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STRATEGIES FOR THE ENHANCEMENT OF THE CATALYTIC PERFORMANCE OF CUTINASE IN NONAQUEOUS MEDIA

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CHAPTER I

Biocatalysis
from ancient to hi-tech applications

Through the history of mankind microorganisms have played a tremendous role in our evolution, influencing our social behavior and economic development. Long before we knew about their existence we used them for food and beverages production creating an evolutionary symbiosis that allowed the evolution of both species. Mesopotamia is commonly referred to as the cradle of applied biocatalysis where we can find several ancient civilizations that developed different fermentation techniques to produce new products. For instance Sumerians and Babylonians practiced beer brewing even before 6000 BC, we find references to wine production in the book of the Genesis, and Egyptians used yeast for baking bread[1,2].

Nevertheless the knowledge about the production of chemical products such as alcohols and organic acids obtained by fermentation is quite recent, and dates from the second half of the 19th century. The work of Louis Pasteur was a great contribution to the understanding of biotransformations. For instance Pasteur was the first to demonstrate the microbial resolution of a racemate, where a microorganism in the presence of a racemic mixture consumes one of the enantiomers preferentially and can even leave the other one untouched. To accomplish this, Pasteur performed the fermentation of an ammonium salt of racemic tartaric acid, mediated by *Penicillum glaucum*, and he obtained (-)-tartaric acid. Later Pasteur also confirmed *Bacterium xylinum* as the agent involved in vinegar production (figure 1.1). His scientific curiosity led him to find out if this microorganism could be involved in other transformations, and in fact he demonstrated that the oxidations of propanol to propionic acid and mannitol to fructose could also be achieved with this particular microorganism. These works of Pasteur are remarkable: in the 19th century he launched the guidelines of modern biocatalysis. The approaches that he followed are the same that are used today when new microorganisms are isolated: let us find out what they are capable of doing for us. The concept is the same, the only difference being that we have better tools to put it into practice

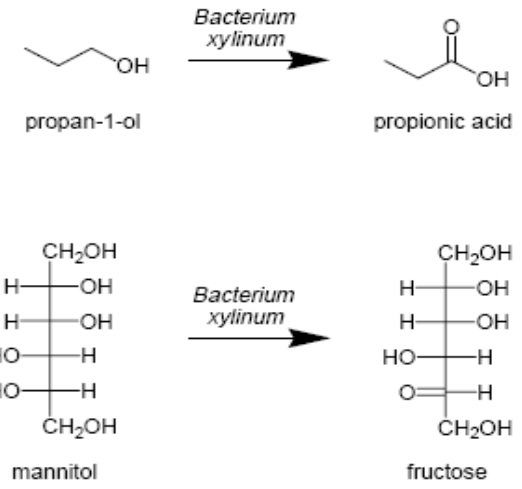

Figure 1.1 – The first microbial resolution of a racemate by Louis Pasteur.

The 19th century was quite profitable for chemistry and biology, with discoveries that launched the foundation of biochemistry and, later, biotechnology. For instance Kirchoff in 1814 found that a glutinous component of wheat could convert starch to sugar. And Payen and Persoz focused their attention on the action of extracts of germinating barley in the hydrolysis of starch to sugars. These works were very important because they were carried out in a very detailed way; in fact their authors postulated some of the basic principles of enzyme action. For instance they demonstrated that just with small amounts of the extracts they were able to liquefy large amounts of starch. They also showed that the extracts were thermolabile and that the active substance could be precipitated form an aqueous solution just by adding an alcohol, and hence be concentrated and purified. Payen and Persoz called this active substance diastase[1,2]. Today we know that these scientists observed the action of a mixture of amylases, which is still being used in several industrial biotransformations.

The pioneer on enzyme production was the Danish scientist Christian Hansen who in 1874 started the first company to commercialize enzyme preparations (Christian Hansen's Laboratory), namely rennet for cheese production. In 1883 this Danish botanist revolutionized beer-making by successfully developing new ways to cultivate yeast, and by refusing to patent his methodology, making it instead freely available to other brewers. He was one of the first to identify different strains of the yeast *Saccaromyces cerevisae*, an over-yeast (floating on the surface of the fermenting beer), and *S. carlsbergensis*, an under-yeast (lying at the bottom of the liquid)[1,2].

The works of Emil Fisher are also an important contribution to this new area. In 1894 Emil Fisher observed in his studies that an enzyme (emulsin) catalyzed the hydrolysis of β-methyl-D-glucoside, while another enzyme (maltase) was active towards α-methyl-D-glucoside. These important results led Fisher to postulate his famous "lock-and key" theory that describes the enzyme specificity principle. As Fisher said, the enzyme and the glucoside must fit each other like a lock and a key (figure 1.3), in order to effect a chemical reaction on each other. Without any information in addition to that derived from his experiments, Fisher was convinced that these so-called enzymes were if fact proteins[1,2]. Today we know that his theory was correct and it is quite easy for us to understand this postulate because we can visualize the enzyme structure and understand the several steps that substrates and enzyme undergo during the catalytic process. But in 1894 Fisher's findings were remarkable. Many consider Fischer to be the most brilliant chemist who ever lived, due to his numerous contributions to science, especially chemistry and biochemistry. Many chemical reactions and concepts are named after him and in 1902 he was awarded the Nobel Prize in Chemistry.[3].

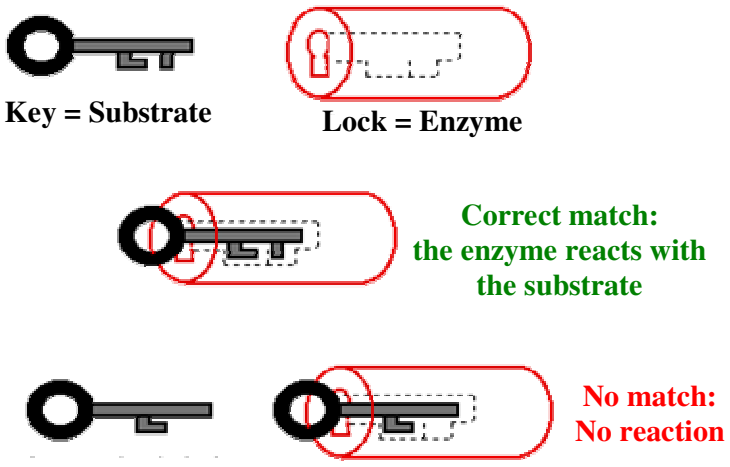

Figure 1.2 – Lock-and-key analogy by Emil Fisher.

These results inspired several other scientists that took Fisher's theories to the next level. One of these works was led by Edward Büchner who in 1898 caused a breakthrough in fermentation and enzymology by showing that the alcoholic fermentation did not required the presence of "such complex apparatus as is the yeast cell". The agent responsible for this transformation was definitely a protein entity which Büchner called "Zymase". Büchner brought to light a new biochemical paradigm that stated that

enzyme catalysis was a chemical process that did not need a living cell to take place. This development settled the foundation for enzyme processing. On that same year Croft-Hill performed the first enzymatic synthesis of isomaltose.[1,2].

At the beginning of the 20th century biocatalysis was giving the first steps towards industrial applications. The major applications of enzymes at this time were in the chill-proofing of beer, the addition of malt extract and in dough making. This took place before it was known that enzymes were proteins, a fact that was established in 1926 only, when Summer was able to crystallize several enzymes like urease and trypsin. Biocatalysis was finally a consolidated area of research[1,2].

The discovery of the double helix by James Watson and Francis Crick in 1953, which led to the identification and elucidation of the role of DNA and RNA in heredity, was one of the great scientific milestones of the last century. This discovery had profound impact on several different areas and definitely transformed the way that we understand life and evolution. It led to the synthesis of recombinant DNA and became the basis for one of the most profiting technologies nowadays: genetic engineering. Such developments quickly made the DNA technology a tool for industrial microbial transformations[1,2,4]. One of the first examples of application of this technology was given by Ensley and co-workers who reported the construction of a strain of *E.coli* that excreted indigo, one of the oldest known dyes [1,2,5]. These scientists discovered that the entire metabolic pathway for the conversion of naphthalene into salicylic acid was coded by a set of genes of *Pseudomonas putida*. Their results were beyond expectation since they found that a subset of these genes were also responsible for the production of indigo. Moreover they showed that indigo formation was a property of a dioxygenase enzyme system that synthesizes *cis*-dihydrodiols from aromatic hydrocarbons. They were able to clone these genes in a more "user friendly" microorganism - *E.coli* - and presented a novel way to produce indigo.[1,2].

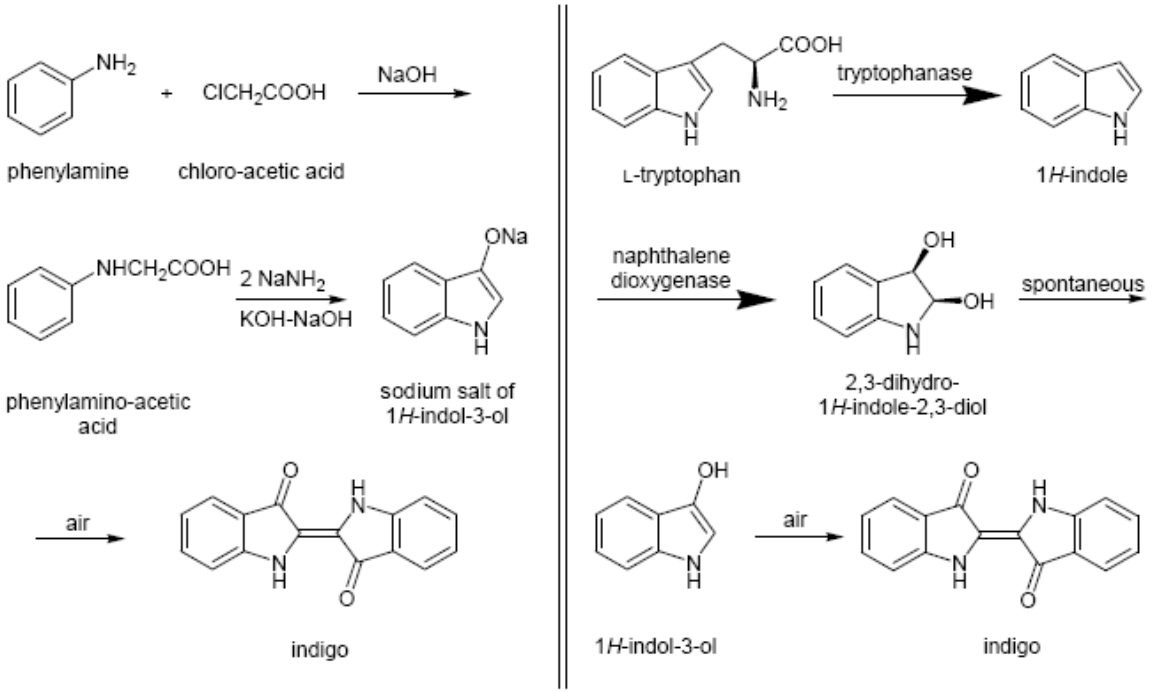

Figure 1.3 - Comparison between chemical and biological routes to synthesize indigo.

In the early 80s, Aleksey Zaks and Alexander Klibanov broke the scientific dogma that enzymes could only work in aqueous media[6,7,8]. The two scientists demonstrated that enzymes could exhibit activity in dry organic solvents. These results were remarkable in that they opened the door to novel applications of biocatalysts. The curiosity of this discovery was that in the early 30s Aleksander Sym, a Polish scientist, had shown this possibility, but nobody paid much attention. Today the reason for this seems easy to understand: no applications were envisaged for his discovery, and so his vision had little consequence. For instance the pharmaceutical industry was giving the first steps and almost all the drugs that were commercialized came from natural sources. What we can say is that Sym's discovery, like many others, was ahead of its time.

But why do enzymes work in nonaqueous media? [6,9,10] Today the answer is simple: they work in such media because when they are placed there they can retain their hydration sphere, the water that is molecularly connected to the protein. This water, which is often called "essential water", acts as a molecular lubricant of the protein structure. In its absence, no enzymatic reaction can take place. The next figure (figure 1.4) helps to understand this effect.

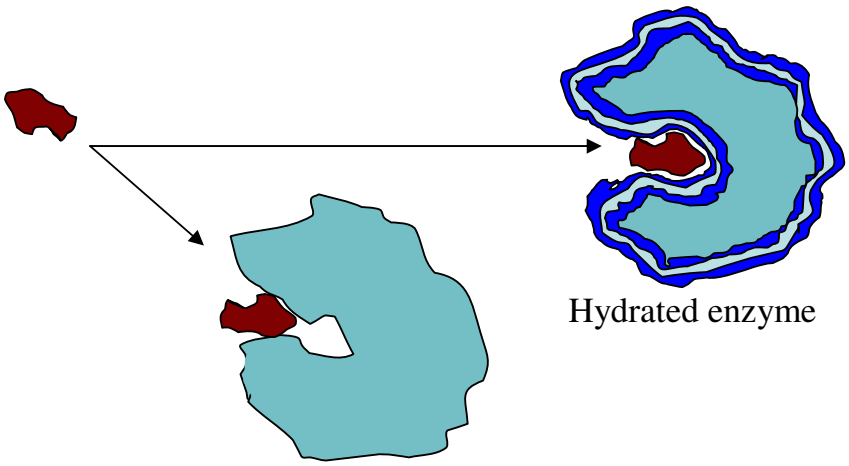

Figure 1.4 – The role of essential water as molecular lubricant of the enzyme structure.

The importance of this discovery derived mostly from the new applications that enzymes could have in the absence of water. For instance in aqueous media enzymes like esterases, lipases and proteases, catalyze the hydrolysis of esters to the corresponding acids or alcohols[74,12]. However in anhydrous solvents these processes are not thermodynamically favorable. Therefore if we replace the nucleophilic agent with an organic compound such as an amine, an alcohol or a thiol, the process leads to an amynolysis, transesterification or thiotransesterification, respectively. These synthetic processes, which are suppressed in water, are the opposite reactions to hydrolysis[12].

By changing the nature of the solvent it became possible to catalyze many organic syntheses with enzymes, and use as substrates different organic molecules whose utilization was formerly restricted because of their low solubility in water. Moreover enzymes in organic media also show different properties compared to aqueous systems[6-11]. Nevertheless the property that most interested researchers and especially industry was the high enantioselectivity that enzymes exhibit in this type of media[13,14,17]. Indeed it is possible to enhance enzyme enantioselectivity simply by changing the solvent where the reaction is performed. This property will be described in a more detailed way in Chapter II.

The use of enzymes in organic synthesis has become one of the routes towards "greener" chemistry since enzymes are able to catalyze reactions in very mild conditions. Also with growing environmental concerns, industry is being forced to change the way it conducts industrial processes and limit the consumption of organic

solvents. In addition to reactions in a solvent-free medium, it was also demonstrated that enzymes were able to catalyze reactions in alternative solvents such as supercritical fluids[15] or ionic liquids[16]. This way enzymes have reinforced their role as an important tool in the emerging green chemistry technology.

