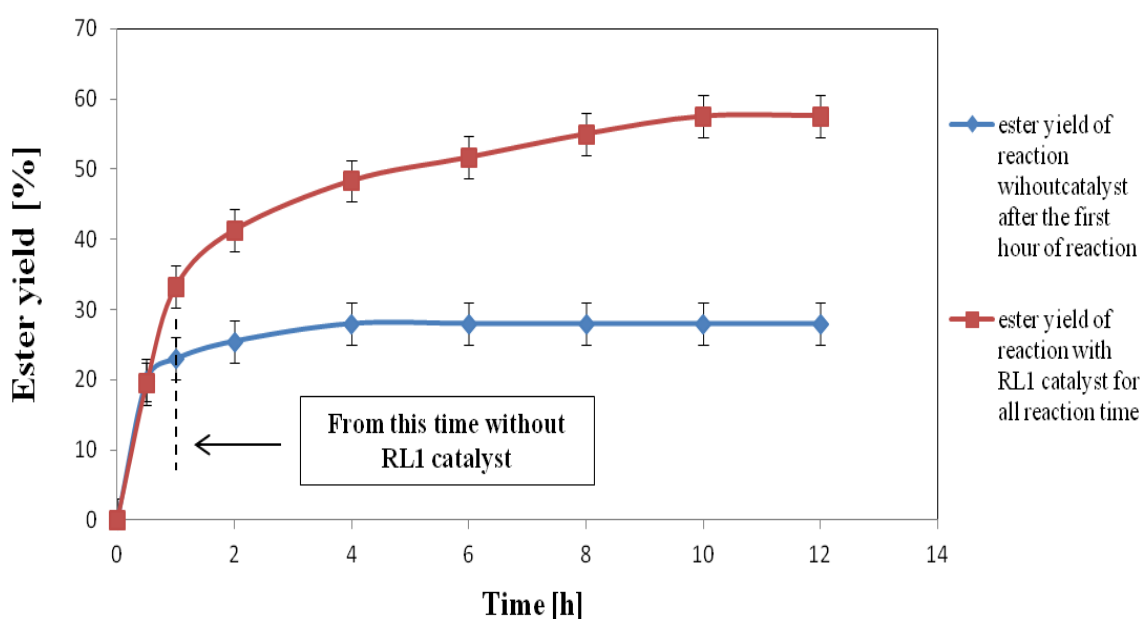
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enzyme, with respect to the free one. This tendency confirms that also for Lipase immobilization in Liposomal shell, covered and protected by silica, the enzyme catalytic center hyper activation occurs, determining better performance of immobilized enzyme with respect to its free and native conformation<sup>16-17</sup>.

Moreover, this comparison also indicates that the reaction occurs in heterogeneous conditions, so no significant enzyme leaching occurs.

In order to accurately verify this aspect, the following experiment has been carried out: after 1 hour of reaction, the reaction was stopped, the solid was separated from the liquid reaction media and the reaction was again carried out without catalyst.

The comparison between the time profile of standard reaction (with RL1 catalyst) and time profile of reaction without catalyst after 1 hour, is reported in **Figure 6.25**.



**Figure 6.25.** Comparison between ester yield obtained in standard reaction condition (RL1 catalyst) and in reaction without catalyst starting from 1 hours. Reaction conditions: 66mM of ibuprofen, 66mM of 1-propanol as a alcohol, 10 ml of isooctane as a solvent, 7% wt of immobilized Lipase with respect to ibuprofen, T= 37 °C. Each data point has been calculated by gas chromatography analysis and represent the average of three experiments.

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It is possible to observe that, since the ester yield does not increase significantly after the first hour of reaction (lower than 5% after RL1 catalyst remove), no enzyme is present in the reaction medium. This indicates that the heterogeneity of the system is maintained during the process, with the occurrence of a non-significant enzyme leaching.

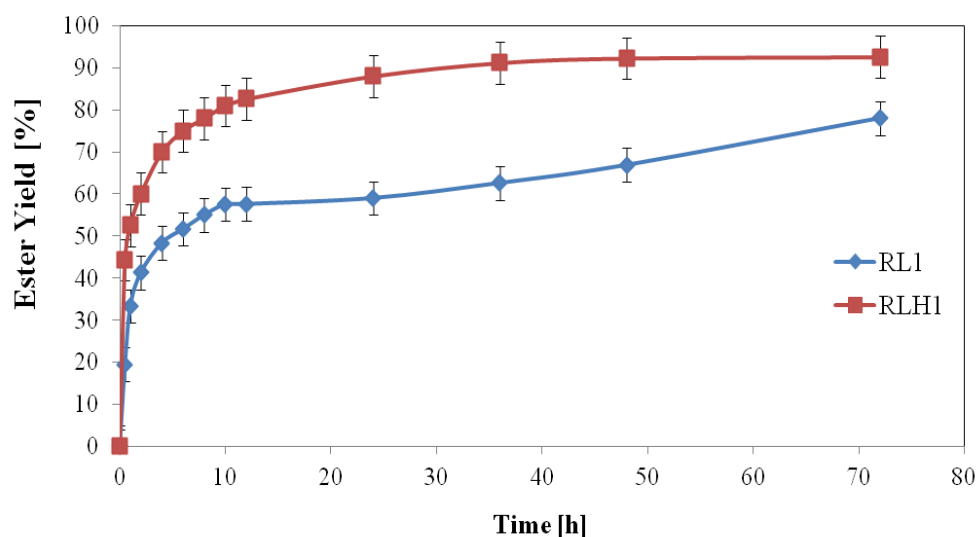
In conclusion, the performed study shows that are many factors that affect the process. It has been demonstrated that the values of the optimal conditions for the esterification of ibuprofen using immobilized *Rhizomucor miehei* Lipase as biocatalyst are:

- Temperature = 37°C;
- Alcohol = 1-propanol;
- Solvent = iso-octane;

using racemic ibuprofen/1-propanol molar ratio equal to 1.

For RL1 catalyst, after 72 h of esterification, enantiomeric excess and ester yield obtained are c.a. 60% and 78%, respectively.

With respect to RL1 sample, the RLH1 sample (nanospheres are synthesized with surfactant) shows a better catalytic performance, under the same optimized conditions. The results of this comparison are reported in **Figure 6.26 and Table 6.7.**



**Figure 6.26.** Comparison between reaction time profiles of RL1 and RLH1 samples. Reaction conditions: 66mM of ibuprofen, 66mM of 1-propanol as a alcohol, 10 ml of isooctane as a solvent, 7% wt of immobilized Lipase with respect to ibuprofen, T= 37 °C. Each data point has been calculated by gas chromatography analysis and represent the average of three experiments

**Table 6.7. Catalytic results of RL1 catalyst and RLH1 catalyst.**

SAMPLE	Yield in ester [%] <sup>a</sup>			ee <sup>b</sup> [%]			TON			TOF		
	1 h	24 h	72 h	1h	24 h	72 h	1h	24 h	72 h	1h	24 h	72 h
<b>RL1</b>	33	58	78	14	37	60	660	1142	1547	660	48	22
<b>RLH1</b>	53	88	93	36	81	89	1042	1745	1836	1042	73	26

<sup>a</sup> Yield in ester is given as a percentage of (S)-ibuprofen esterified after the reaction time.

<sup>b</sup> Enantiomeric excess

First of all, RLH1 sample shows a faster reaction kinetics in the early hours of reaction: after 4 hours RLH1 ester yield is equal to 70% with respect to 48% in the case of sample RL1. Moreover, all parameters at the same detected reaction time, are higher for RLH1 sample, with respect to RL1 sample.

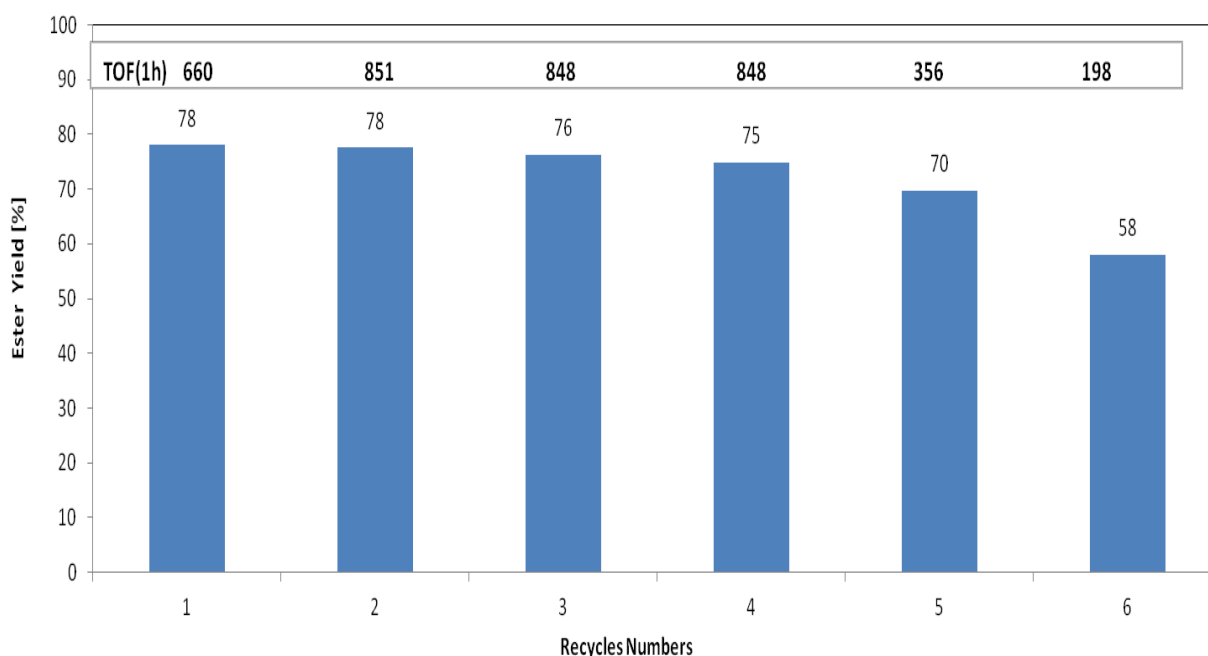


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This catalyst shows excellent catalytic performance, better than RL1 catalyst, due to the action performed by the surfactant shell, that most probably, facilitates the mass transfer of substrate and products.

### 6.2.6 Stability and productivity of catalysts

Finally, for industrial use it is very important the stability and productivity of the catalyst. So, the residual esterification activity under optimal condition of RL1 and RLH1 catalyst, after repeated catalytic uses, has been analyzed. In **Figure 6.27** the stability results after 6 reaction cycles for RL1 catalyst have been reported.



**Figure 6.27.** Performance of RL1 catalyst after 6 reaction cycles. Reaction conditions: 66mM ibuprofen with 66 mM of 1-propanol in 10 ml of isooctane, 7% wt of immobilized Lipase with respect to ibuprofen, for 72h for each cycle at T=37°C, has been carried out.

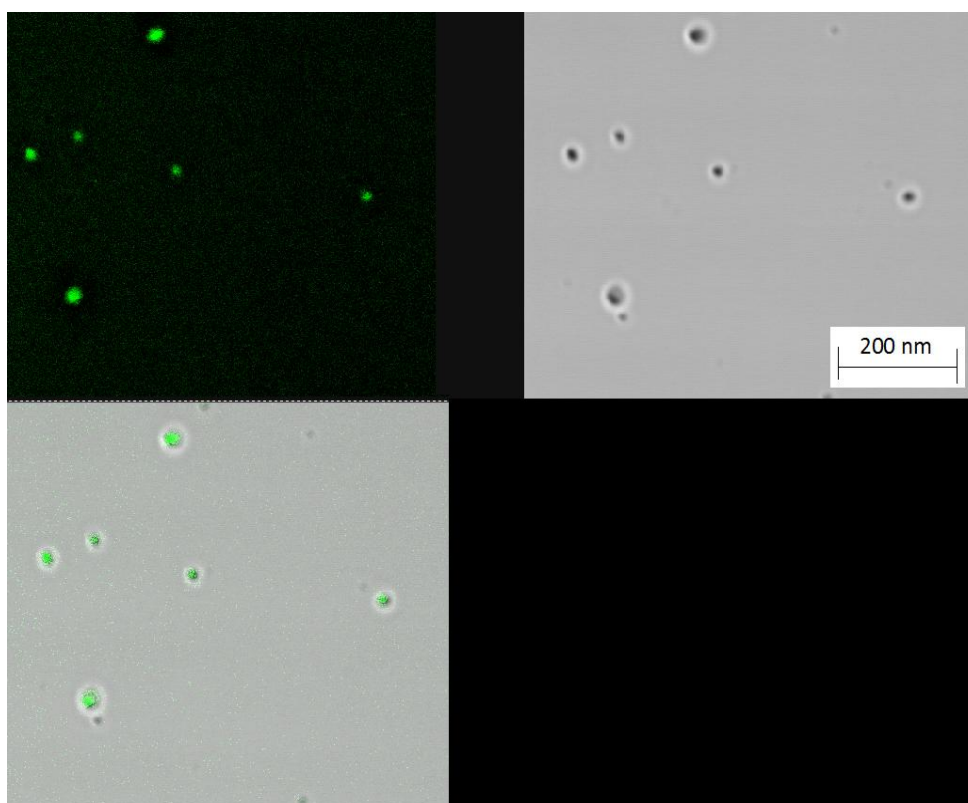
These data indicate that lipase immobilized into hybrid nanosphere is stable after repeated operations. In fact, immobilized lipase retained its activity with moderate loss:

- lower than 3% after 4 reaction cycles;
- lower than 11% after 5 reaction cycles.

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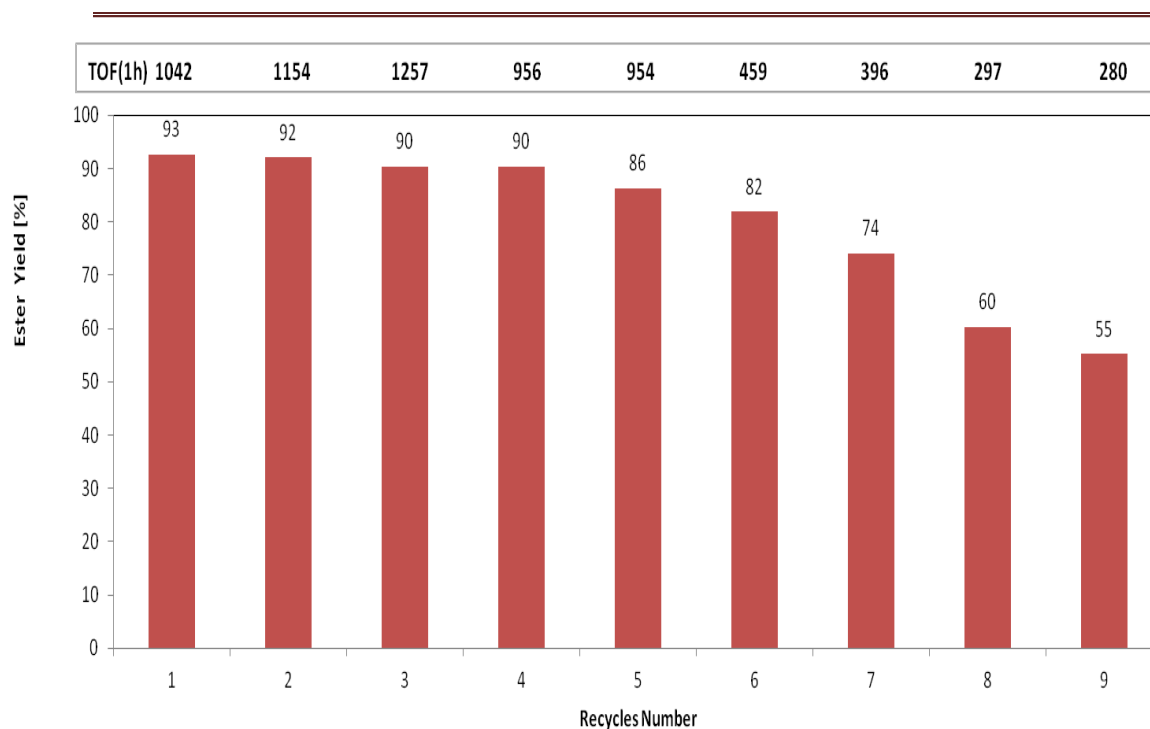
After six cycles the immobilized lipase retained almost 70% of its initial esterification activity.

The reaction should be again carried out for other cycles (>6) because, as corroborate by the Fluorescence Confocal Microscopy analysis results on RL1 catalyst after 6 reaction cycles (**Figure 6.28**), the enzyme is still present in the nanospheres.



**Figure 6.28** . Fluorescence Confocal Microscopy of sample RL1 after 6 reaction cycles

In **Figure 6.29** the residual esterification activity of RLH1 catalyst, under optimal conditions, with repeated catalytic uses has been studied.