Today our challenges are extending the range of enzymes suitable for application and searching for new solutions for synthesis, especially of chiral compounds. The traditional methods for identifying new enzymes suitable for these applications are based on the screening from strain collections by enrichment, or from soil samples[17-19]. We can find in the literature several remarkable examples of these approaches[17,6]. But these techniques are becoming obsolete towards our need to obtain new and better solutions[20], due to the fact that with this methodology, only a tiny fraction of the biodiversity that is present can be accessed. For instance the number of culturable microorganisms from a sample is estimated at merely 0.001-1 %, depending of course on their origin, which means that 99 % of the biodiversity escapes our efforts to identify new biocatalysts.

In order to solve these limitations, new strategies have been put forward to include the excess on non-culturable biodiversity in biocatalysis[19-23]. One of these new strategies is the **metagenome approach**, which basically consists in the direct extraction of the DNA from the non-cultivated microbial consortia. This DNA is then cloned and expressed and the distinct enzymatic activities are identified by suitable assay methods (figure 1.5), a simple concept whose major advantage is the large number of biocatalysts that can be found. On the other hand phylogenetic analysis revealed that new subclasses of enzymes can be identified, which show very broad evolutionary diversity and thus the chance of identifying biocatalysts with unique properties is substantially increased.

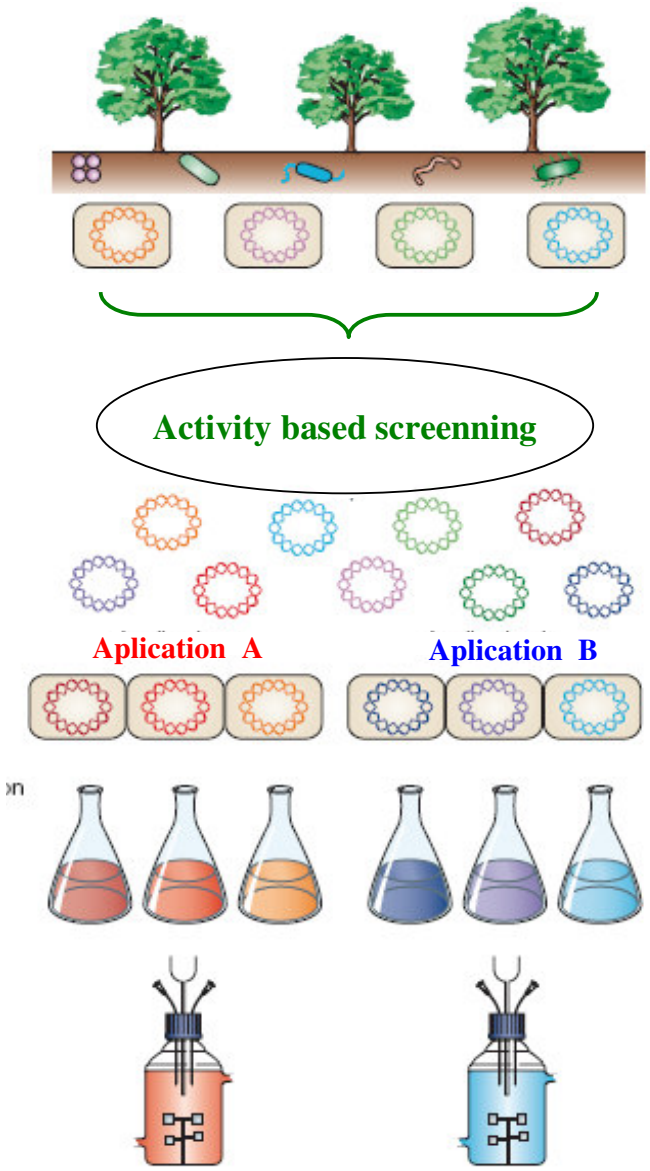

Figure 1.5 – Schematic representation of a metagenome approach (adapted from reference 19).

The most remarkable example of the metagenome approach was the discovery of more than 130 nitrilases from more than 600 biotope-specific environmental DNA libraries[22,23]. But more impressive even was the diversity of catalytic possibilities generated by this approach. For instance the application of these novel nitrilases revealed that 27 enzymes afforded mandelic acid in 90% ee in a dynamic kinetic resolution, and one nitrilase afforded (R)-mandelic acid in 86% yield and 98% ee. Also, aryllactic acid derivatives were accepted with high conversion and selectivity. The best enzyme gave a 98% yield and 95% ee for the *(R)*-product, and 22 enzymes gave the opposite enantiomer with 90-98% ee.

Before these studies, there were available 20 nitrilases, obtained by classical isolation approaches. The metagenomic approach changed that situation dramatically, proving that today it is one of the best approaches to look for new biocatalysts and new catalytic solutions in nature. These studies were taken up by Diversa, the largest and more prominent specialist biotech company for the commercialization of metagenome technologies. Diversa has partnered with other companies for the discovery and development of enzymes, among them DSM, Syngenta, BASF and Genencor International. The German company BRAIN has adopted a similar strategy and is using metagenome technology to collaborate with the German manufacturer Henkel in the screening of novel glycosyl hydrolases for use in laundry applications[19].

These examples reflect the extreme importance of this technology in the development of new industrial solutions. In addition to the successful cases presented before, it is expected that this technology will be improved since there are still some limitations and difficulties related the expression and screening processes that have to be overhauled.

To make the manipulation easier, all the metagenomic libraries described here were constructed in E.*coli*. However the successl of this approach is related with the class of enzyme that is screened for. For instance a soil sample gave 98 positive hits for lipase/esterase activity but when a different enzymatic activity was screened the number of positive hits considerably decreased[20,21]. This might be due to the difficulties in expressing specific genes in E.*coli*, and of course with their scarcity in the metagenome pool or even in some cases to the combination of both. To overcome these limitations several approaches have been tried, for instance expressing the metagenome in different hosts, like *Bacillus subtillis*, *Streptomyces* spp. *Pseudomonas* spp. or even in eukaryotic systems. Nevertheless this option is also limited by the low throughput that is associated with alternative cloning hosts and serves mainly as an option for large inserts (>30 Kb)[19-21]. A different strategy based on the stimulation of the metagenomic pool towards the expression of a desired activity is one way to overcome the difficulties. In this case the abundance of the target genes in the metagenomic DNA pool is increased through microbial enrichment, using low concentrations of externally added nutrients prior to DNA extraction. This strategy has been pursued by the Icelandic biotech company Prokaria[24].

Another interesting approach that have been presented as an alternative to host-cell expression systems is **sequence based screening**[20]. This strategy is not dependent on the expression of the cloned genes in heterologous hosts, but rather on the conserved DNA sequences of target genes. In this case hybridization or PCR are performed on the deduced DNA consensus. For instance if we have a certain type of enzyme activity that we want to screen in a DNA pool, what we must to do is compare the sequences of the known enzymes that can exhibit that type of activity and obtain a consensual sequence between them. With this sequence we perform hybridization with the DNA sequences in the DNA pool and the ones that hybridize have a high probability to code for the desired activity. With this technique several novel enzymes from biotechnologically relevant enzyme classes were identified, such as xylases and polyketide synthases[19,25]. However this approach has some limitations, namely related with the availability of the consensual sequences that are fundamental to apply this technique. In addition, the technique cannot be applied to all biocatalysts, multimeric enzymes being outside its range of application.[25].

Another effort to improve the frequency of screening hits was developed by Wanatabe[26] and their colleagues, and consists in **substrate induced gene expression screening (SIGEX)**. This technique is based of the fact that catabolic genes are generally induced by substrates or metabolites of catabolic enzymes, which means that their expression is controlled by regulatory elements located proximately in many cases. In this strategy SIGEX screens the clones harboring the desired catabolic genes that are expressed in the presence of certain substrates but are not expressed in their absence. This kind of selection is possible because of the way the expression vector is constructed. In this case an operon-trap vector (p19GFP) is used, in which the cloning site divides the *lac* promoter and the *gfp* structural gene. The genomic libraries are constructed on this vector and the second step is to remove the self-ligated clones and the ones that express *gfp* constitutively. These false positives are removed by IPTG induction in the absence of substrates. The expression of catabolic genes in cloned metagenomic DNA is determined by *gfp* in the presence of the substrate and the positive clones are separated on agar plates and characterized. The last step uses fluorescence activated cell sorting (FACS), which is a very efficient technique to separate the *gfp* expression cells, i.e. the clones with the desired catabolic genes (figure 1.6)[21,26].

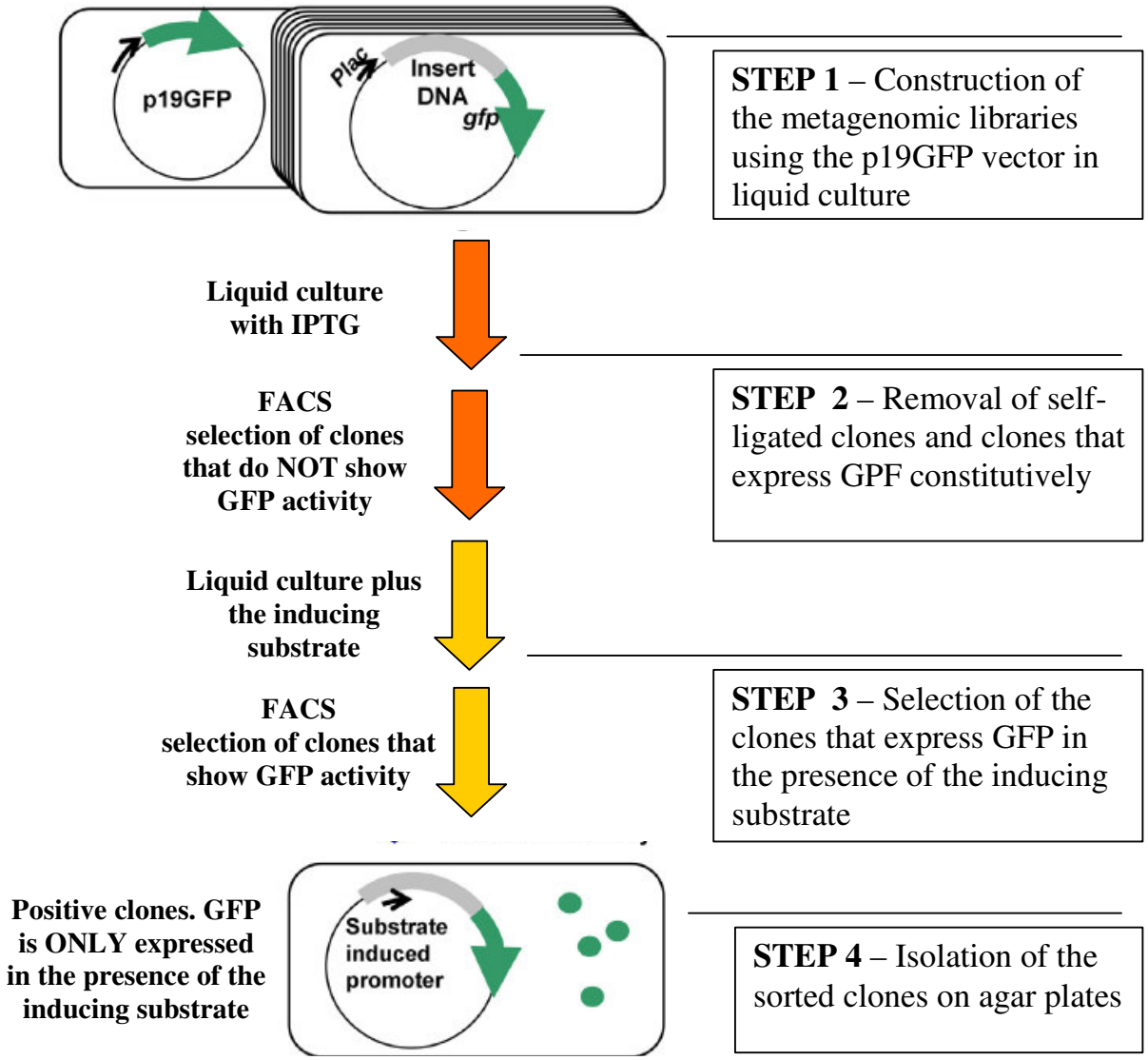

Figure 1.6 – Schematic diagram of the SIGEX process (adapted from reference 21).

Watanabe and his colleagues[26] constructed a library of 33 clones induced by benzoate, and two clones were expressed by naphthalene from a pool with 152000 clones. They also showed that the enzyme Bzo71-8 P450 obtained form the same library was a novel enzyme. SIGEX has tremendous advantages to be used as a screening method in metagenome screening since it provides a more efficient and economical approach to select the desired enzymatic activity[20,21,26]. On the other hand the fact that the possibly to screen for hydrocarbon induced genes is an additional advantage since these genes are quite difficult to express using conventional techniques. In addition SIGEX does not

require special colorimetric substrates that are frequently used in the traditional screening methods.

However the application of SIGEX has also some limitations[19,21]. The first one is related with the fact that SIGEX is only sensitive to the structure and orientation of genes with desired traits, thus missing in this way the genes that are expressed constitutively in nature. Moreover this approach also misses the active clones that have a transcription terminator between the catabolic genes and the following *gfp* gene. For these cases the functional-based screening methods have been used with relative success. Especially for this reason SIGEX is not suitable to be applied to metagenomic libraries harboring large insert DNA, due to the abundance of termination factors. At conditions where SIGEX can be applied, it is the most interesting and efficient technique at the moment for the screening of metagenomic libraries[21]. When SIGEX requirements are not met, other methods are preferable: the screening method should simultaneously account for the type of enzymatic activity and for the type of library that is present[21].

Despite the large investment that the pharmaceutical industry has made in R&D, the number of pharmacologically active new chemical identities (NCEs) has not increased proportionately. Most notable is the decrease in the number of new antibiotic drugs that are discovered and reach the market.[19]. This fact has two main causes. The first is related with the fact that big pharma currently finds antibiotics economically unattractive to develop, and directs its main investment to the development of drugs for chronic diseases like obesity and high cholesterol. This could have dramatic consequences in the future, due to global antibiotic resistance. The second cause is the fact that natural compounds and their derivatives represent a large percentage of all newly approved anti-infective drugs. [19,27]. The problem is that these compounds are mainly secondary products of the activity of bacteria and fungi. Since most microorganisms cannot be easily cultivated, the discovery and characterization of novel active compounds is further compromised. Metagenomics has also been used to deal with this problem. The first targets of this approach were polyketide synthases due to the fact that these enzymes are involved in the synthesis of a broad class of antibiotics including erythromycin, ephithilone and rifamycin[19-21]. The strategy consisted in designing primers that hybridized with very conservative regions of polyketide synthase genes, and amplified novel polyketide synthase genes directly from the soil. From this

type of screening, 11 new polyketide synthase homologues were obtained that contained significant sequence similarity to polyketide synthase genes from cultured microorganisms.

As shown above, the metagenomic approaches have changed the way we conceive a bioprocess, in that in the past we fitted the process to the biocatalysts that were available, whereas right now we can start by defining what we want to produce and then look for a suitable and specific biocatalyst that suits the process. This strategy should be able to keep offering creative solutions for the industrial production of both known and new compounds.