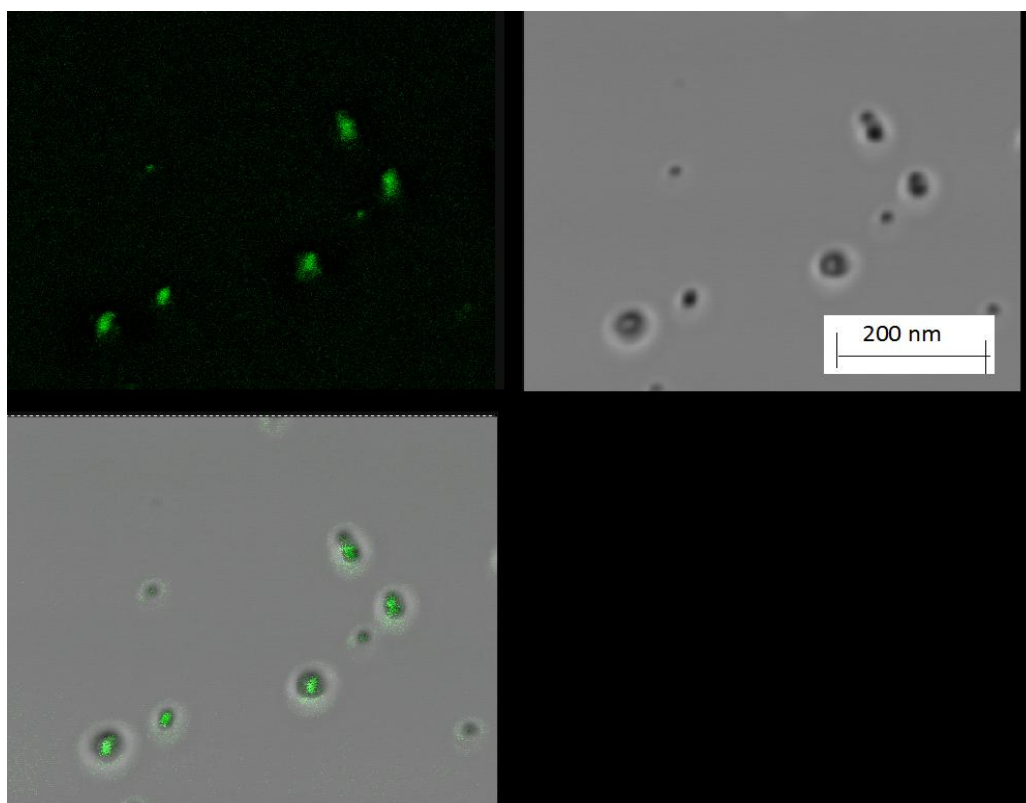
**Figure 6.29.** Performance of RLH1 catalyst after 9 reaction cycles. Reaction conditions: 66mM of ibuprofen with 66 mM of 1-propanol, in 10 ml of isooctane, 7% wt of immobilized Lipase with respect to ibuprofen, for 72h for each cycle at T=37°C, has been carried out.

These data indicate that also lipase immobilized into hybrid nanosphere with silica shell prepared by surfactant is stable after repeated operations. RLH1 sample retained its activity with moderate loss:

- lower than 3% after 4 reaction cycles;
- lower than 18% after 8 reaction cycles.

After nine cycles the RLH1 sample retained almost 60% of its initial esterification activity.

The reaction should be again carried out for other cycles (>9) because, as corroborate by the Fluorescence Confocal Microscopy analysis results on RLH1 catalyst after 9 reaction cycles (**Figure 6.30**), the enzyme is still present in the nanospheres. In fact, it remains linked to the phospholipids layer inside the liposome. This corroborates the hypothesis by which the catalytic efficiency is also affected by the action performed by the surfactant shell.

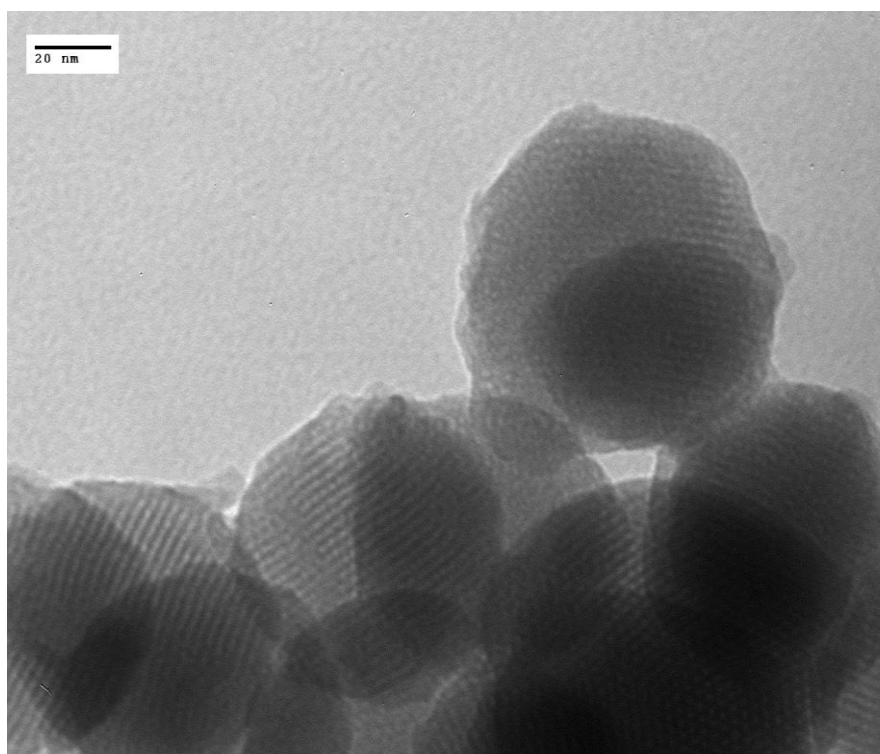


**Figure 6.30.** Fluorescence Confocal Microscopy of sample RLH1 after 9 reaction cycles

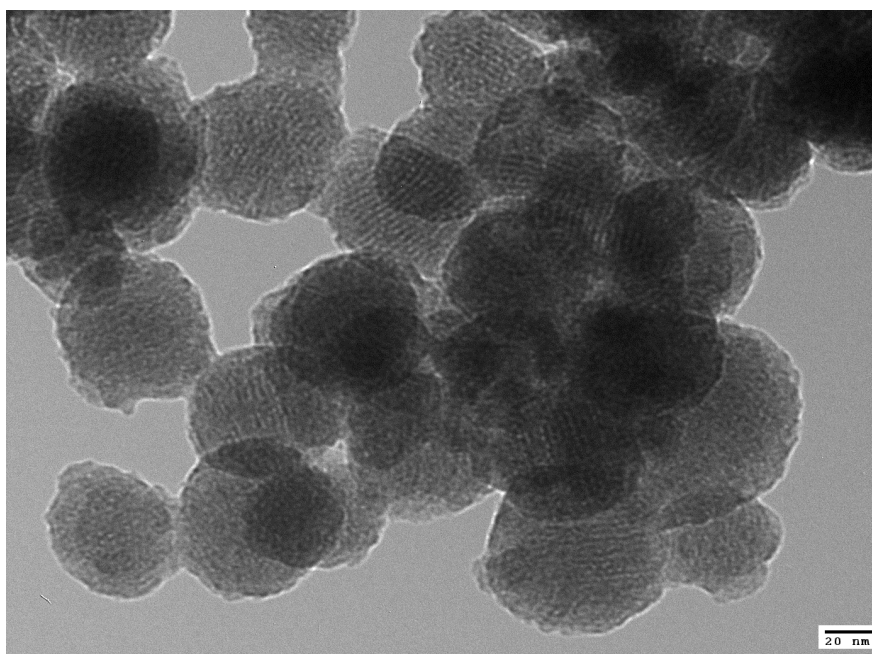
Furthermore, after 9 reaction cycles, the external silica shell of nanospheres of RLH1 catalyst maintains its external structure (**Figure 6.31 a-b**).

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(a)



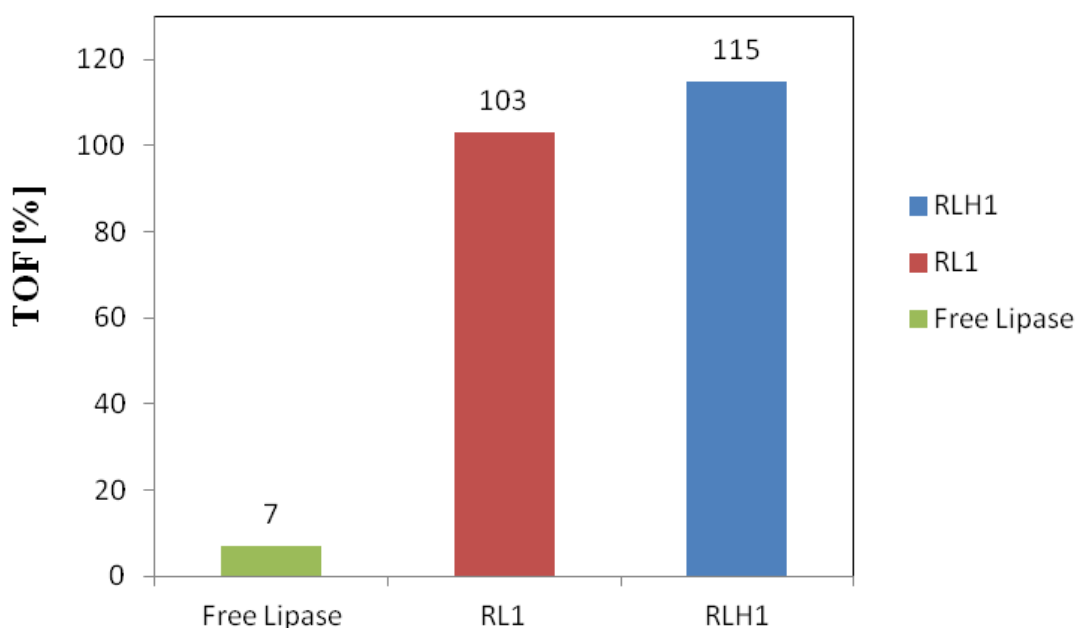
(b)



**Figure 6.31 (a)-(b).** TEM images of RLH1 sample after 9 cycles of reaction (different resolution)

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Finally, the productivity of Lipase immobilized in both system (RL1 and RLH1) is higher than that of free enzyme, as demonstrated by the TOF values of respective catalysts: after 6 reaction cycles for RL1 catalyst and after 9 cycles of reaction for RLH1 catalyst (**Figure 6.32**).



**Figure 6.32.** Comparison between TOF numbers of RL1 catalyst after 6 cycles of reaction, RLH1 catalyst after 9 cycles of reaction, and of Free Lipase (after 9 cycles of reaction). Each cycle is of 72 h.

Particularly, the TOF numbers of NS1 and NSH1 catalyst are c.a.15÷16 times higher than that of Free Lipase after 9 reaction cycles. These results confirm that the desired open conformation of enzyme after immobilization into hybrid nanospheres has been obtained with great success because the immobilized enzyme results much more active and, then, productive with respect to its free form.

Finally, it is important to notice that the stereospecificity of *Rhizomucor miehei* Lipase, both in its free and immobilized form, does not change and the enzyme, under all the reaction conditions tested, reacts only with the S(+)enantiomer of (R,S)-ibuprofen.

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### 6.3 Comparison with literature results.

As commented in Chapter II, there are very few works where immobilized *Rhizomucor miehei* Lipase has been used as catalyst in the esterification of racemic ibuprofen.

Therefore, we compared our results with those reported in these few previous works, where commercial Lipozyme IM<sup>11,12</sup> (*Rhizomucor miehei* immobilized in a porous granular weak base anion resin, Duolite A 568) has been used.

Moreover, the comparison has been carried out with the results that the authors (Sanchez *et al* and Lopez-Belmonte *et al*) have obtained by their optimized conditions.

In particular, Sanchez *et al* studied the effect of ibuprofen / alcohol molar ratio and the temperature on the esterification reaction of racemic ibuprofen, maintaining constant enzyme amount, type of alcohol (1-butanol) and of solvent (iso-octane). Their optimum conditions are:

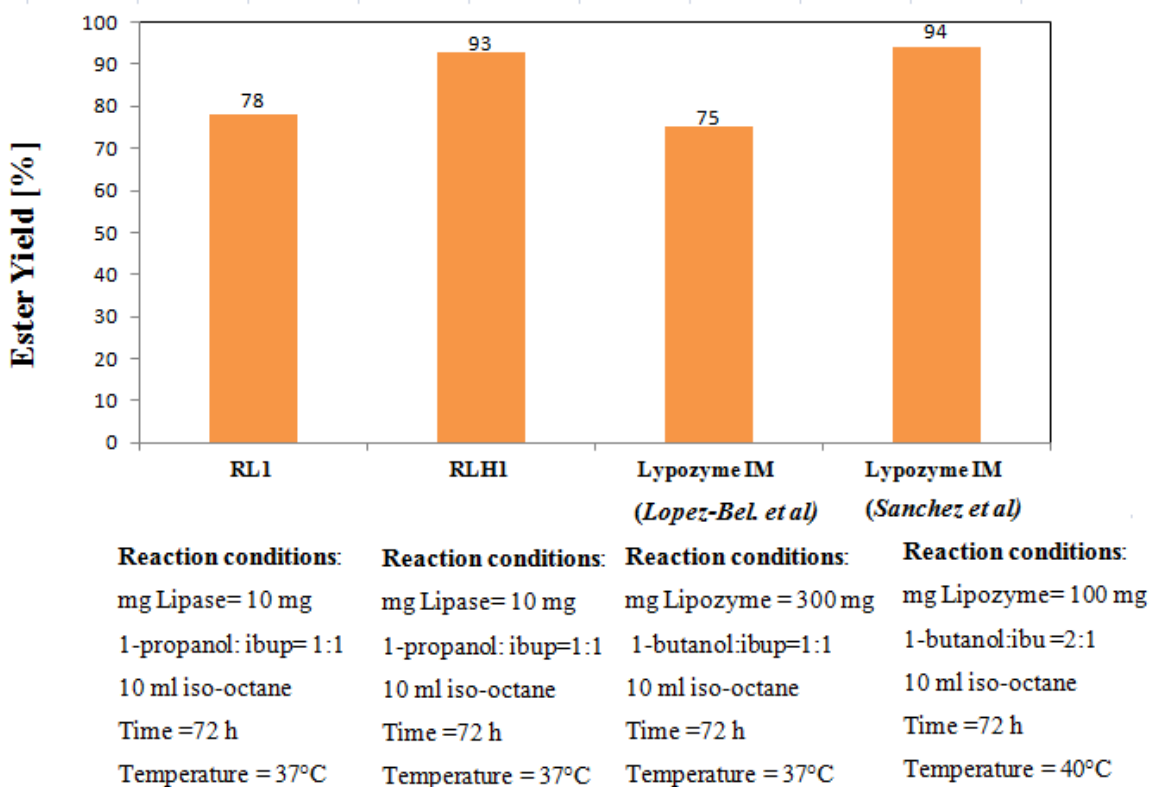
- 10 ml of iso-octane;
- 100 mg of enzyme;
- 50 mM of ibuprofen (0.103 gr);
- 100 mM of 1-butanol (92  $\mu$ l);
- 1-butanol : ibuprofen = 2:1;
- Temperature 40 °C;
- Time 72 hour.

While, Lopez *et al*, studied the effect of nature of the solvent, enzyme amount, the temperature and type of the alcohol. Their optimized conditions are:

- 10 ml of iso-octane;
  - 300 mg of enzyme;
  - 125 mM of ibuprofen (0.258 gr);
  - 125 mM of 1-butanol (114  $\mu$ l)
  - ibuprofen : 1-butanol = 1:1;
-

- Temperature 37 °C;
- Time 72 hour.

The comparison of the best performance of Lipozyme IM, tested by Sanchez *et al* and by Lopez-Belmonte *et al*, and the best performance of our catalysts, RL1 and RLH1, have been summarized in **Figure 6.33**.



**Figure 6.33.** Comparison among catalytic performance of our catalysts (RL1, RLH) and commercial Lipozyme catalyst used in published works.

It is possible to observe that, at the same reaction conditions, the RL1 ester yield is comparable to that obtained by Lipozyme IM, while the RLH1 ester yield is higher (see Lopez-Belmonte et al results).

The catalytic performance of Lipozyme increase in the reaction experiments carried out by Sanchez et al. because a double amount of alcohol has been used. Also, in the Lopez-Belmonte et al and Sanchez et al. work, the higher quantity of enzyme has been used (10 or 30 times higher



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than those used in our works) Therefore, it is surely possible to consider the catalytic performance of our synthesized heterogeneous biocatalysts, RL1 and RLH1, better than that obtained by commercial Lipozyme IM, in both published works.

Finally, it should be interesting to compare the productivity of our catalysts with that of Lipozyme but, neither Lopez-Belmonte et al. nor Sanchez et al. studied the reusability of Lipozyme catalyst. Moreover, it should not have been possible to do this comparison also because the Novo Nordisk Co. does not supply any information about the exact amount of enzyme contained into organic matrix of Lipozyme.

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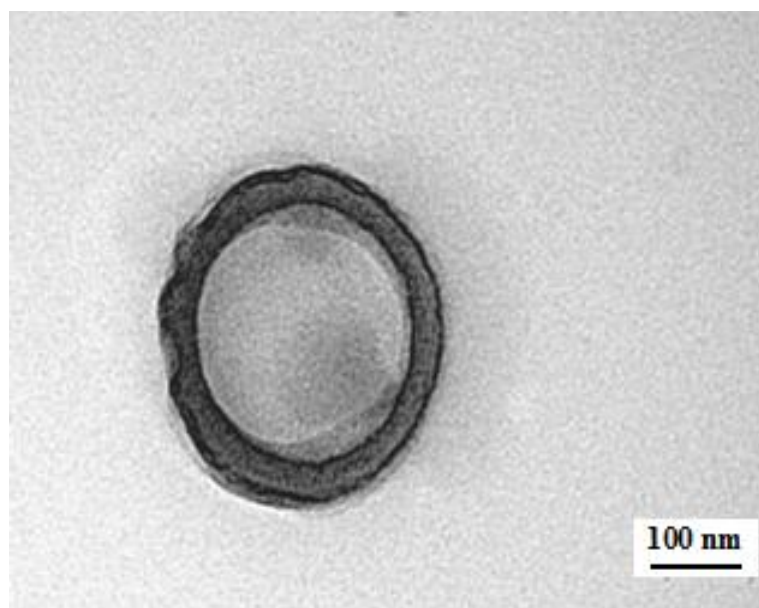
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# **CONCLUSIONS**

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Enzyme-containing liposomes are interesting systems in which biocatalyst can be immobilized retaining its free and stable conformation, due to the biocompatible microenvironment inside the liposome membrane. The chemical stability of organic nanospheres could be improved by external silica coverage.