But if metagenomics is the future, we still cannot neglect what we achieved without this approach. Until now we have developed several commercial enzyme preparations that are very competitive in many industrial processes, namely enzymes produced by NOVO and AMANO that have a very high catalytic efficiency, and can be used in several applications that go from laundry to enantiomeric resolution.

What scientists have tried to do is to improve the catalytic properties of the existing enzymes, in order to increase enzyme activity, stability and selectivity towards some industrially relevant targets. The strategies adopted for this purpose also involve molecular biology, since they are directly associated with the modification of enzyme structure and function. They were inspired by Darwin's theory of natural evolution since they involve the evolution of a certain gene towards a specific property, for instance so that an enzyme can be more selective for a group of substrates of even an enantiomer. The first reports of a directed evolution approach were by Stemmer and co-workers[28,29] in 1994, and by Francis Arnold and co-workers[30,31] in 1996.

The concept of directed evolution is a simple one that is based in three step design experiments. The first step consists in creating diversity, the second one in expressing this diversity, and the third one in the screening for the desired property. These steps are repeated in several cycle,s in order to reach the desired property (figure 1.7).

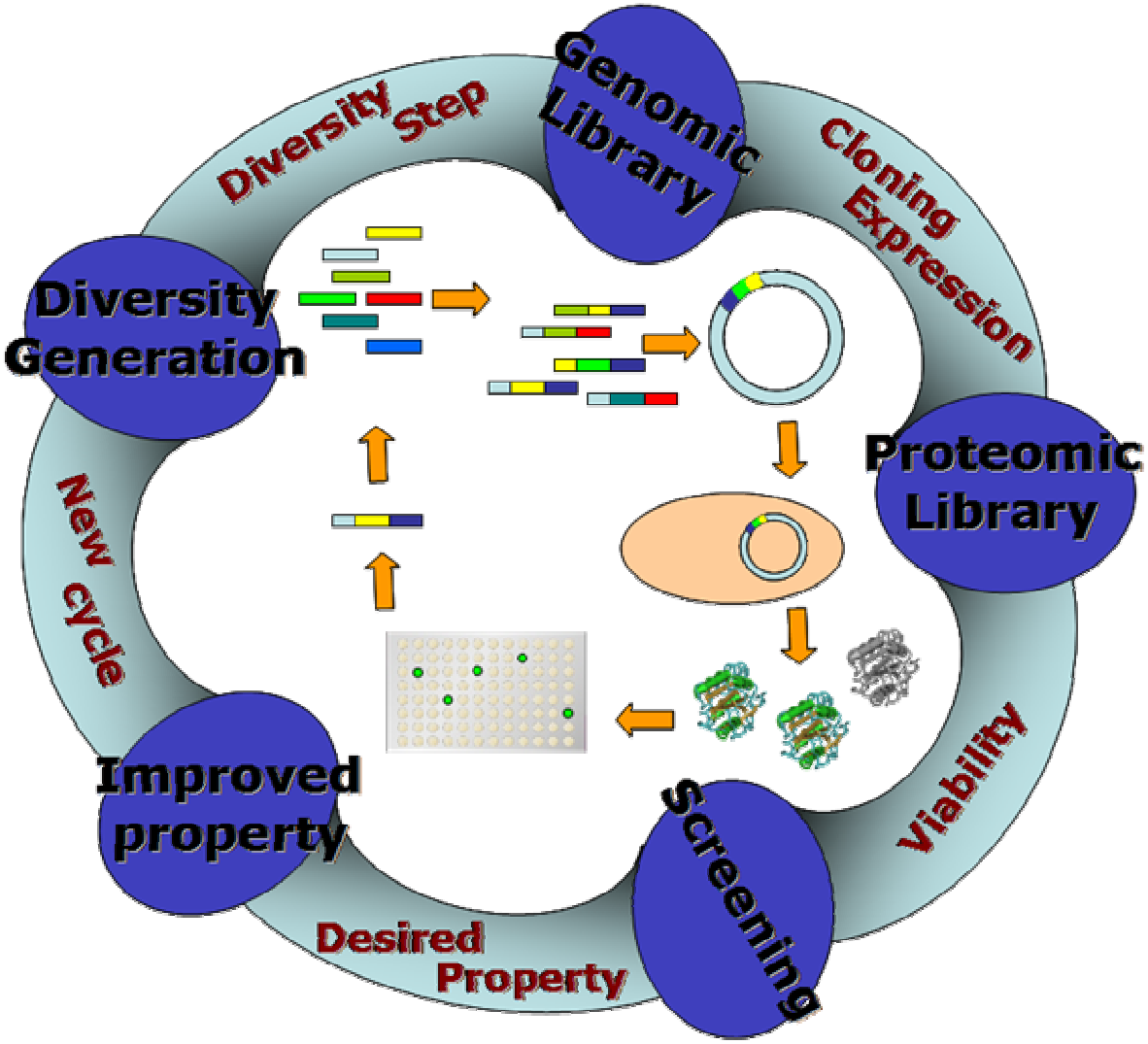

Figure 1.7 – Schematic representation of a directed evolution strategy.

Directed evolution has maturated during the last decade and has became a key technology in the field of molecular enzyme engineering, especially because it allows the modification of a protein to obtain a desired property, without the need of knowing the protein structure. We can find in the literature several excellent reviews that report on the evolution of these strategies[32-37].

The creation of diversity is the crucial step in this technology. This can be achieved by isolating homologous though not identical genes available in nature, or by artificially generating genes, introducing random mutations into a target gene. The subsequent recombination of this diversity has proved to be a very effective strategy for combining advantageous mutations and sorting out the deleterious ones. The first technique to be used to create molecular diversity was **error-prone-PCR** (epPCR), which exploits the

lack of proofreading of polymerase, which can be accomplished by simply changing the concentration of magnesium chloride during the PCR process[32]. In this way the diversity is generated as a function of the errors committed by polymerase. The fact that epPCR introduces random point mutations makes this technique unsuitable to effect block changes that are essential for an efficient sequence evolution. Instead epPCR is a useful technique when just a few cycles of mutation have to be applied. Nevertheless several successful results were obtained with this approach. One of the best examples was demonstrated by Arnold and You[31] who with this technique were able to increase the activity of subtilisin in the presence of a high concentration of the solvent dimethylsulfoxide (DMF). This enzyme is quite stable in DMF but exhibits a very poor activity in it. However after just one cycle of directed evolution, a mutant was obtained containing four different point mutations, which showed a 40-fold increase in activity relative to the wild-type enzyme.

The breakthrough in directed evolution was achieved by the development of **DNA shuffling** that overcame the limitations of the epPCR mentioned before[30,32]. In this case the diversity is generated from a homologous set of genes that are recombined under specific conditions. There is a first step of homology generation. Variants in this step can be obtained by simply combining different homologous genes, such as genes that code for an enzyme expressed in different organisms. These genes are then digested with DNAase I to yield fragments with 50-100 bp range which are then submitted to 40-60 rounds of PCR but without flanking primers. By self-priming the annealed fragment, the DNA chain grows. The extended fragments dissociate during the next step of the PCR cycle, and grow further by self-priming after annealing. In the end a library of chimeric genes is obtained, resulting from the random recombination of their ancestors, that are ready to be screened for the desired function. One of the best examples in the literature of this technique was given by Crameri et. *al*[38] who demonstrated that by shuffling together 4 different genes of cephalosporinase, an important commercial enzyme, was possible to increase the enzyme activity up to 540 fold in just one single round of shuffling The best clone exhibited 33 amino acid point mutations and resulted from seven crossovers of the starting genes. The wide evolutionary distance between the best clone and the individual parents clearly suggests that this particular solution would never have been found using random mutagenic techniques such as epPCR.

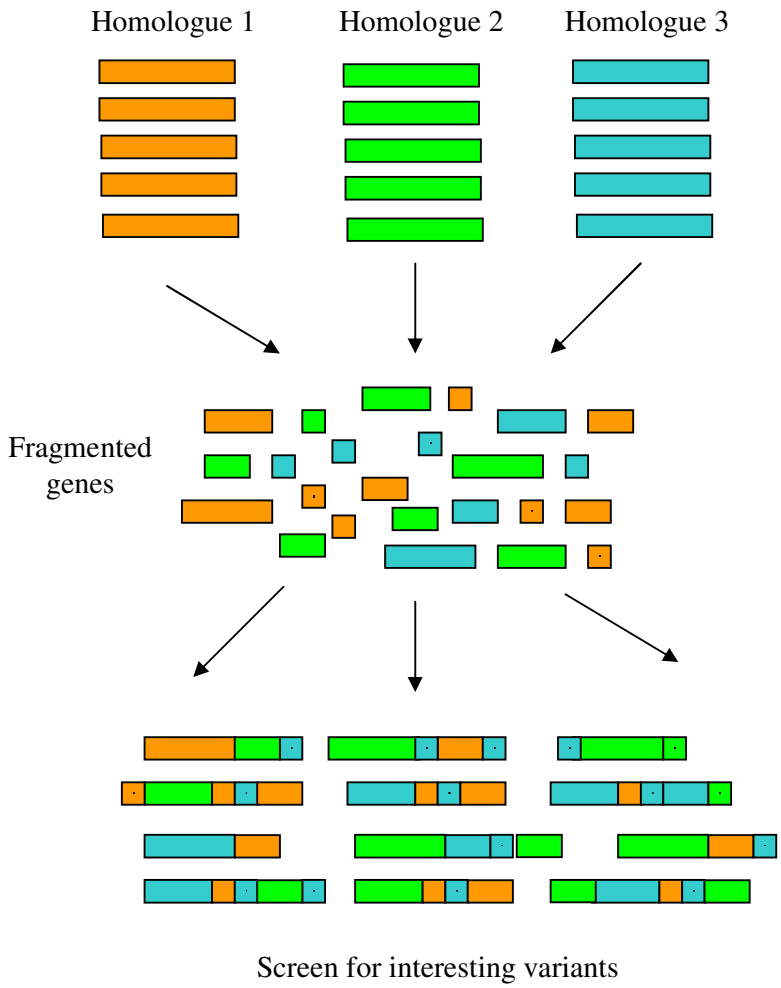

Figure 1.8 - Schematic representation of DNA shuffling. Several homologous genes are fragmented, creating a pool of short fragments. These fragments are allowed to recombine, creating chimeric genes that are made up of a combination of the original genes.

Another good example was reported by Ness et *al*[36] who explored the molecular breeding of the subtilisin family. Subtilisins are commercially important serine endoproteases with a wide range of applications, including food and leather processing, and laundry detergent formulations for stain hydrolysis. Those authors have used a DNA shuffling technique to recombine 25 subtilisin genes with the gene that codes for Savinase®, a highly engineered enzyme. A small set of variants was analyzed for 5 distinct properties, including activity at different pH values, thermostability and solvent stability. In a pool of 654 clones the authors saw improvements in up to 12 % of these clones to any given property. In addition several enzymes had improved multiple

properties, and new combinations of properties were generated. The most interesting thing was that these new combinations were not present in any of the parent genes. This type of family-shuffling approach has also been demonstrated with success in the recombination of several genes from herpes simplex virus that code for thymidine kinase .[39].

Often the desired properties or combinations of several different properties do not exist among natural enzymes, likely because these combinations have not been selected in nature. For example good activity a 23 ºC and thermostability are not required to be present in the same organism, and activity at low pH will not have been selected for alkalophilic species. What this means is that we can in fact look for any desired property in one enzyme, simply by combining the gene that codes for the desired enzyme with a pool of familiar genes. In this way we are increasing the frequency of recombination and the number of possibilities that can be generated by evolution towards the desired property. The example of subtilisins suits perfectly the concept of *in vitro* evolution[38].

In nature evolution occurs thought the combined forces of mutation, recombination and selection. The examples above show that DNA shuffling is a good starting point for transferring the evolutionary process to the lab bench.

DNA-shuffling is still the preferred method in most directed evolution experiments. Recently other methods have been presented that show to be quite good options to create the much desired diversity (table 1.1). Most of these techniques are improvements of DNA-shuffling by itself, and often incorporate changes in the way that diversity is generated before the recombination step[32].

Table 1.1 - Some evolution techniques derived used in ourdays.

Method	Features and problems	Ref.
Family shuffling	Creates diverse, highly functional libraries; closely related genes are required; requires separation of small DNA fragments and libraries contain large percentage of unshuffled clones	Stemmer, 1994 [28,29]
Staggered Extension Process (StEP)	Comparable diversity to family shuffling but with no fragment purification required; same problems as family shuffling	Zhao, 1998[39]
Combinatorial Library Enhanced by Recombination in Yeast (CLERY)	Similar diversity to family shuffling; limited to protein screening in yeast	Abecassi, 2000[40]
Single-stranded DNA shuffling	Higher proportion of shuffled clones than family shuffling.	Kikuchi, 2000[43]
RandomChimeragenesis on Transient Templates (RACHITT)	All generated library members are shuffled; more crossovers than possible with PCR methods	Coco, 200[41]
Sequence Homology-Independent Protein Recombination (SHIPREC)	No requirement for sequence homology; requires size separation of DNA prior to screening; only one crossover per gene	Sieber, 2001[45]
Combination of THIO-ITCHY and family shuffling (SCRATCHY)	More diverse family created than either method alone; useful for generating shuffling candidates where higher sequence homology is required than available genes.	Lutz, 2001[44]
Exon shuffling	No homology required; high percentage of functional clones; limited to intron-containing genes; diversity is proportional to the number of exons	Kolkman, 2001[42]
Gene Site Saturation Mutagenesis (GSSM)	All single amino acid substitutions explored. Technically out of reach for most researchers.	US Patent 6171820

Many different enzymes have been subjected to optimization via direction evolution, including amylases, laccases, phytases, and cellulases[46]. Substrate specificity, thermal stability, and organic solvent resistance, but also more difficult properties such as cofactor-independence or enantioselectivity were evolved using directed evolution techniques[30,35,39,47-49]. Several of these approaches combined different evolution techniques to improve the enzyme desired properties. One of the most interesting examples of this combination was given by Jaeger and Reetz for the increase in enantioselectivity of *Pseudomonas aeruginosa* lipase[34].

The combination of metagenomic approaches together with directed evolution techniques have been recently proposed by Lorenz and co-workers[50]. This elegant approach starts with the amplification of specific partial gene sequences using conserved and degenerated oligonucleotides, called metagenome sequence tags (MST's). Subsequently, shuffling of the cloned fragments and PCR-amplification generates

biocatalyst genes of increased diversity, as shown for dehalogenases and haloperoxidases.

We have looked at the enormous potential of molecular approaches in the discovery and evolution of several types of enzyme functionalities, which makes it possible to improve enzymes without knowing anything about their structure. Nevertheless these methods are still too expensive and time consuming, especially when it comes to screening methods. In the last 7 years many efforts have been made to improve screening techniques in order to design faster and more selective screening processes. An extremely accurate screening is important in the design of an evolution experiment since, in the end, you get what you screen for. In this way the success of the techniques is strictly related with the availability of a good screening procedure.