In order to improve the enzymatic processes in organic and pharmaceuticals chemistry, in this thesis work we have studied the optimal synthesis parameters of organic-inorganic nanospheres containing lipase. The optimization of synthesis methodology, obtained by changing several synthesis parameters, has shown that higher immobilized enzyme amount and the best nanospheres morphology (with dimension of c.a. 200 nm) have been obtained when the SiO<sub>2</sub>/Liposome ratio is equal to 2 and the Lipase/Liposome mixing time is equal to 2 hours (**Figure 1**).

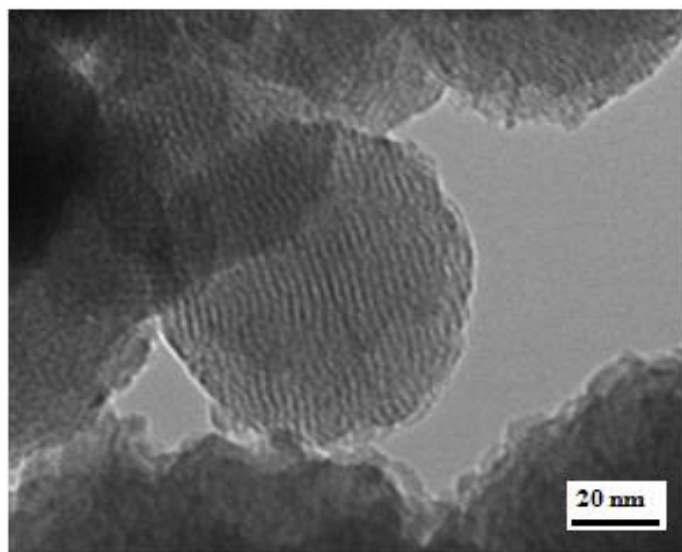


**Figure 1a.** TEM analysis of RL1 catalyst.

The optimized procedure has been used to prepare also hybrid nanospheres where hexadecylamine has been used as surfactant during the polymerization of silica shell. In this last case, nanoparticles, with dimension of 20÷70 nm, show a typical worm-hole structure with the same

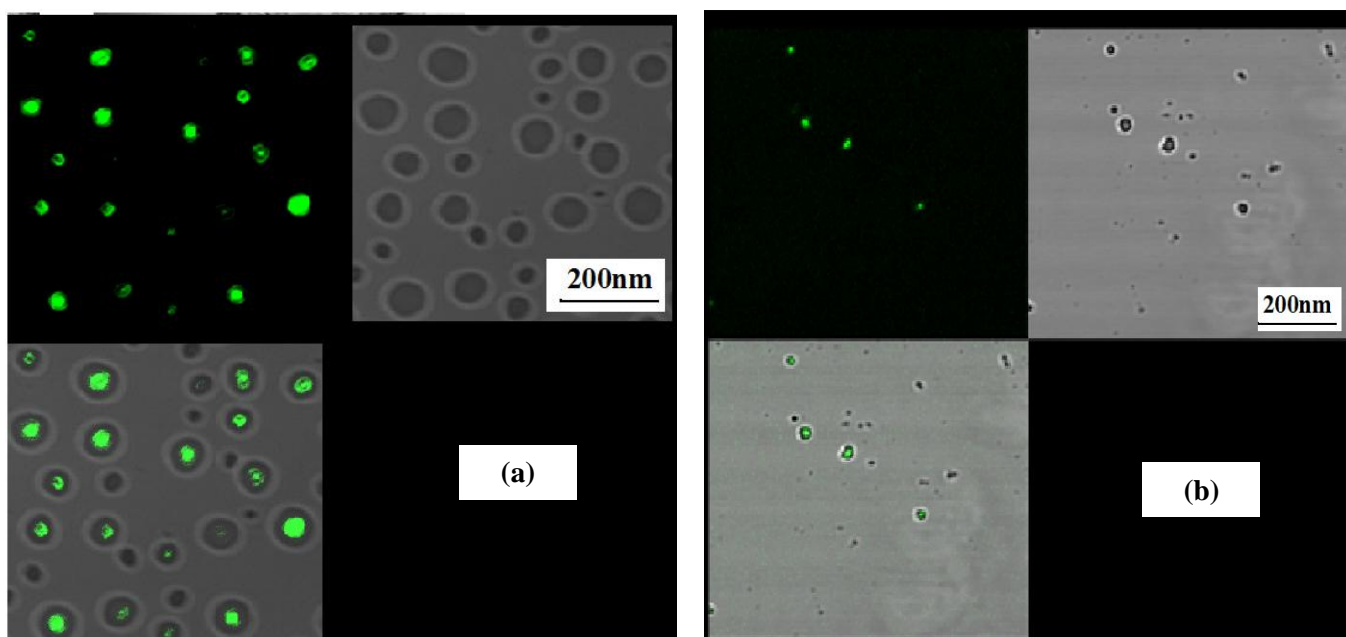
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arrays that those observed with Hexagonal structure, in which the channels are always parallel to the support surface (**Figure 2**).



**Figure 2** TEM analysis of RLH1 catalyst.

Both catalysts, RL1 and RLH1, show the suitable position of enzyme: in the core of the nanospheres, inside the liposomal phase, as demonstrated by fluorescence confocal microscopy results (**Figures 2 (a) e (b)**).



**Figures 2.** Fluorescence Confocal Microscopy images of sample RL1(**a**) and of RLH1(**b**).

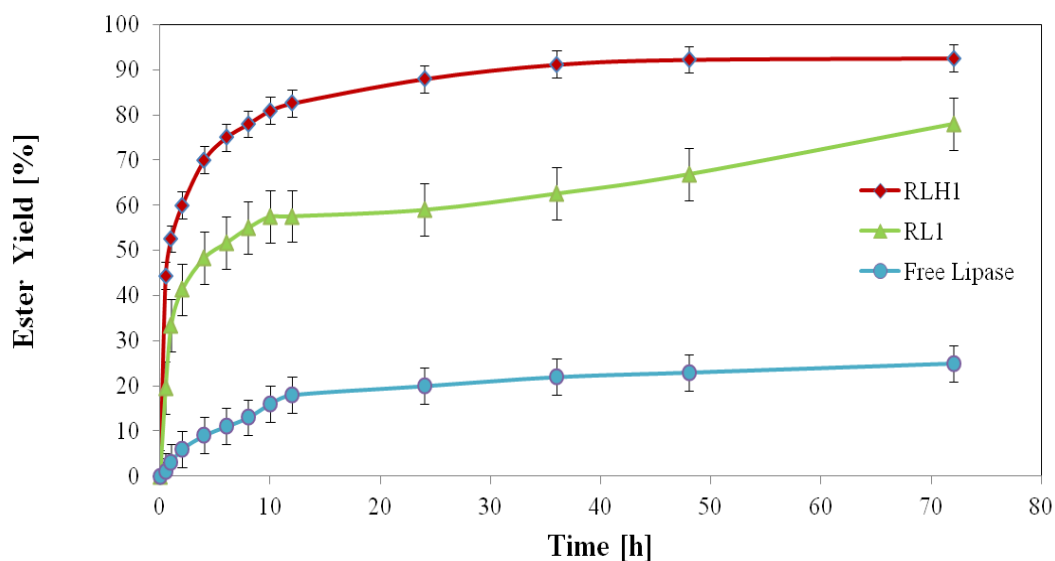
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In order to evaluate their catalytic performance, the optimized hybrid nanospheres (RL1 and RLH1 samples) have been used in the enantioselective esterification of ibuprofen.

The performed study shows that there are many factors which affect the enantioselectivity and conversion of esterification reaction catalyzed by immobilized *Rhizomucor miehei* lipase. Particularly significant influence has been observed by temperature, type of the solvent and nature of the alcohol on the substrate conversion degree. The optimal conditions for the esterification of ibuprofen using hybrid nanospheres containing *Rhizomucor miehei* Lipase as biocatalyst are:

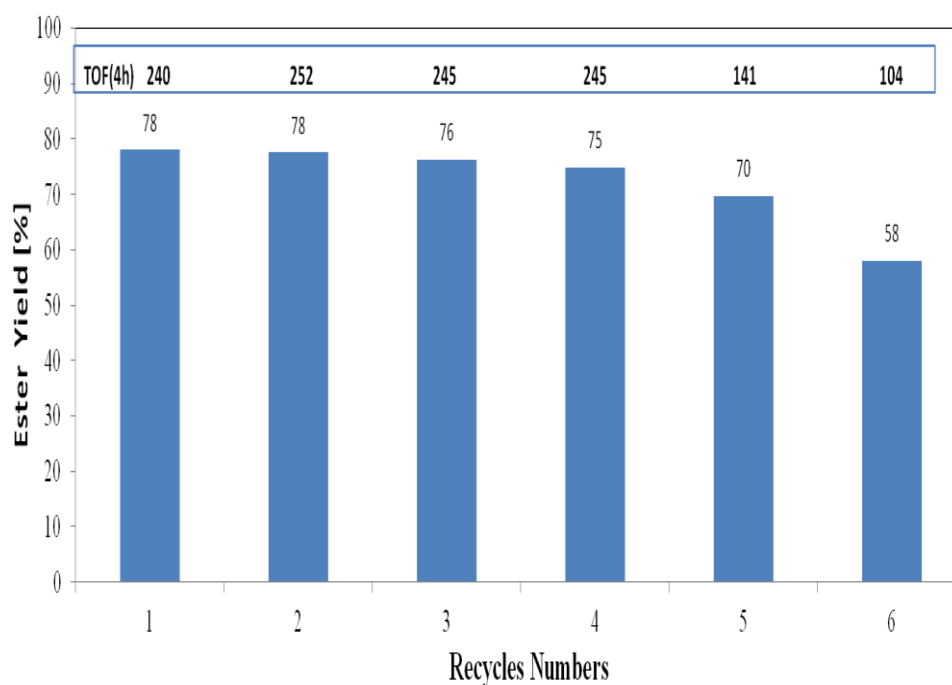
- Temperature = 37°C;
- Alcohol = 1-propanol;
- Solvent = iso-octane.

After 72 h of esterification, enantiomeric excess and ester yield values are, respectively, 60% and 78% for RL1 catalyst, and 85% and 93% for the RLH1 catalyst. RLH1 sample (nanospheres are synthesized with surfactant) shows better catalytic performance, under the same optimized conditions, compared to RL1 and to Free Lipase, due to the action performed by the surfactant shell that, most probably, facilitates the mass transfer of substrate and products (**Figure 3**).

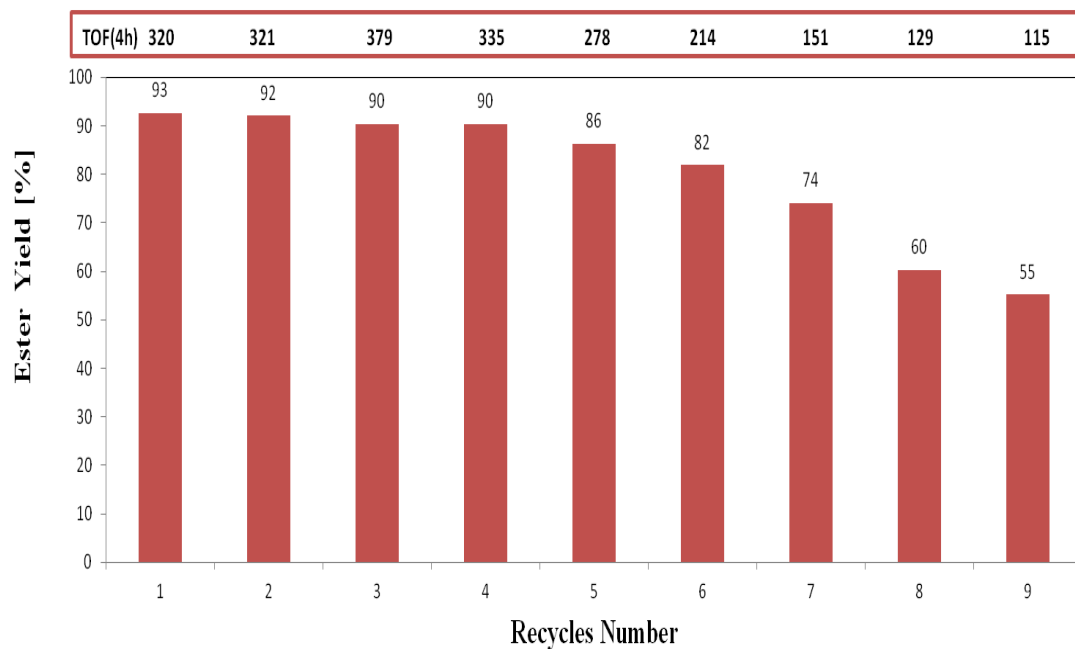


**Figure 3.** Comparison between reaction time profiles of RL1, RLH1 and Free Lipase. Reaction conditions: 66mM of ibuprofen, 66mM of 1-propanol as a alcohol, 10 ml of isooctane as a solvent, 7% wt of immobilized Lipase with respect to ibuprofen, T= 37 °C. Each data point has been calculated by gas chromatography analysis and represent the average of three experiments

The residual esterification activity of RL1 and RLH1 catalyst, after repeated catalytic uses, is very high (**Figure 4** and **Figure 5**).

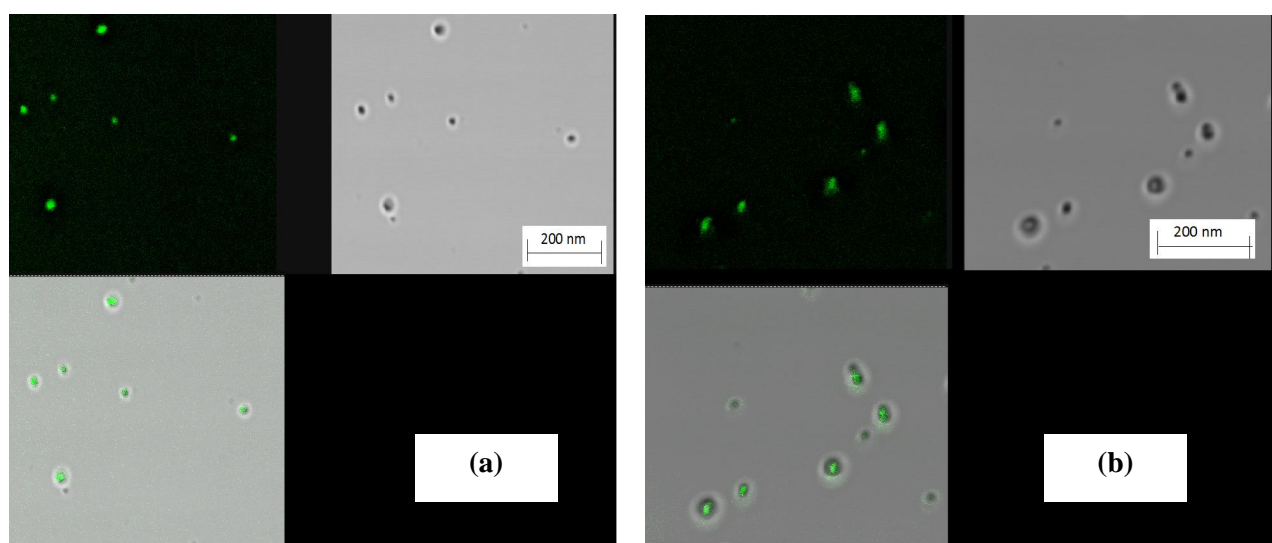


**Figure 4.** Performance of RL1 catalyst after 6 reaction cycles. Reaction conditions: 66mM ibuprofen with 66 mM of 1-propanol in 10 ml of isooctane, 7% wt of immobilized Lipase with respect to ibuprofen, for 72h for each cycle at T=37°C, has been carried out.



**Figure 6.** Performance of RLH1 catalyst after 9 reaction cycles. Reaction conditions: 66mM of ibuprofen with 66mM of 1-propanol, in 10 ml of isooctane, 7% wt of immobilized Lipase with respect to ibuprofen, for 72h for each cycle at T=37°C, has been carried out.

The data reported for both catalysts indicate that lipase immobilized into hybrid nanospheres is stable after repeated operations and no significant enzyme leaching occurs, as corroborated by Fluorescence Confocal Microscopy of catalyst after several reaction cycles (**Figure 6(a)** and **(b)**).

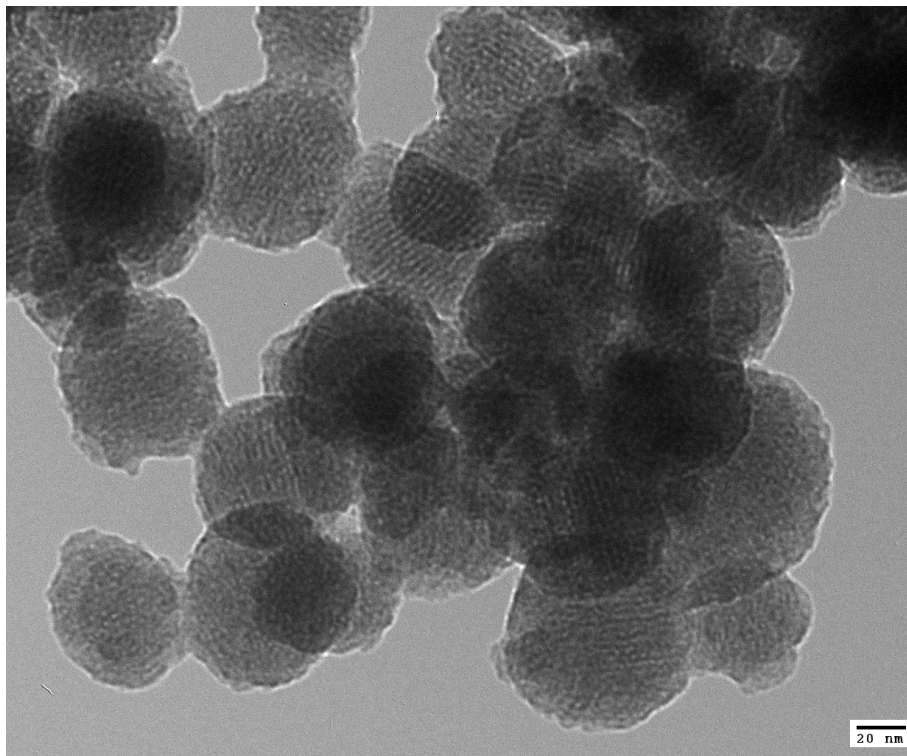


**Figures 6.** Fluorescence confocal microscopy images: **(a)** of RL1 after 6 reaction cycles and **(b)** of RLH1 after 9 reaction cycles.