The problem begins when the screening for the desired enzyme functionality is complex or requires the use of substrates that are not soluble in conventional aqueous media. This is particularly true for properties such as enzyme enantioselectivity. Reetz and co-workers have explored this topic in a very detailed way, bringing to light some screening techniques that improve the screening ratios and efficiency[34,35]. In their several publications in this field they have made use of high-throughput spectroscopic techniques, such as NMR and IR spectroscopies, and special GC and electrophoresis techniques[51]. Nevertheless the size of the libraries also increased as a direct result of the improvement in recombination approaches. Therefore and in spite of the efforts involved, the great limitation in the generalization of these techniques is still the screening for the desired information.

One way to solve this problem is to rationalize the information obtained so far from molecular evolution experiments. For instance, what have we learned about enzyme function so far? Can we correlate the amino acid substitution with the improvement in the desired property that we submit to evolution? Is there a standard or is the system completely chaotic? These are all relevant questions but we can only have an idea of how to start the rationalization if we know more about the enzyme, i.e. its structure. If we know the structure the protein of interest we can start thinking of a better way to evolve it in a certain way. This can be accomplished by detecting *hot-spots* for the desired improvement, i.e. amino acids whose change has a clear impact on the

improvement of the desired property. On the other hand we previously showed that many successful mutants obtained in the course of directed evolution processes have several modifications of their amino acid sequence relative to the wild-type enzyme, which makes the processes of rationalization even more complex. The idea right now is to put the *in vitro in silico*. This requires special tools since it is completely impossible to predict or correlate the impact that certain mutations have on enzyme function. For instance, it is impossible to evaluate the impact on enzyme activity of a mutation located far away from the enzyme active site. Over the last decade the field of bioinformatics has approached the problem of enzyme evolution in several ways, focused both on rationalization and on the generation of diversity.

Recombination strategies have attracted the attention of several groups towards the design of molecular models to predict the shuffling events during the recombination procedure[52-58]. This is quite important to understand and consequently improve the DNA manipulation for library creation. Although the critical step to obtain the desired function is the screening step, the diversity is obtained via DNA recombination. Thus without sufficient diversity in the underlying combinatorial DNA library, all the evolution processes can be compromised. Therefore being able to predict the impact that protocol setups have on the level and type of diversity generated can ultimately determine the success or failure of a directed evolution project. The eShuffle approach was reported for the first time by Moore *et* al.[54,55]. These authors have studied *in silico* the impact that fragmentation length, annealing temperature, sequence identity, and number of shuffled parental sequences have on the number, type, and distribution of crossovers along the length of full-length reassembled sequences. In the eShuffle framework, annealing events during reassembly were modeled as a network of reactions, and equilibrium thermodynamics along with complete nucleotide sequence information was employed to quantify their conversions and selectivities. The authors based their models on the experimental data obtained from several distinct works[36,38.] One of those works was the cephalosporinase evolution study mentioned earlier[38]. One remarkable aspect of this study was the absence of any fragment of either of the four genes shuffled in the chimeric DNA sequence of the best two mutants. Moore et *al.*[54,55] looked at this question and modeled the experimental data. They suggested two possible situations: the fact that gene did not contribute to the increase in enzyme activity with the particular substrate tested, and the fact that the gene was not present simply because the

pieces of that gene were disproportionately misrepresented in the library, due to the lack of sufficient long stretches of near-perfected identity with the other three genes. To put it more simply: of genes 1, 2, 3 and 4, gene 3 was the one absent in the chimeric sequence. Moore et *al*. determined that the identity of each one of the four genes against the remaining three was 70 %, 70 %, 65 % and 59 % respectively. The simulation results predicted that 36 % of the naïve gene sequences contained at least one crossover. But when the authors analyzed the fraction of crossover bearing sequences containing at least one piece from each of the four genes, they saw that gene 3 had the lowest percentage. The results were 85 %, 95 %, 7 % and 19 %, and even though gene 3 was not the one with the lowest sequence identity, it was by far the one with the lowest percentage of crossover. This could explain why it was not present in the chimeric sequences of the two best mutants.

The same authors also verified that the annealing temperature was also responsible for the crossover generation[54,55]. The authors saw that this effect was correlated with the length of the fragments that were present in the medium. Thus the crossover of small fragments of 15 bases was highly increased at high temperatures due to the entropic contribution of the free energy of annealing, which dominates at these temperatures, blurring in this way the distinction between homo and heteroduplexes causing an increase in the total number of crossovers. On the other hand the number of crossovers of long fragments is more favorable at lower annealing temperatures. These results suggest that in a good experiment of evolution, a good compromise should be established between the fragment length and the annealing temperature, in order to increase the diversity via crossover.

Another field explored by bioinformatics is the rational design of proteins. One of the factors that promoted this type of approach was the increase in the number of available enzyme structures[59-62]. In this case the goal of the modeling is to know how the structure affects enzyme properties and how certain structural modifications can affect those properties. Some authors mention this approach as a direct competitor to the *in vitro* evolution design. But both approaches appear to be excellent ways to perform evolution and create diversity, as well as good tools to rationalize the interplay between structure and function. Much can be gained by using a combined approach rather than

the two techniques separately and there are several successful examples in the literature that confirm it.

One of the properties most explored by rational design has been enzyme stability, and several attempts have been made to improve and understand this property. The rational approaches include improvement of the packing of the hydrophobic core of the enzyme, the introduction of disulfide bridges, stabilization of helix dipoles, the engineering of the enzyme surface, salt and point mutations aimed at reducing the entropy of the unfolded state.

Entropic stabilization is one of the most recurrent strategies to increase enzyme stability via rational design and is based on the introduction of mutations that decrease the entropy of the unfolded state but do not introduce unfavorable strain in the folded protein structure[63]. One good example of this strategy was reported by Van den Burg et *al*[63] With a limited number of modifications, the authors were able to increase the stability of a thermolysine-like protease (TLP-ste) from *Bacillus stearothermophlius,* which is considered a moderately stable enzyme. The strategy to achieve this hyperstable enzyme involved replacing residues in TLP-ste by residues found at equivalent positions in thermostable variants, as well as rationally designed mutations. In this study an extremely stable mutant enzyme was obtained that was able to work at 100 ºC in the presence of denaturing agents. Most of the mutations responsible for this stabilization effect were related with the reduction of the entropy of the unfolded state, such as the introduction of two rigidifying mutations (Gly-Ala and Ala-Pro substitutions) and the introduction of one extra disulfide bridge

One of the main issues when efforts are made towards improving one property, such as enzyme stability, is not to affect adversely enzyme activity or selectivity. The increase in stability is often related with the increase in the protein rigidity, and lack of structural flexibility can have a negative effect on enzyme activity. Recently Reetz and co-workers[64] presented an interesting strategy to overcome this problem, consisting on putting together rational and evolutionary tools in order to find target motives in the enzyme structure to direct an evolutionary approach.

The authors first selected the sites for amino acid replacements in order to avoid the screening of large libraries. These sites were chosen based on several systematic structural studies regarding mesophilic and thermophilic enzymes, which pointed out the factors that mostly contribute to an increase in structure rigidity and a resulting increase in enzyme stability. These factors include salt bridges, hydrogen bonds, and π–π interactions at crucial positions in the protein, in addition to hydrophobic interactions and disulfide bridges, all of which help to prevent unfolding at various stages of denaturation[64]. The challenge for these authors was to achieve significant degrees of thermostabilization in an efficient manner without losing enzyme activity at room temperature. Once they identified the potential sites, they developed a model that established a compromise between the rigidity and the atomic displacement parameters obtained from the X-ray data, namely the B factors. Therefore, they targeted solely those amino acids in a protein that display the highest B factors, corresponding to the most pronounced degrees of thermal motion and thus flexibility. These sites, comprising one or more amino acids, were then chosen for saturation mutagenesis which generated small focused libraries. The gene of the best hit was subsequently used as a template for a second round of saturation mutagenesis. From this strategy the authors obtained a considerably small library with 8000 clones, and the result was an impressive enhancement or thermostability without compromising the enzyme activity or even its selectivity.[64]

Until this point we focused on the importance of computational strategies for the improvement of enzymatic properties, either by understanding the factors that affect the diversity generation of an evolutionary process, or by modulating the several contributions that are relevant to one specific property. Nevertheless these strategies have several limitations that are partially overcome when combined with *in vitro* evolution approaches. The idea of putting together *in silico* diversity creation with *in silico* screening processes is the aim of several groups[53,57,65,66]. This approach is based on the development of computational algorithms and is still an emerging area with a high potential in protein design.

For instance, combinatorial randomization of only five residues generates a library of 205 possibilities ($3{,}2 \times 10^6$ mutants), too large a number for manual screening. Thus, to increase the power of rational and combinatorial modification of enzyme activities,

computational methods have been developed based on protein design algorithms. These methods can either perform a virtual screening of a vast library or can be applied to the design of enzyme active sites[65,66].

Hayes et al.[6] developed a strategy for the computational screening of large libraries, called Protein Design Automation (PDA), which predicts optimal sequences space that can adopt a desired fold. PDA was used for pre-screening large, virtual libraries of mutants (10^{23}), and led to a decrease in the number of sequences of interest by many orders of magnitude. PDA allows all, or a rationally defined set of residues, to change. The optimal sequence is chosen based on its lowest conformational energy, and is used to identify other near-optimal sequences through Monte Carlo simulated annealing. The mutations that occur most frequently define the library which is going to be experimentally screened.

With the use of PDA, the authors identified 19 residues of interest in TEM-1-β-lactamase, generated *in silico* $7x10^{23}$ combinatorial mutants of these residues, and chose cut-offs to define the library of roughly 200 000 lowest-energy mutants that were then generated experimentally by mutagenesis and recombination. The advantage of PDA is that it can model a vast sequence of diversity and allows for multiple mutations to be identified simultaneously, which is particularly beneficial when the effect of multiple mutations is synergistic (non-additive).

The fusion between rational and evolutionary approaches is a reality nowadays and with the combination of both approaches it has been possible to achieve very interesting results so far. The evolution of biocatalysts will be in part what researchers improve in this merging area. Recent advances in other areas, namely DNA synthesis, made possible the synthesis of large DNA sequences in a fast, inexpensive and accurate way, which constitute one more powerful tool to develop this area. In the future the biocatalyst could be a result of *in silico* screening for the desired property, followed by the synthesis of an artificial gene that will subsequently be expressed in a free host-cell system.

The way that we use life to improve and development high-tech solutions is about to be changed. An emerging area called **synthetic biology** brings a completely different

concept of biological systems engineering[66-69]. The vision and applications of this area will influence many other scientific and engineering areas, as well as affect various aspects of daily life and society. The goal of synthetic biology is to extend and modify the behavior of living organisms and engineer them to perform completely new tasks. One excellent analogy to conceptualize the goal and the methods of synthetic biology is the computer engineering hierarchy (figure 1.9).

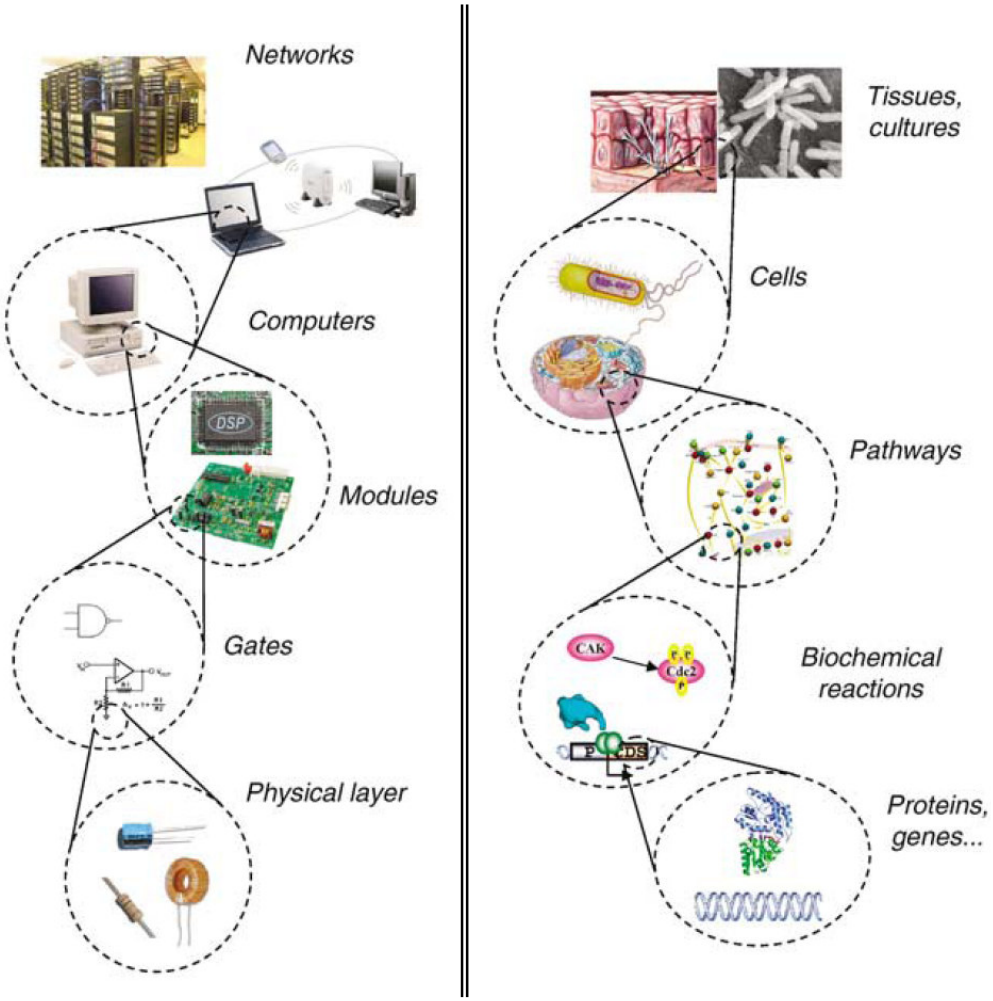

Figure 1.9 - A possible hierarchy for synthetic biology is inspired by computer engineering (adapted from reference 68).

Inside the hierarchy each constituent is a part that contributes to a high complexity system. The implementation of a new action or behavior on top of the hierarchy level is implemented with a bottom-up strategy. So in the cells, we have at the bottom of the hierarchy the DNA, RNA, proteins, and metabolites (including lipids and carbohydrates, aminoacids, and nucleotides), that are analogous to the physical layer of transistors,

capacitors, and resistors, in computer engineering. The next layer is the device layer that comprises biochemical reactions that control not only the flow of information, but also the physical processes. These units are equivalent to engineered logic gates that perform computation in a computer. The module in cells consists in a diverse library of devices to assemble complex pathways that function like integrated circuits. The connection between these modules allows the synthetic biologist to extended or modify the cell behavior in a programmed way. Although single cells can perform several complex tasks, we can achieve more sophisticated and coordinated ones with a population of communicating cells that work like a computer network[68].