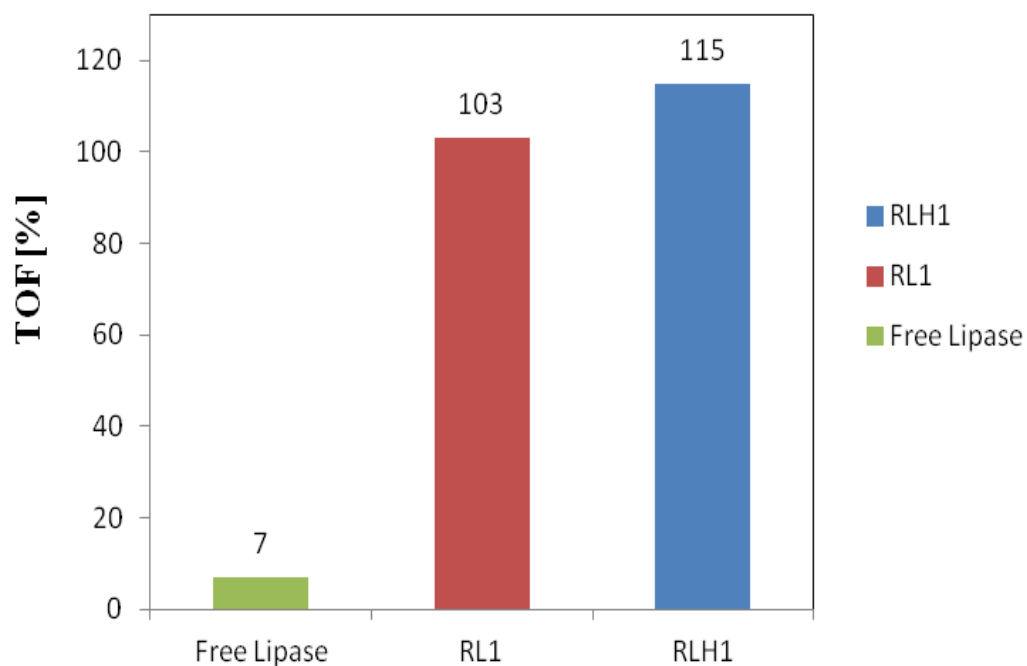
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Moreover, after 9 reaction cycles the external silica shell of nanospheres of RLH1 catalyst maintains its external structure (**Figure 7**).



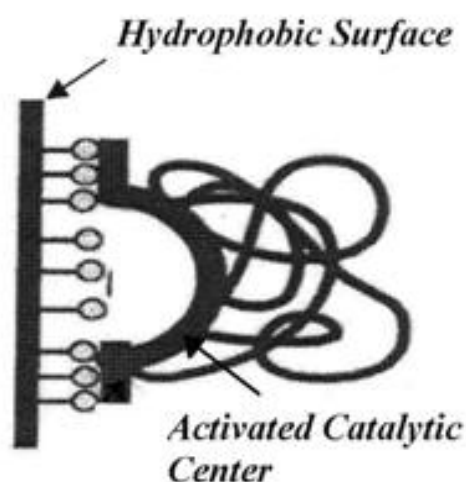
**Figure 7.** TEM image of RLH1 sample after 9 cycles of reaction.

Consequently, the productivity of Lipase immobilized in both system (RL1 and RLH1) is higher than that of free enzyme, as demonstrated by the TOF values of respective catalysts: after 6 reaction cycles for RL1 catalyst and after 9 cycles of reaction for RLH1 catalyst (**Figure 8**).



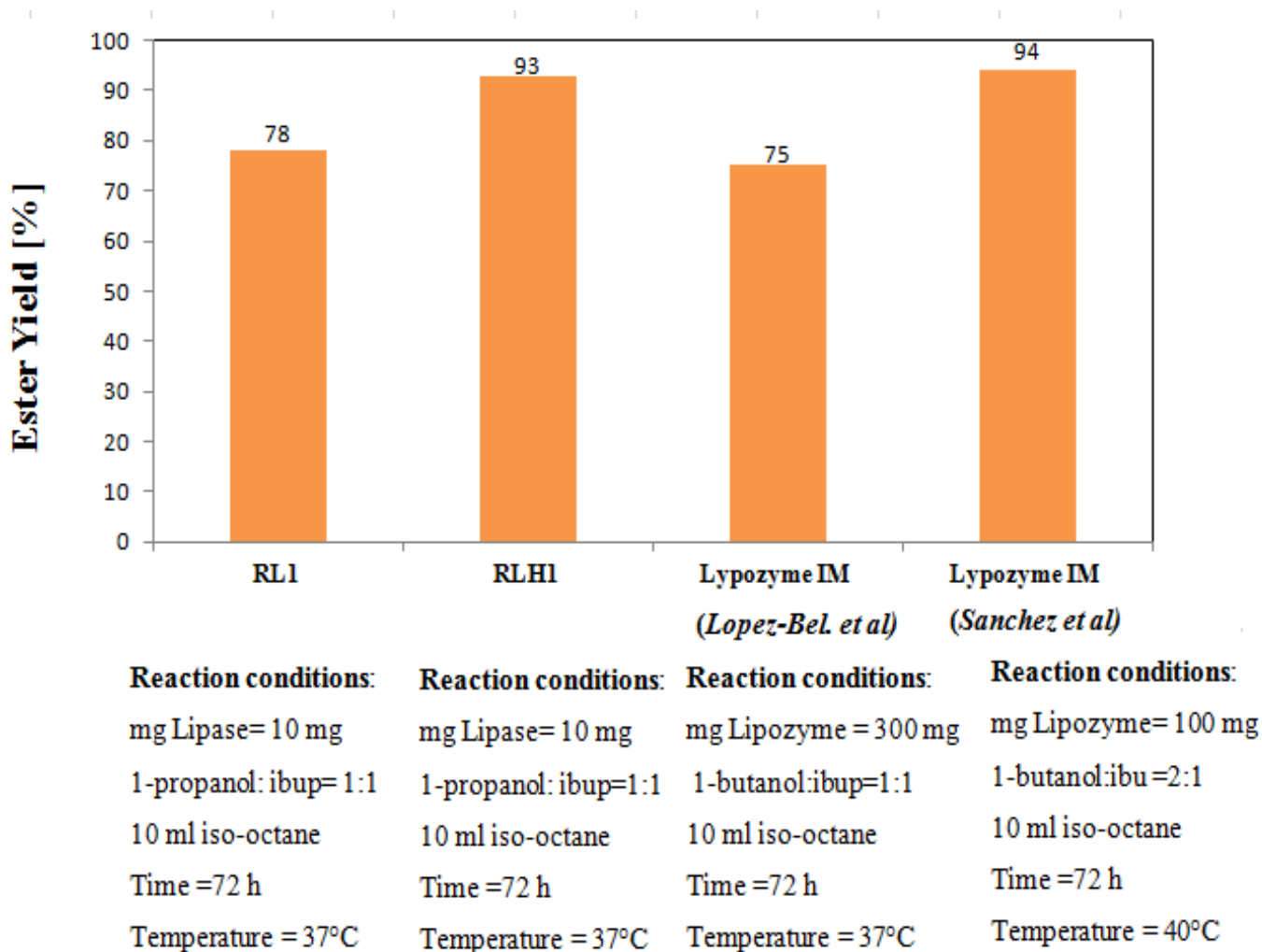
**Figure 8.** Comparison between TOF numbers of RL1 catalyst after 6 cycles of reaction, RLH1 catalyst after 9 cycles of reaction, and of Free Lipase (after 9 cycles of reaction). Each cycle is of 72 h.

Particularly, the TOF numbers of RL1 and RLH1 catalyst are c.a.15-16 times higher than that of Free Lipase after 9 reaction cycles. These results confirm that the desired open conformation of enzyme after immobilization into hybrid nanospheres has been obtained with great success (**Figure 9**).



**Figure 9.** "Open Form" (Active Form) of *Rhizomucor miehei* Lipase

Finally, we compared our results with those reported by Sanchez *et al* and Lopez-Belmonte *et al* (**Figure 10**).



**Figure 10.** Comparison among catalytic performance of our catalysts (RL1, RLH) and commercial Lipozyme catalyst used in published works.