This innovative approach brings a completely new set of problems that entail a set of design problems and solutions. This is because biological devices and modules are not independent objects, and are not built in the absence of a biological environment. With this in mind synthetic biologists have seriously considered cellular modification in order to run the desired function. The major concern in this approach so far is the lack of information about inherent cellular characteristics, for instance the effects of gene expression noise, mutation, cell death, undefined and changing extracellular environments. Also interactions within the cellular context currently prevent us from engineering single cells with the same confidence that we can engineer computers to do specific tasks[67-69].

But the answer to control the predictability and reliability of these biosynthetic networks can be found inside is own concept, and could be achieved either by using a large numbers of independent cells, or by synchronizing individual cells through intercellular communication. Even more interesting is to use intercellular communication systems in order to coordinate tasks across heterogeneous cell populations, which is a highly sophisticated behavior. The concept has to focus on multicellular systems to achieve overall reliability of performance[67-69].

The concept of a device is an abstraction overlaid on physical processes that allows for the decomposition of systems into basic functional parts. Thus complex systems have been designed through the combination of basic design units that represent biological functions. These biological devices have the basic function of processing inputs to produce outputs, and to accomplish this they have to control the information flow,

perform metabolic and biosynthetic functions, and interface with other devices that can be inside or outside of its own environment. The application of this new technology of "programming" cells is achieved by "reprogramming" their sets of biochemical reactions including transcription, translation, protein phosphorylation, allosteric regulation, ligand/receptor binding, and enzymatic reactions.[67-69].

Since this field is in its very infancy, there are only a few examples that constitute in fact novel biodevices. A new application was obtained by coupling gene regulatory networks with biosensor modules and biological response systems. Kobayashi et *al.* [67] interfaced a toggle switch with the SOS pathway detecting DNA damage as the biosensor module, and biofilm formation as response output. The exposure of the engineered cells to transient UV irradiation caused DNA damage, triggering the toggle switch to flip to the state that induced the formation of biofilms (figure 1.10).

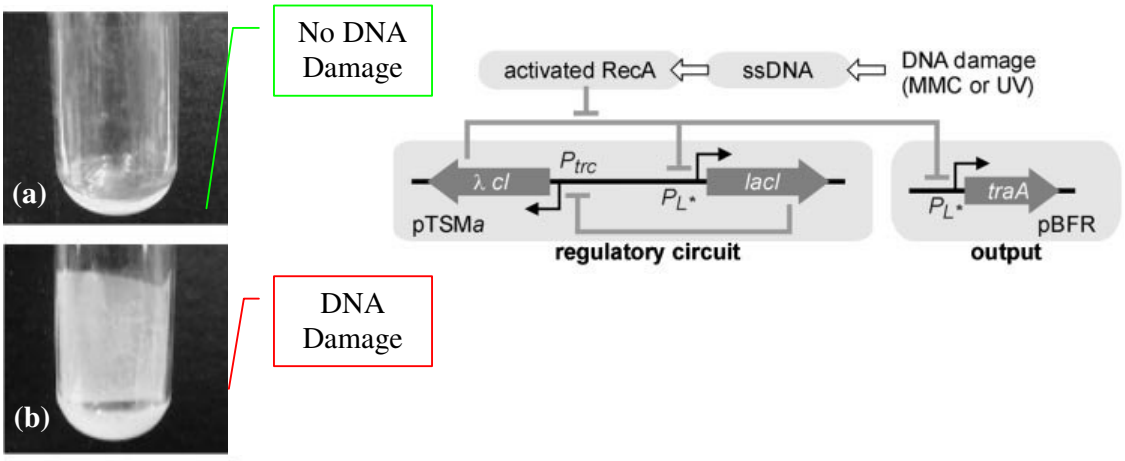

Figure 1.10 - An example of programmed phenotype in strain A2. The diagram represents the engineered genetic circuitry. The genetic toggle switch module (pTSM*a*) controls the expression of *traA* from plasmid pBFR in response to DNA damage. The result of the activation of the pTSM*a* switch is the formation of the biofilm, which is represenedt in picture (b) .(adapted from reference 67).

Recently, Anderson et al,[70] engineered *E. coli* cells that were able to invade specific mammalian cells exhibiting tumorigenic properties. In this study the interaction of *E. coli* with mammalian cells was modified in order to be dependent on the heterologous environmental signal. To do this the authors built different plasmids containing different genes that could promote the invasion in response to an external signal. They characterized invasin, which is a protein of *Yearsinia pseudotuburculosis*, as an output

module that enables *E.coli* to invade the cancer cells. But this invasion should be specific and in order to accomplish this they placed this module under the control of heterologus sensors. For that purpose, the authors used as promoters the *Vibrio fischeri* lux quorum sensing circuit and the hypoxia-responsive fdhF. The result was simple (figure 1.11).

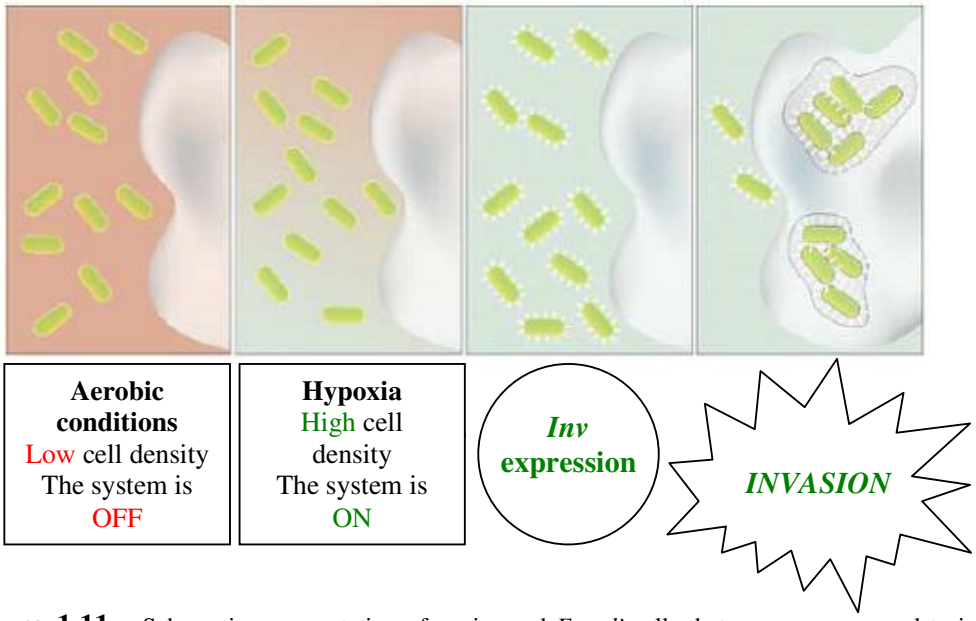

Figure 1.11 – Schematic representation of engineered *E. coli* cells that were programmed to invade mammalian cells exhibiting tumorigenic properties.

Biocatalysis is always a good area to demonstrate new concepts, and synthetic biology confirms this statement. It is easy to imagine a biofilm completely programmable to respond to different inputs, which can be for instance a chemical stimulus or an environmental change. The resulting outputs are products of the biocatalytic cascade involving the biochemical sets mentioned earlier, and can be materialized in the form of a substance of pharmaceutical interest, such as a protein complex, or even in the form of an electric current. The function of this biofilm will be the result of the behavior that we set on the top of the hierarchy, and the way that it accomplishes that function will be a result of the programme set at the bottom, i.e., the DNA.

We can have glance at this idea if we consider the study of Lovley and co-workers[71-73] where these authors present a very interesting way to produce energy using a biofuel cell(figure 1.12). They used the oxidative metabolic pathways to oxidized sugars and to

generate electrons. They demonstrated that this concept could also occur in soil, and they developed a fuel cell that could use the microorganisms fauna presented in the soil to produce electricity. Now imagine that we could put both concepts together. We could produce electric energy in a very cheap way from a myriad of natural resources everywhere in this planet. Maybe this is one the futures of biocatalysis. Of course there are severe limitations to the implementation of this technology, but those limitations are our future challenges.

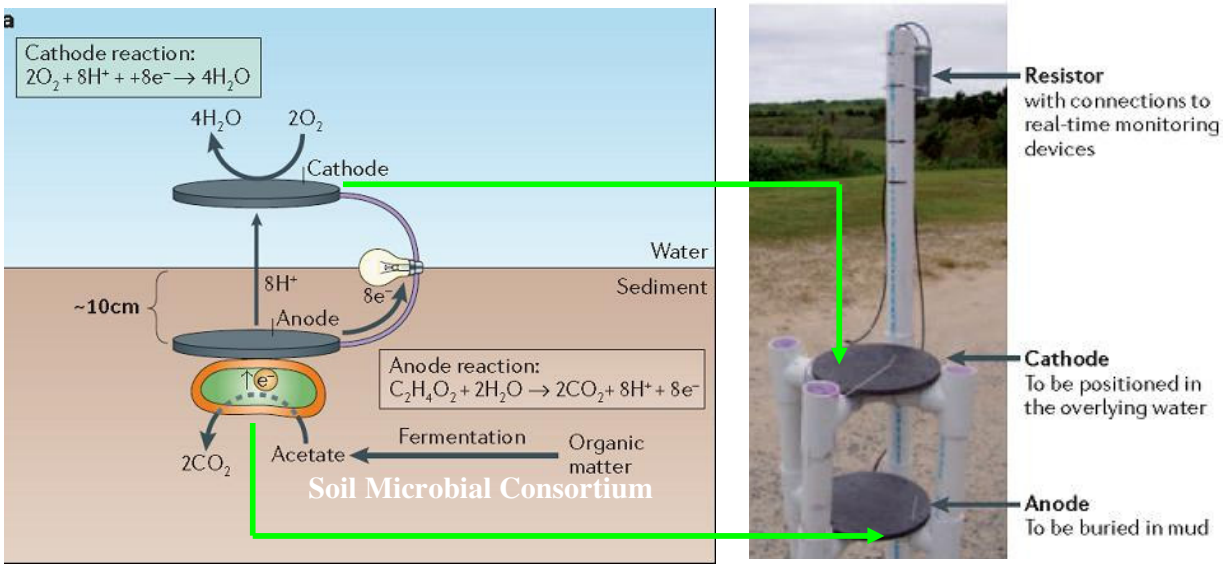

Figure 1.12 – Example of microbial fuel cell developed by Lovley et al. (adapted from reference 71).

Today we can consider biocatalysis as a melting pot of different disciplines that go from organic chemistry to molecular biology. This soup of different areas of knowledge and expertise puts biocatalysis on the top of the areas with industrial priorities.

In summary the aim of this chapter was to give a global idea of biocatalysis and its impact in science and society along the times. We have used this technology even before we were aware of the underlying science, we have uncovered some of its mysteries and taken it beyond the most optimistic expectations, and nowadays we rationally incorporate it in many applications whose scientific basis most of those who benefit from them completely ignore. This is in fact the best way to evaluate the success of a technology.

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CHAPTER II

Biocatalysis:
the key to chiral resolutions.

As we saw in the previous chapter the high enantioselectivity that enzymes exhibit has always been pointed out as one of the major advantages of enzymes for industrial applications, and makes biocatalysis one of the most suitable approaches towards the chiral resolution of organic racemic mixtures. Therefore a considerable number of researchers have put a lot of effort into understanding and improving enzyme enantioselectivity, considered by many to be the hallmark of biocatalysis.

This subject attracted particular interest in the 80s when is became clear that enzymes could be active in dry organic solvents and could perform organic syntheses in these media. It was also shown that in nonaqueous media enzyme enantioselectivity responded to the medium properties. Thus organic solvents did not only provide a way of doing biocatalytic synthesis, but could also tune enzyme selectivity.

Having this in mind the aim of the present chapter is to give an idea of the high potential of enzymes in performing chiral resolutions. To accomplish this it is necessary to go trough fundamental aspects of this property, such as the elucidation of the molecular mechanisms behind enantioselectivity, several successful examples of application, the means that the scientific community developed to improve this enzymatic property.

In general, all the strategies mentioned in section 4 of the previous chapter to improve catalytic performance can also be applied to enzyme enantioselectivity, and some examples relating to enantioselectivity were already mentioned. One of the classes of enzymes that have been extensively explored in this respect is that of serine hydrolases, which includes the lipases, esterases and proteases. For instance lipases are considered excellent biocatalysts to prepare optically active compounds such as alcohols, carboxylic acids, esters, amines, thiols, hydroperoxides and ketones, and have been used is the asymmetric synthesis of natural products[1-5]. Lipases also exhibit a good stability and activity in nonaqueous media and are in fact the enzymes that were more helpful in understanding and developing enantiomeric resolutions. Thus, most of the applications and fundamental studies that are going to be presented here are mostly related with lipases.

The first thing that we should have in mind when studying a property related with enzyme selectivity is the catalytic mechanism. Since lipases are serine hydrolases the catalytic mechanism is well described by figure 2.1.

The active site of serine hydrolases is formed by an oxyanion hole and a catalytic triad formed by a serine (Ser), a histidine (His) and an aspartic acid (Asp). The switch of this active center is the His, since it works as a proton acceptor from the serine residue. This switch is in fact controlled by the pKa of the His residue (pKa=6), which means that only above this pKa the histine is able to accept the proton from the serine. This step is crucial for the active site catalytic function since the serine can only act as a nucleophile when its proton is set aside by histidine.

The best way to understand this mechanism is with an example. Here we present the transesterication of a generic ester with a generic secondary alcohol. In this case four transition sates and two tetrahedral intermediates are formed, in order to accomplish the reaction. The stabilization of tetrahedral intermediates is vital for the reaction progress, and occurs through electrostatic interactions between the imidazolium of His and the carboxylic group from the Asp, as well as by the hydrogen bonding between the acyl donor and the amine group form of the oxyanion hole. The acylation of the enzyme by an acyl donor gives the acyl enzyme-intermediate (E-Ac), and subsequent nucleophilic attack by an alcohol (R-OH), as can occur in a nonaqueous medium, yields an ester and free enzyme.

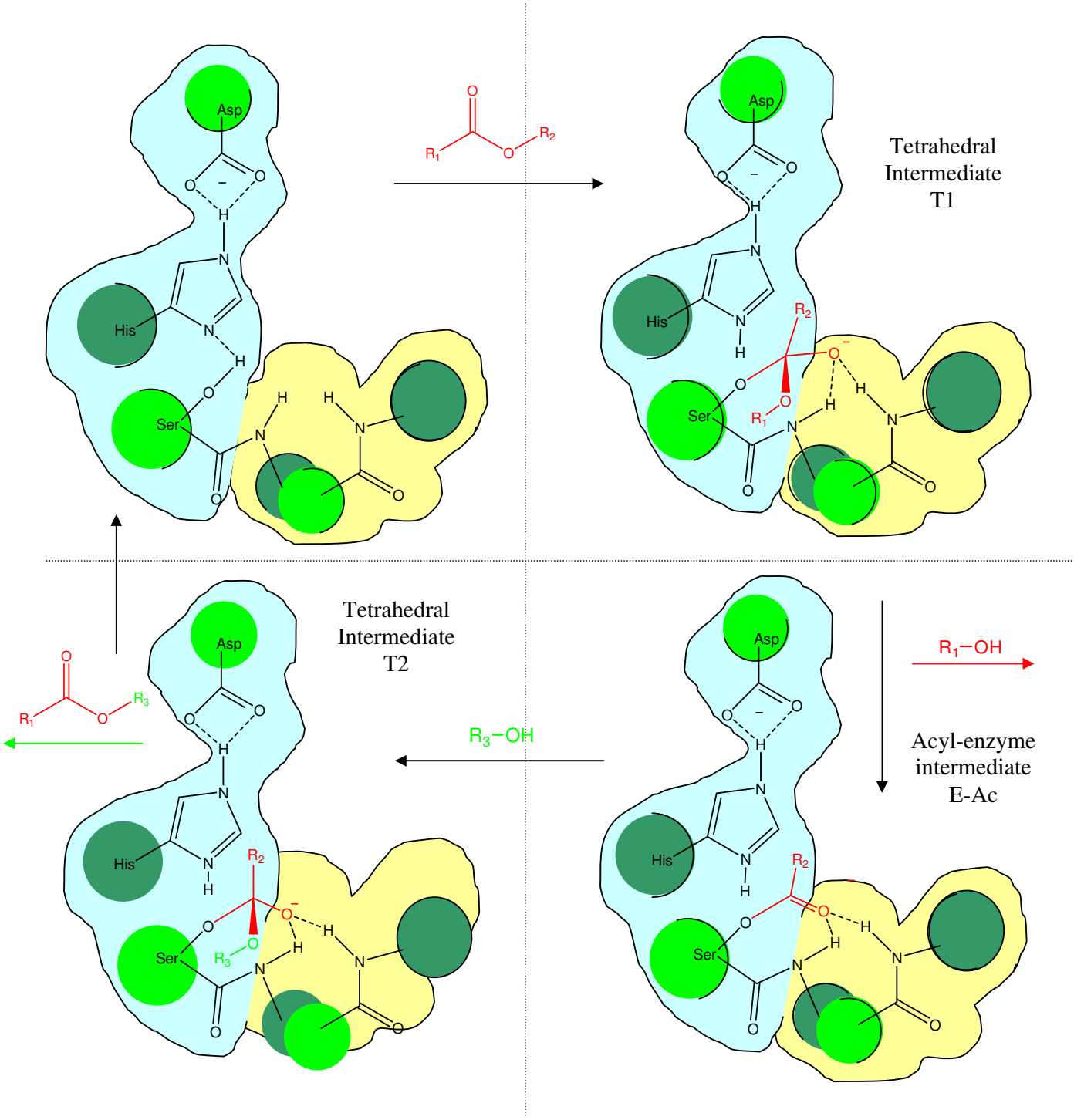


Figure 2.1 - The catalytic cycle of a lipase/esterase for the transesterification of a generic ester with a generic alcohol.

Before going trough the reasons why enzymes exhibit enantioselectivity towards some substrates, there are two concepts that should be understood: the enantiomeric excess (ee) and the enantiomeric ratio (E).

The enantiomeric purity is expressed in terms of the ee value and is defined as

$$\%\ \text{eeR} = \frac{\text{R-S}}{\text{R+S}} \text{x}\, 100 \qquad \text{(For R>S)}$$

R is the concentration of the (*R*)-enantiomer and S the concentration of the (*S*)-enantiomer. Therefore for a racemic compound the ee value is zero, whereas for an enantiomerically pure compound the ee value is 1 (or 100% ee). Since lipases are chiral structures they posses the ability to distinguish between both enantiomers of certain racemic mixtures. The parameter that quantifies this is the enantiomeric ratio, E. The E value is defined as the ratio of specificity constants for the two enantiomers:

$$E_{RS} = \frac{(K_{cat}/K_M)_R}{(K_{cat}/K_M)_S} \text{x}\, 100$$

k_{cat} is the rate constant and K_M the Michaelis-Menten constant. One of most important contributions in this respect was made by Sih and his colleagues[6] who developed the next equation for E as a function of the ee of the product (ee_p) and the unreacted substrate (ee_s), and the conversion (c). Therefore, for a reversible enzymatic reaction the E value is expressed by:

$$\begin{aligned} \text{E} &= \frac{\ln[1-(1+K)c(1+\text{ee}_\text{p})]}{\ln[1-(1+K)c(1-\text{ee}_\text{p})]} \\ &= \frac{\ln[1-(1+K)(c+\text{ee}_\text{s}\{1-c\})]}{\ln[1-(1+K)(c-\text{ee}_\text{s}\{1-c\})]} \end{aligned}$$

K is the equilibrium constant. When the reaction is irreversible or the reverse reaction is negligible (K = 0), this equation is reduced to:

$$\text{E} = \frac{\ln[1-c(1+\text{ee}_\text{p})]}{\ln[1-c(1-\text{ee}_\text{p})]} = \frac{\ln[(1-c)(1-\text{ee}_\text{s})]}{\ln[(1-c)(1+\text{ee}_\text{s})]}$$

Where (c) can be expressed by:

$$c = \frac{ee_s}{ee_s + ee_p}$$

Thus E can also be expressed in terms of ee_s and ee_p:

$$E = \frac{\ln\left[\frac{1 - ee_s}{1 + (ee_s/ee_p)}\right]}{\ln\left[\frac{1 + ee_s}{1 + (ee_s/ee_p)}\right]}$$

To calculate the E value we have to measure two of the three variables ees, eep, and the extent of conversion (c). A nonselective reaction has an E value of 1, while an E value above 100 is the minimum for an acceptable resolution[3].

Figure 2.2 shows how the substrates have to bind to the active site for the reaction to take place. This fact suggests that the dimensions of the chemical groups can modify the way the substrates can be accommodated inside the active site. For instance too large groups can make the stabilization of the tetrahedral intermediates difficult, since these groups have to find a pocket of suitable dimensions to fit in, if the reaction is to take place. This explains why esterases exhibit low activity towards long chain acyl donors or alcohols[7-8].

This type of assumption has led to the design of the first rationalization methods to understand enantioselective discrimination based on stereochemical rules. These models were put forward in the late 80s and early 90s with the aim to predict the enantioselectivity of the most common enzymes in industrial process[7, 9-13]. In 1991 Roman Kazlauskas[7] and co-workers presented more than 130 examples for several enzymes, namely *Burkholderia cepacia* lipase (formerly known as *Pseudomonas cepacia* lipase), *Candida rugosa* lipase and cholesterol esterase that were in agreement with the rules based on the size of the substituents bound to the chiral center of substrate. (figure 2).Today this rule is frequently called the “empirical rule” and is basically used to predict the enantioselectivity of some enzymes towards chiral alcohols and acids.

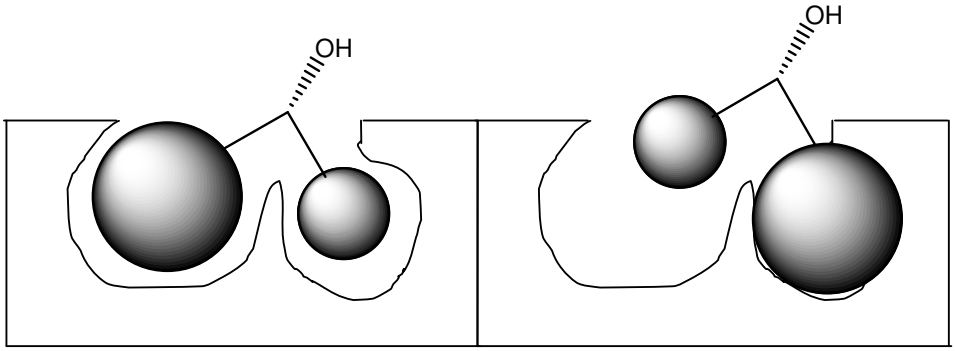


Figure 2.2 – "empirical rule"; (a) - the fast reacting enantiomer; (b) - the slow reacting enantiomer

This model was quite important to design the first attempts to improve enzyme enantioselectivity towards a desired racemic mixture. This substrate engineering approach is based on the subtle modification of the substrate to change the enzyme enantioselectivity in a given reaction. Having in mind the previous model, this approach is no more than the attempt to move the chiral centre to pockets that favor the stabilization of one of the enantiomers. This is in fact a kinetic engineering approach since the structure of the enzyme is not affected, and the only change that is effected is the accommodation of the substrate in the active site.

The success of this approach was confirmed by several authors[14,15]. One of these approaches was related with the oxidative kinetic resolution of *sec*-alcohols using *Rhodococcus ruber* DSM 44541. In this case it was found that the introduction of C=C bond units into the side chain could improve the reaction enantioselectivity[14.] The enantioselectivities towards *rac*-2-pentanol and *rac*-3-octanol were E=16.8 and E=13.3, respectively, and were improved to E>100 and E=50 just by placing a C=C bond adjacent to the carbonyl group.

Two other authors, Magnusson and Holmquist[15], presented a different approach called engineered substrate-assisted catalysis. They used for this purpose *Candida antarctica* lipase B (CALB) as a model system, and also have created a mutant of this enzyme with a substitution in the active center. They replaced a threonine, Thr40, which is involved in the stabilization of the transition state, by a valine residue (figure 2.3). The aim of this strategy was to replace the side chain hydroxyl group of Thr40 by one placed in the substrate to recreate the equivalent interaction found in the wild-type enzyme–substrate pairing. In fact, ethyl-2-hydroxypropanoate turned out to be a good substrate for the

mutant since it was able to increase the selectivity towards the (*S*)-isomer (E = 22), relative to the wild-type enzyme (E=1.6).

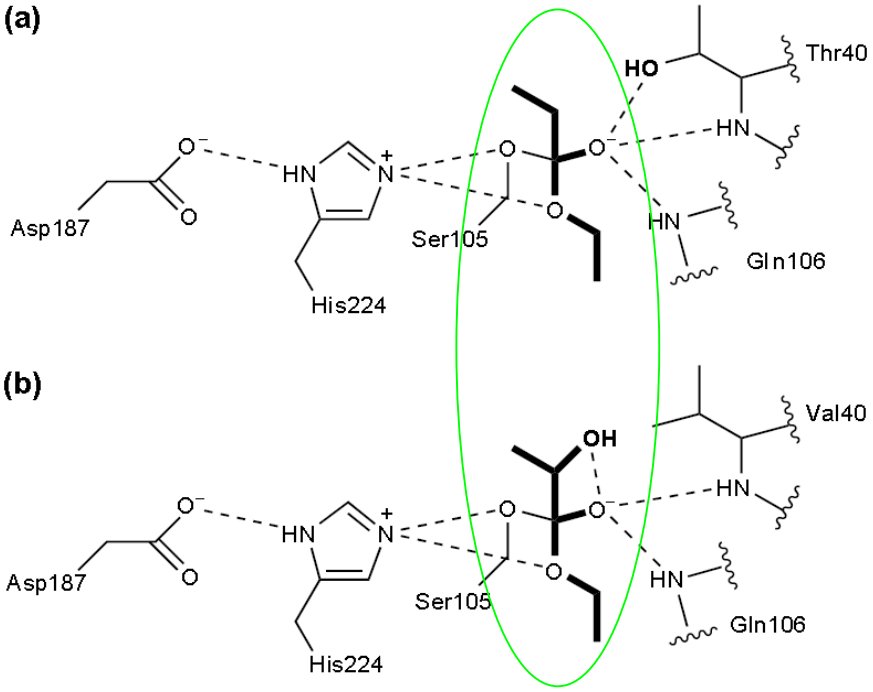
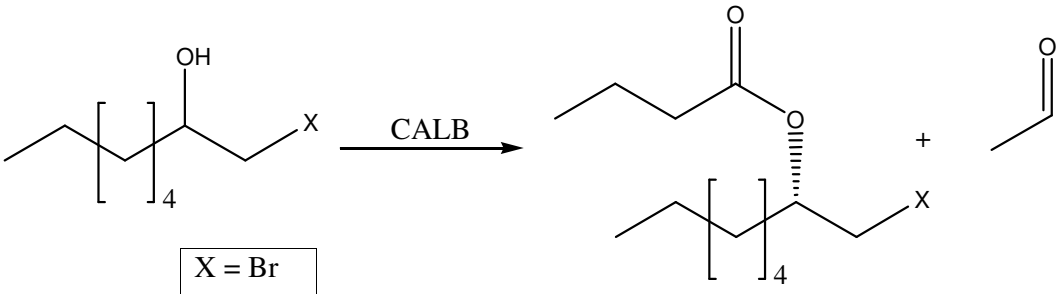


Figure 2.3 - The active site of CALB. (a) Transition-state stabilization in the wild-type enzyme. (b) Substrate-assisted transition-state stabilization in a Thr40Val mutant. The substrate is represented by a thick black line and hydrogen bonds are indicated as dashed lines. This figure was adapted from reference 16.

This type of approach led to the development of several models with the aim of understanding the contributions involved in enantiomeric discrimination at the enzyme active center [17-19]. Indeed this research provided the guidelines to the development of rational protein engineering, or rational design, which is an important tool today.

Karl Hult and co-workers[20-29] are one of the groups that have contributed more to the development of this area. Most of their studies have been focused on *Candida antarctica* lipase B, which is a preferred enzyme for many industrial applications. This group has proposed a model to explain the mechanism of enantiorecognition by CALB towards secondary alcohol enantiomers. This molecular model was based on the transition states of the fast and the slow enantiomers, as well as on the experimental kinetic resolution of sec-alcohols[27-29]. The authors concluded that steric interaction was important but it was not the only factor that contributes to the enantiomeric

discrimination. For instance they showed that CALB exhibits a quite different enantioselectivity towards aliphatic alcohols and their almost isosteric halohydrin analogues (figure 2.4). They correlated this difference with the unfavorable interaction between the halogen atom and the stereoselectivity pocket of the enzyme. These results led them to a rational design approach in order to create mutants that could exhibit a different enantioselectivity toward these substrates.



X = Br
X = Cl
X = CH_3

Figure 2.4 – Enzymatic kinetic resolution of sec-alchohols with *Candida antartica* lipase B. this reaction scheme as adapted from reference 26.

Hult and co-workers proposed several mutants on specific sites, namely Thr40, Thr42, Ser47 and Trp104 (Trp = tryptophan), which define the stereoselectivity pocket of CALB. They excluded the Thr40 due to the importance of this residue on the stabilization of the oxyanion formed in the transition state. This approach is completely different from the one presented before by Magnusson and Holmquist, who considered the modification on this residue an advantage to increase enzyme enantioselectivity in their substrate engineering approach. This fact clearly suggests that there are multi-solutions to improve the enantioselectivity by a rational design approach. Hult and co-workers only considered modifications in those sites toward smaller residues, due to limited space in the stereoselectivity pocket, but their aim was actually to decrease the electronegativity of the pocket. The decrease in the repulsion felt by the halogenated substrates was meant to decrease the enantioselectivity towards these particular substrates. To accomplish this, Hult and co-workers introduced basic residues in those sites, in order to increase the affinity of the new residues towards the halogen group. Their results confirmed that their rationalizations were correct, i.e. that an increase in the volume of the stereoselectivity pocket could decrease the enantioselectivity, while

changes in electrostatic potential increased the enantioselectivity. In fact, the Thr42Val, Ser47Ala (Ala = alanine) single mutations and the Thr42Val/Ser47Ala double mutation only affected the stereoselectivity towards the halohydrins. The E value for aliphatic alcohols remained the same when compared to the wild-type. These results show that the change in the E value obtained for the mutants is strictly related with electrostatic contributions. The model proposed by Hult and co-workers was the first to consider the contribution of entropy to the enantioselectivity.