Since the catalytic conditions of these previous studies are comparable or more disadvantageous (higher amount of alcohol and mg of enzyme used) than ours, it is possible to conclude that our catalysts show better catalytic performance than that of its competitor: commercial Lipase, Lipozyme.

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In conclusion, the results obtained in this thesis works largely open to the possibility, in the next future, to use a high-efficiency catalytic system for various enzymatic processes, like multi-enzymatic cascade reactions, biosensors and drug delivery.

**Table A.**

Amino acids classification based on their chemical properties.

Structure	Name	Abbreviation	
1. Neutral amino acids			
(a) nonpolar, aliphatic			
$\begin{array}{c} \text{H} \\   \\ \text{H}_3\text{N}^+-\text{C}-\text{H} \\   \\ \text{COO}^- \end{array}$	Glicina	Gly	G
$\begin{array}{c} \text{H} \\   \\ \text{H}_3\text{N}^+-\text{C}-\text{CH}_3 \\   \\ \text{COO}^- \end{array}$	Alanina	Ala	A
$\begin{array}{c} \text{H} \quad \text{CH}_3 \\   \quad / \\ \text{H}_3\text{N}^+-\text{C}-\text{CH} \\   \quad \backslash \\ \text{COO}^- \quad \text{CH}_3 \end{array}$	Valina	Val	V
$\begin{array}{c} \text{H} \quad \quad \text{CH}_3 \\   \quad \quad / \\ \text{H}_3\text{N}^+-\text{C}-\text{CH}_2-\text{CH} \\   \quad \quad \backslash \\ \text{COO}^- \quad \quad \text{CH}_3 \end{array}$	Leucina	Leu	L
$\begin{array}{c} \text{H} \quad \text{CH}_3 \\   \quad   \\ \text{H}_3\text{N}^+-\text{C}-\text{CH}-\text{CH}_2-\text{CH}_3 \\   \\ \text{COO}^- \end{array}$	Isoleucina	Ile	I
(b) polar, aliphatic			
$\begin{array}{c} \text{H} \\   \\ \text{H}_3\text{N}^+-\text{C}-\text{CH}_2-\text{OH} \\   \\ \text{COO}^- \end{array}$	Serina	Ser	S
$\begin{array}{c} \text{H} \quad \text{OH} \\   \quad   \\ \text{H}_3\text{N}^+-\text{C}-\text{CH}-\text{CH}_3 \\   \\ \text{COO}^- \end{array}$	Treonina	Thr	T
$\begin{array}{c} \text{H} \\   \\ \text{H}_3\text{N}^+-\text{C}-\text{CH}_2-\text{C}-\text{NH}_2 \\   \quad \quad \parallel \\ \text{COO}^- \quad \quad \text{O} \end{array}$	Asparagina	Asn	N
$\begin{array}{c} \text{H} \\   \\ \text{H}_3\text{N}^+-\text{C}-\text{CH}_2-\text{CH}_2-\text{C}-\text{NH}_2 \\   \quad \quad \quad \parallel \\ \text{COO}^- \quad \quad \quad \text{O} \end{array}$	Glutamina	Gln	Q

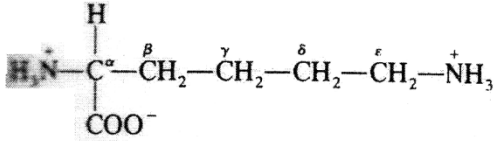
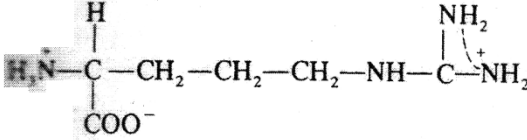
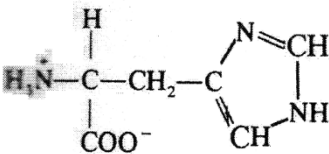
**Table A.**

Amino acids classification based on their chemical properties.

Structure	Name	Abbreviation	
<i>(c)</i> Aromatic			
$\begin{array}{c} \text{H} \\   \\ \text{H}_3\text{N}^+ - \text{C} - \text{CH}_2 - \text{C}_6\text{H}_5 \\   \\ \text{COO}^- \end{array}$	Fenilalanina	Phe	F
$\begin{array}{c} \text{H} \\   \\ \text{H}_3\text{N}^+ - \text{C} - \text{CH}_2 - \text{C}_6\text{H}_4\text{OH} \\   \\ \text{COO}^- \end{array}$	Tirosina	Tyr	Y
$\begin{array}{c} \text{H} \\   \\ \text{H}_3\text{N}^+ - \text{C} - \text{CH}_2 - \text{C} \\   \quad \quad \quad   \\ \text{COO}^- \quad \quad \quad \text{Indole ring} \\   \\ \text{H} \end{array}$	Triptofano	Trp	W
<i>(d)</i> Sulfur-containing			
$\begin{array}{c} \text{H} \\   \\ \text{H}_3\text{N}^+ - \text{C} - \text{CH}_2 - \text{SH} \\   \\ \text{COO}^- \end{array}$	Cisteina	Cys	C
$\begin{array}{c} \text{H} \\   \\ \text{H}_3\text{N}^+ - \text{C} - \text{CH}_2 - \text{CH}_2 - \text{S} - \text{CH}_3 \\   \\ \text{COO}^- \end{array}$	Metionina	Met	M
<i>(e)</i> containing a secondary amino group			
$\begin{array}{c} \text{CH}_2 \\ / \quad \backslash \\ \text{H}_2\text{N}^+ \quad \text{CH}_2 \\   \quad \quad   \\ \text{H} - \text{C} - \text{CH}_2 \\   \\ \text{COO}^- \end{array}$	Prolina	Pro	P
2. Acid amino-acid			
$\begin{array}{c} \text{H} \\   \\ \text{H}_3\text{N}^+ - \text{C}^\alpha - \text{CH}_2^\beta - \text{COO}^- \\   \\ \text{COO}^- \end{array}$	Aspartato	Asp	D
$\begin{array}{c} \text{H} \\   \\ \text{H}_3\text{N}^+ - \text{C}^\alpha - \text{CH}_2^\beta - \text{CH}_2^\gamma - \text{COO}^- \\   \\ \text{COO}^- \end{array}$	Glutamato	Glu	E

**ble A.**

Amino acids classification based on their chemical properties.

Structure	Name	Abbreviation
3. Basic Amino acids		
	Lisina	Lys K
	Arginina	Arg R
	Istidina	His H

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## **PARTICIPATION AT EVENTS:**

- Forum on Innovation in zeolites and ordered porous Materials (Zeoforum)-Valencia -Spain (3/12/2012-4/12/2012)
- 2<sup>a</sup> Scuola Hispano-Italiana di Catalisi -Sevilla -Spagna-(24/06/2013-25/06/2013);
- SECAT 2013 (Meeting of the Spanish society of catalysis)- Sevilla-Spain (26/06/2013-28/06/2013)
- Course of "Identification, assessment and control of chemical risk factors in the workplace". Organized by Consejo Superior De Investigaciones Científicas (CSIC) - Valencia -Spain (3/07/2014)

## **CONGRESS:**

- F. Verri, U. Diaz, A. Macario , A. Corma, G. Giordano; *HYBRID ORGANIC-INORGANIC NANOSPHERES AS CARRIER SYSTEMS OF BIOACTIVE MOLECULES*; 5<sup>th</sup> Czech-Italian-Spanish Conference on Molecular Sieves and Catalysis (CIS-5)- Segovia - Spain (16/06/2013 - 19/06/2013).
  - F. Verri, U. Diaz, A. Macario , A. Corma, G. Giordano; *HYBRID ORGANIC-INORGANIC NANOSPHERES: A NEW SYSTEM FOR STABLE AND ACTIVE BIOCATALYSTS*; XVII National Congress of Catalysis and XI National Congress of Science and Technology of Zeolites (GIC 2013)- Riccione - Italy - (15/09/2013 - 18/09/2013).
  - F. Verri, U. Diaz, A. Macario , A. Corma, G. Giordano; *ENZYME-CONTAINING LIPOSOMES AS ACTIVE MOLECULES FOR HYBRID AND HETEROGENEOUS BIOCATALYSTS PREPARATION*, 5<sup>th</sup> International Symposium Advanced Micro-and Mesoporous Materials- Golden Sands-Bulgaria (6/09/2013-9/09/2013)
  - F. Verri, U. Diaz, A. Macario, A. Corma, G. Giordano; *PREPARATION AND CHARACTERIZATION OF HYBRID NANOSPHERES CONTAINING LIPASE FOR CHIRAL DRUG BIOTRANSFORMATION*, accepted as a oral presentation in 4<sup>th</sup>
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International Conference on Multifunctional, Hybrid and Nanomaterials - Sitges (Barcelona)-Spain (9/03/2015-13/03/2015).

**PUBLICATIONS:**

- F.Verri, U. Diaz, A. Macario, A. Corma, G. Giordano; "PREPARATION AND CHARACTERIZATION OF HYBRID NANOSPHERES CONTAINING LIPASE FOR CHIRAL DRUG Biotransformation", has been submitted to *Journal of Materials Chemistry*.



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