A very elegant example to improve enzyme enantioselectivity through a rational approach was reported by Raushel and co-workers[30] for the phosphotriesterase of *Pseudomonas diminuta*. In this example the understanding of the stereochemical specificity of this particular enzyme was the starting point for the rational design strategy. The stereochemical pocket of this enzyme has three biding subsites with different sizes. These authors found that by introducing small modifications with the aim of altering the dimensions of these sites, they could produce dramatic changes in enzyme enantioselectivity. One of the best results was obtained with ethyl phenyl p-nitrophenyl phosphate (figure 2.5), a substrate that the enzyme hydrolyzed with E=21, preferring the *(S)*-enantiomer. But when they introducedc the Gly109Ala (Gly = glycine) mutation with the aim of reducing the size of the smaller subsite, the enantioselectivity towards the (*S)*-enantiomer increased 3-fold. This improvement in selectivity was related with the increase in K_{cat}/K_m for the fast reacting enantiomer and the subsequent decrease in K_{cat}/K_m for the slow reaction one. The true success of this work was obtained when these authors performed a size reduction of the larger subsite (His257Tyr; Tyr = tyrosine) together with an increase in the size of the small subsite (Ile106Gly/Phe132Gly/Ser308Gly; Ile = isoleucine; Phe = phenylalanine). The result was the inversion of the enantioselectivity, i.e. the (*R)*-enantiomer, which was the slow reacting one, became favoured over the (*S)*-enantiomer by a factor of 80.

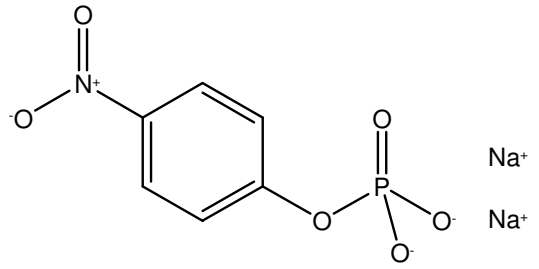


Figure 2.5 – An example of a *p*-nitrophenyl phosphate compound used on the approach reported by Raushel and co-workers[30]

A more complex example of this type of rational approach has been proposed by Pleiss and co-workers[31,32], who made a comparison between the structure of several lipases and esterases and found that lipases differ in a certain motif located in the active site. This motif is called the *GX or GGGX* motif, where G denotes glycine and X denotes any aminoacid located in the oxyanion binding pocket of lipases and esterases. This motif is responsible for the stabilization of the tetrahedral intermediate during catalysis. Later Bornscheuer and co-workers[33] showed that mutations in this motif could effect changes in activity and enantioselectivity towards tertiary alcohols. In fact their modelling study of p-nitrobenzylesterase from *Bacillus subtilis* (BsubpNBE) showed that mutations in one of the glycines of this motif (Gly105Ala) was responsible for more than a three-fold improvement in enantioselectivity for the resolution of (*R,S*)-2-phenyl-3-butin-2-yl acetate.

The impact of medium parameters on enzyme enantioselectivity has also been a target for molecular modelling. A good example of this type of approach was brought again by Pleiss and co-workers[32] who evaluated the impact of pressure on the enantioselectivity of *Candida Rugosa* towards menthol. The aim of their study was to understand why *Candida Rugosa* exhibited a decrease in enantioselectivity along with the increase in medium pressure in aqueous an organic media. It is known that distinct regions of the protein respond differently when submitted to pressure. For instance, helical and loop regions have shown higher compressibility and volume fluctuation than β-sheets[34,35]. These variations could impact on enzyme structure and ultimately be reflected on enzymatic properties. This is easier to understand when the property in case is activity or stability. These authors found that enantioselectivity could also be affected by these changes in enzyme structure. They explained that the geometry of the stereocenter of

this enzyme was affected to favor the formerly nonpreferred enantiomer, explaining in this way why E gradually decreased with the increase in pressure.

The rationalization of the several parameters that can affect enantioselectivity is quite important because it makes it easier to predict and consequently to modify that property towards the desired kinetic resolution. In the previous example the authors directed their attention to the impact of pressure on enzyme enantioselectivity. In nonaqueous media, that and other medium parameters such as temperature, water activity, solvent polarity or enzyme ionization state have been explored in the first strategies to improve enantioselectivity. For instance the discovery that organic solvents could enhance and in some cases even reverse enantioselectivity was a tremendous achievement[36-38]. The medium engineering solvent approach is still quite important today and many successful examples have been published so far. It is still one of the first ways to evaluate and improve enantioselectivity when a new enzyme or a new reaction setup is used.

One such example was provided by Westcott and co-workers[36]. In this work the authors showed that the α-chymotrypsin enantioselectivity towards the transesterification of methyl 3-hydroxyl-2-phenylpropionate (figure 2.6) with propanol could be enhanced just by changing the type of solvent. More interesting, the authors showed that the enzyme strongly prefers the *(S)*-enantiomer of the substrate in solvents like di-isopropyl ether or cyclohexane, and that this preference is inverted when the reaction is performed in acetonitrile or methyl acetate. This work was made is the middle 1990s and was surprising at the time, but such effects were shown to be a general characteristic of some enzymes[39-41], and in some cases could be rationalized quite easily. For instance the α-chymotrypsin inversion mentioned before was rationalized taking into account the different transition states formed by the *(R)*- and the *(S-)* enantiomers in the different solvents. The structural modelling of both transition states showed that the solvent promoted changes in the sterochemical pocket, creating different reaction pathways with a profound effect on the transition state stability, which was ultimately reflected on enzyme enantioselectivity[38].

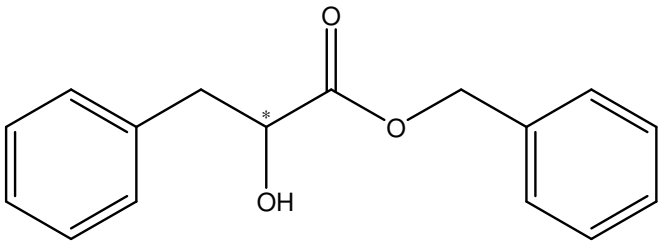


Figure 2.6 - Hydroxyl-2-phenylpropionate. This was the coupond was the target substrate of Westcott and co-workers[36] study.

The solvent effect is also responsible for controlling two other types of enzyme selectivity: regio- and chemo-selectivity. The first one is related with the preference of the enzyme for the position of a specific functional group in the substrate molecule. This preference was shown to be present in *Burkolderia cepacia* lipase[41] when two differently positioned ester groups were placed in the same aromatic molecule substrate. This enzyme also showed the same effect towards sugar hydroxyl groups, and in this case the enantioselectivity can even been reversed just by changing the position of the OH group on the sugar molecule. The chemo-selectivity is the preference that an enzyme exhibits for one type of reaction over a set of other plausible reactions. For many lipases and proteases, the degree of preference for a hydroxyl group relative to an amino group has been found to be strongly dependent on the solvent[36, 41-43].

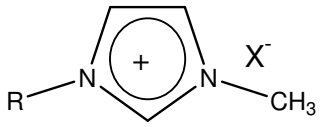
The solvent engineering approach was fully explored and we can find examples for most of the enzymes used in biocatalytic transformations. Thus this approach only attracted new scientific interest when ionic liquids started to compete with organic solvents as reaction media. Ionic liquids are one of the pillars of green chemistry technology. Very simply they are organic salts which are liquid at room temperature. Unlike traditional solvents, which can be described as molecular liquids, ionic liquids are composed by ions (figure 2.7 shows the structures of the commonly used ionic liquids). Their unique properties such as nonvolatility, nonflammability, and excellent chemical and thermal stability have made them an environmentally attractive alternative to conventional organic solvents, on the road to higher sustainability. Ionic liquids have low melting points (<100 ºC) and remain as liquids within a broad temperature window (<300 ºC)[46-48]. Ionic liquids are known as tailored or design solvents, due to the fact that it is relatively easy to change the intrinsic properties of these solvents. For instance we can change the polarity of the solvent just by changing the anion or the cation of its structure. This type of design strategy has been explored in biocatalysis in order to

enhance several enzymatic properties. Obviously enantioselectivity has been the property of major interest[45-52]. Although ionic liquids have tremendous applications in several different areas, in this chapter only their impact on enzyme enantioselectivity is going to be highlighted. A more detailed description of this new class of solvents will be presented in Chapter IV of the present thesis.

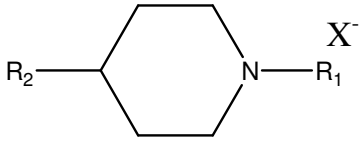
There are many excellent examples of enantioselectivity improvement related with ionic liquids design, but only the most relevant ones will be referred here since the concept underlying this approach is similar to that of medium engineering in organic solvents[47,48,50-55]. The first example of enantioselective biocatalysis in ionic liquids was presented by Kragl and co-workers[46] who reported the screening of nine different lipases in ten different ionic liquids for the classical kinetic resolution of *rac*-1-phenylethanol by transesterification with vinyl acetate. This study revealed that ionic liquids could be a very good alternative to organic media since the studied enzymes showed good activities and in some cases improved enantioselectivities compared with the same reaction in methyl *tert*-butyl ether (MTBE). Another observation was that quite different reactivity patterns were observed in the different ionic liquids.

Abbreviation	R group	X^-
[MMIm][$MeSO_4$]	CH_3	$CH_3OSO_3^-$
[EMIm][BF_4]	C_2H_5	BF_4^-
[EMIm][Tf_2N]	C_2H_5	$(CF_3SO_2)_2N^-$
[BMIm][BF_4]	n-C_4H_9	BF_4^-
[BMIm][PF_6]	n-C_4H_9	PF_6^-
[BMIm][TfO]	n-C_4H_9	$CF_3SO_3^-$
[BMIm][Tf_2N]	n-C_4H_9	$(CF_3SO_2)_2N^-$
[BMIm][$MeSO_4$]	n-C_4H_9	$CH_3OSO_3^-$
[BMIm][$EtSO_4$]	n-C_4H_9	$C_2H_5OSO_3^-$
[BMIm][NO_3]	n-C_4H_9	NO_3^-
[BMIm][lactate]	n-C_4H_9	$CH_3CH(OH)COO^-$
[HMIm][PF_6]	n-C_6H_{13}	PF_6^-
[OMIm][BF_4]	n-C_8H_{17}	BF_4^-
[OMIm][PF_6]	n-C_8H_{17}	PF_6^-
[M*O*EMIm][BF_4]	$CH_3OCH_2CH_2$	BF_4^-
[PPMIm][[PF_6]	$C_6H_5CH_2CH_2CH_2$	PF_6^-

3-alkyl-1methyl-imidazolium cation



1-alkyl-pyridirinium cation



Alkylammonium cation

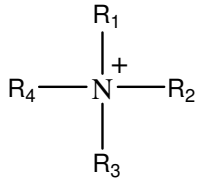


Figure 2.7 – Some of the ionic liquids most commonly used in biocatalysis.

Following this type of approach, Kim and co-workers[54] reported that the use of ionic liquids in lipase-mediated kinetic resolution of racemic alcohols could markedly enhance enantioselectivity. In this case they tested several transesterification reactions of vinyl acetate with four different alcohol substrates in [bmim][BF4] and [bmim][PF6], employing lipases such as *Candida antarctica* lipase B and *Burkolderia cepacia*. The results were excellent since the enzymes always exhibited higher enantioselectivity when compared with THF (tetrahydrofuran) or toluene, and in some cases this increase was up to 25 times.

The positive effect that ionic liquids have on enzyme enantioselectivity has also been shown for proteases. Zhao and Malhotra[56] demonstrated that the ionic liquid N-ethyl pyridinium trifluoroacetate [epy][OTf] could be a good substitute for organic solvents in the resolution of N-acetyl amino acid esters performed by *Bacillus licheniforms* alcalase (figure 2.8). Using the solvent mixture ionic liquid [epy][OTf]-water with the composition (15:85) instead of acetonitrile–water at the same composition, the reaction proceeded with higher enantioselectively in all cases studied (86–97 % ee). But the most interesting result obtained by these authors consisted in the fact that the production of two L-amino acids (L-serine and L-4-chlorophenylalanine), which could not be achieved in acetonitrile–water using alcalase, was successfully accomplished in the ionic liquid medium.

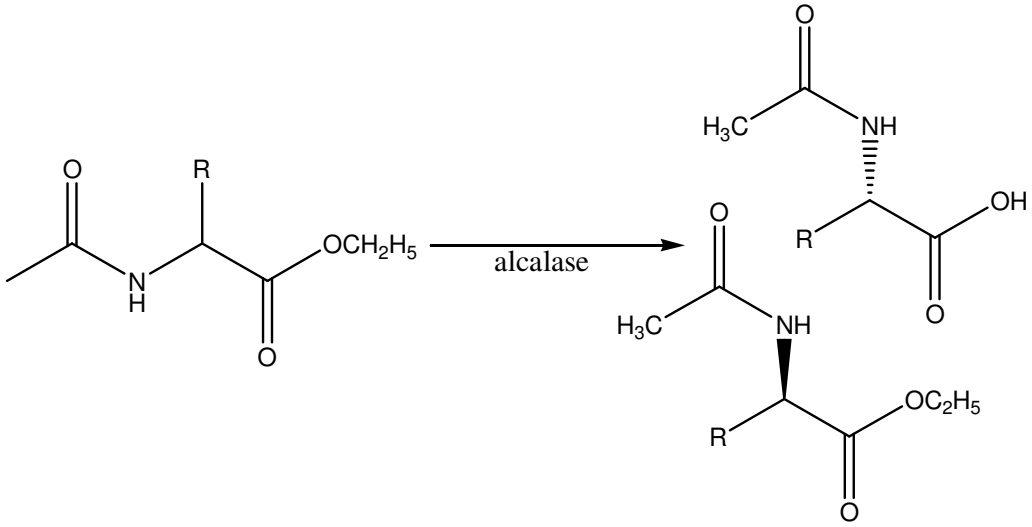


Figure 2.8 - Enantioselective hydrolysis of *N*-acetyl amino acid esters by alcalase (adapted from Zhao and Malhotra[56]).

All the examples relating to solvent engineering show that there is not a generic solvent to tune enantioselectivity, whether it is an organic solvent or an ionic liquid. Thus every time that we need to design a new synthetic process we should always perform a careful choice of the solvent that is going to be used as reaction medium. This selection still has to be done experimentally, since the molecular and theoretical models related with this property are still in their infancy.

When a solvent engineering approach is carried out, one of the parameters that have to be taken into account is the water activity. Whenever a reaction is performed in nonaqueous medium water activity must be monitored, especially when the purpose is to evaluate enzymatic properties in different solvents. This is due to the importance that water has on enzyme dynamics. As we saw in the previous chapter of this thesis, water acts as a molecular lubricant of the enzyme structure. The effect of water activity or water content on enzyme enantioselectivity is a controversial subject. Some authors[79,80,84,85] refer that increasing water content can produce an enhancement in E, whereas others[81] mention exactly the opposite, and still others say they saw no effect of water content on E[78,82,83]. The problem is that some of the studies take into account water concentration rather than water activity, which is directly correlated with enzyme hydration. With this in mind, Bovara and co-workers[78] did a consistent study on the effect of water activity on enantioselectivity. These authors found that water activity did not have any impact on the enantioselectivity of several lipases . Later other authors[77], including ourselves[57], have tried to use this "rigidity switch" to enhance the enantioselectivity of several enzymes, but with no success. Most of the examples reported in the literature show that water is responsible for changes in enzyme activity, but not enantioselectivity. This is quite interesting because it seems to suggest that whereas the flexibility introduced by water on the enzyme structure is crucial for enzyme activity, changes in the flexibility of the enzyme molecule as a whole are either not reflected on the regions of the enzyme more involved in enantiomer discrimination, or if they are, then the dynamics of the active site is not crucial for enzyme enantioslectivity.

The addition of different chemical species to the reaction medium, such as salts or metal ions, has also been explored as a method to increase enantioselectivity in nonaqueous media. A good example of this strategy was published by Okamoto and Ueji[58] who

reported that the addition of lithium chloride could significantly increase the enantioselectivity of *Candida antarctica* in the resolution of several 2-(4-substitute-phenoxy)-propionic acids with butanol (figure 2.9). They found that the addition of a small amount of that salt (0, 5%) could enhance the enantioselectivity almost 200 times. This fact was explained by the increase in the reaction rate of the fast reacting enantiomer in the presence of the lithium salt.

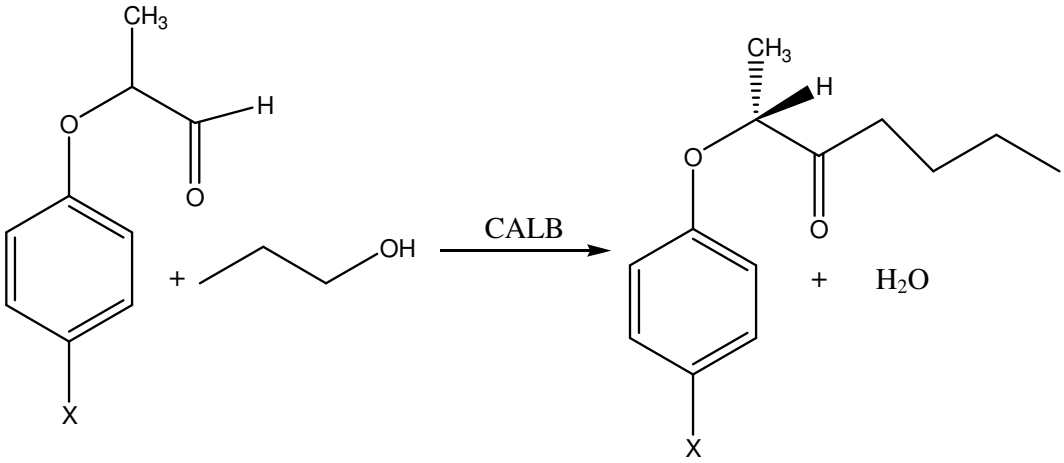


Figure 2.9 – Enantioselective resolution of 2-(4-substitute-phenoxy)-propionic acids. Reaction scheme used by Okamoto and Ueji[58].

Temperature is one of the most critical parameters in biocatalysis, since it can promote severe changes on enzymatic properties. Enzyme stability is the best example to evaluate the impact of this medium parameter, since is quite intuitive to understand why the enzyme suffers denaturation with an increase in temperature. But temperature can also be used to increase enzyme enantioselectivity, and this strategy is accepted as a simple and theoretically reliable one. There are examples in the literature where good E values were obtained by decreasing temperature to as low as -80 ºC, a temperature at which the lipase used was still active.

Water is essential for enzyme activity. One obvious question is what happens to the water bound to the enzyme at very low temperatures? What is the impact on the enzyme of lowering temperature below that at which water normally freezes? If we think of bulk water, when temperature decreases we expect to find it in several states[59]. Below 273 K, bulk water can be in a supercooled state if crystallization is somehow prevented. Nevertheless crystalline hexagonal ice is formed if the temperature decreases to 235 K, and if we then rapidly decrease temperature to approximately 100 K, glassy water is

formed. Between the glass transition and the crystallization state, water is commonly referred as being in an "ultra-viscous" state. The low-temperature behavior of water that is in the vicinity of a protein surface is considerably different. For instance, the water that belongs to the first hydration shell (figure 2.10) does not crystallize; this water is referred to as "unfreezable". Beyond this hydration shell water can in fact be crystallized on slow-cooling, or vitrified at faster cooling rates. Lüsher and co-workers[60] studied the water behavior in the hydration shell of myoglobin though calorimetry and infrared spectroscopy. They found that the transition temperatures for second hydration shell water were higher than for bulk water. This indicates that second hydration shell water is structurally and dynamically modified by the presence of the protein surface with which it interacts via water molecules of the first hydration layer. Water dynamics and protein dynamics are thought to be highly correlated. Indeed, hydrated proteins undergo a so-called "dynamical transition" at temperatures similar to those at which water undergoes phase changes. Above the dynamical transition, anharmonic motions on the protein are activated, allowing the protein to shift between conformational states. This flexibility is important for protein function.

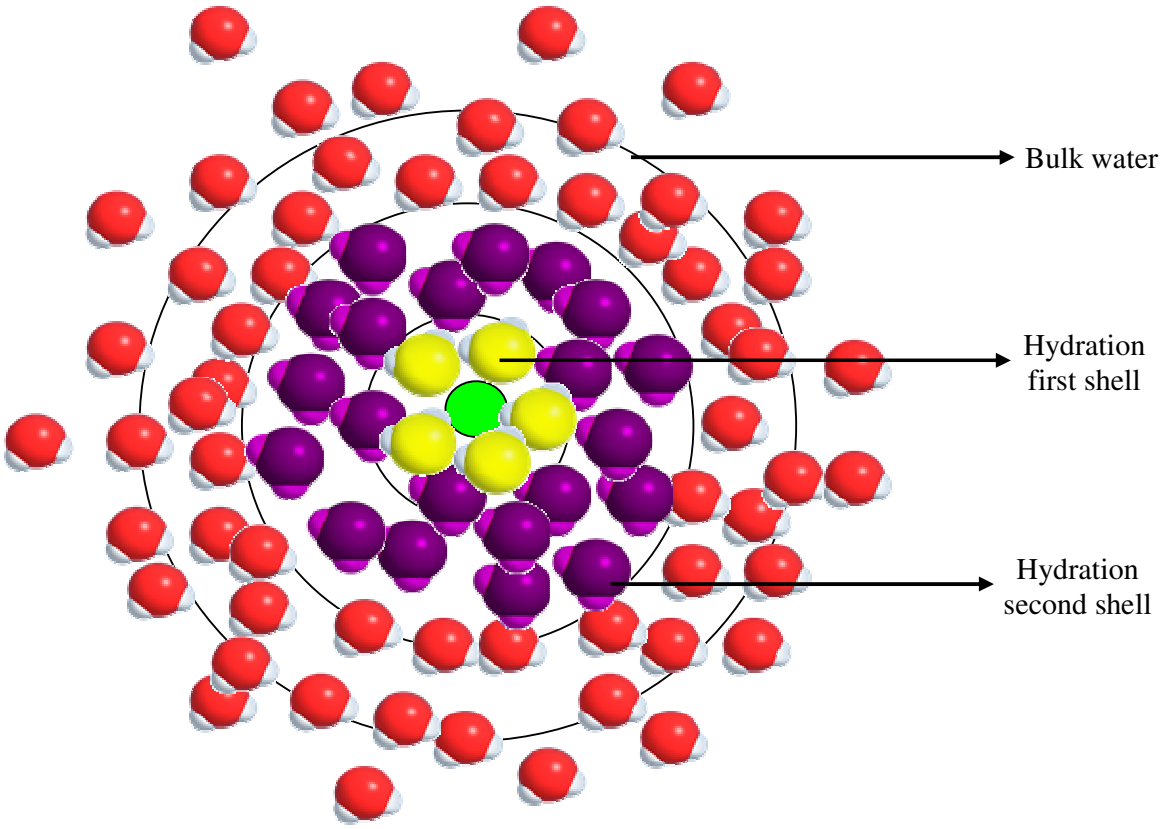


Figure 2.10 – A very simple illustration of hydration shell of a protein

Using a modified Eyring equation, we can experimentally evaluate the effect of temperature on enzyme enantioselectivity.

$$\ln E = \frac{-\Delta\Delta H}{RT} + \frac{\Delta\Delta S}{R}$$

The racemic temperature (T_r) is the temperature at which E=1 and the enzyme does not discriminate between enantiomers. In some cases there has been shown to exist an inversion temperature (T_{inv}), i.e. the plot of ln E vs. 1/T exhibits two linear regions. The point of intersection defines two sets of activation parameters, one above $T>T_{inv}$ and one below. Some authors[61, 62] have hypothesized that the existence of an inversion temperature is related with structural changes on the enzyme caused by the low temperature, or by the solvent, or both.

We can find in the literature several examples of the manipulation of temperature to increase enantioselectivity[61-68]. Most of the examples reported involve low-temperature reactions, where enzymes are required to work at sub-zero temperatures. Sakai and co-workers were among the first to present and elucidate this concept[63-68]. These authors reported that *Burkolderia cepacia* catalysed the kinetic resolution of 3-phenyl-2H-azirine-2-methanol at -40ºC, with a 6-fold increase in E relative to room temperature, which represented a dramatic enhancement in the selectivity of this enzyme. Their results highlight a correlation between the alcohol type and the impact of temperature. The low temperature method has been found to be a very effective way to enhance enzyme enantioselectivity. Nevertheless, it also causes a pronounced decrease in enzyme activity. For instance in the resolution of solketal by *Pseudomonas fluorescens*, E increased from 9 to 55 while enzyme activity decreased almost 10 times[68]. To overcome this limitation, these authors have implemented immobilization strategies.

The enzymes we have been referring to were from mesophilic microorganisms, which means that these enzymes have an ideal temperature for function between 25-40 ºC. Would different effects of temperature on E be observed for enzymes from microorganisms that live in extreme environments? For instance can we increase E for a thermophilic enzyme just by setting the reaction temperature to near room temperature values? Or if we think of psychrophilic enzymes, can an increase in E be obtained at

even lower temperatures? A positive answer has already been found for the above question on thermophilic enzymes[69-70]. In fact, the studies on thermophilic enzymes were conducted previously to studies on mesophilic enzymes. In the late 1980s, Pham and co-workers[69-71] conducted one such study with an alcohol dehydrogenase from *Thermoanaerobacter ethanolicus*, a microorganism capable of using higher sugars, such as xylose, xylan, cellobiose or even cellulose as a carbon source for alcohol production. At its optimum working temperature, which is around 55 ºC, that alcohol dehydrogenase exhibited a preference for (*R)*-2-butanol. But by reducing temperature to 26 ºC, this preference could be inverted. The authors obtained the same kind of effect with 2-pentanol, and were able to determine that the preference of the enzyme for (*S*)-2-pentanol should disappear at the racemic temperature of 75 ºC. This was not confirmed experimentally, however, because the enzyme was not stable at such high temperature. The authors correlated enzyme enantioselectivity with the different contributions from the activation enthalpy and entropy for the two enantiomers.

Not many psycrophilic enzymes have been used in studies based on the low temperature method. This may not be as surprising as it might seem[72-76] Psycrophilic organisms had to evolve in order to survive in extremely low temperature environments. This evolution is in some cases related with the development of alternative isolation systems which can be found, for instance, in some fishes, like the Omul, an endemic specie of Lake Baikal. Evolution is also reflected at the metabolic level, i.e. the enzymes that catalyze vital functions on metabolic pathways have to retain their activity in such environments. Thus the enzymes of psycrophilic organisms have in general three main differences when compared with mesophilic ones. The first one is a higher ion-pairing content, which is responsible for the reduction of protein folding due to the hydrophobic effect. Usually these enzymes have a higher content in arginine residues. Secondly, these enzymes have a lower number of hydrogen bonds and salt-bridges, and thus have higher flexibility. Finally these enzymes have a great accessibility to the active site in order to overcome the reduced diffusivity of substrates that is observed at very low temperatures. All three facts but especially the last two can explain why these enzymes are not so attractive for enantiomeric resolutions. However, high flexibility and highly accessible active sites make enantiomeric resolutions difficult, but not impossible. In fact, the most exploited enzyme in the present chapter and one of the most studied enzymes in biocatalysis has a psycrophilic origin. *Candida antarctica* lipase B, which NOVO

commercializes as NOVOZYM 435, is a recombinant enzyme obtained from a lipase isolated from the yeast *C. antarctica*. This yeast was isolated in Lake Vanda in Antarctica. Conceptually, it is very interesting that the origin of the enzyme should be reflected on the way that temperature affects its enantioselectivity. This could be an important parameter to explore. It is known that extremophilic enzymes work at very high or very low temperatures because their structures make that possible. This means that the major contributions to enantioselectivity in this case probably come from structural motifs.

More needs to be learned on the impact of enzyme dynamics on enzyme enantioselectivity. Molecular modelling and rational design studies have been providing insight into the clarification of that relationship. Nevertheless a severe limitation of these studies is the fact that we cannot rationalize about modifications that are made far from the enzyme active site. For instance directed evolution approaches have led to great improvements in enzyme enantioselectivity, but the rationalization of these achievements is impossible to do with the current state-of-art. We understand the importance of the enzyme stereochemical pocket on enantioselectivity. It is reasonable to expect that the impact of mutations far from the enzyme active site have to be poured through the protein structure up to the active site, and this is impossible to simulate with the techniques currently available. This is a little like the butterfly effect in chaos theory. Correlating protein structure and function is proving to be more complex than might have been expected.

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CHAPTER II

Experimental Section

A brief introduction to the experimental section

This chapter explores the enantioselectivity of cutinase towards secondary alcohols. In the beginning the goal was to enhance cutinase enantioselectivity in nonaqueous media. To this end our first approach consisted in a medium engineering approach where parameters like water activity and ionization state were explored. This first work gave us the idea that it is not so easy to affect cutinase enantioselectivity, at least in the case of the substrates/reactions tested. Modelling studies offered a rationale to explain the differences in E values obtained for two similar secondary alcohols. Rather than a drawback, the results from our first study led us to devise a strategy to change cutinase enantioselectivity. An attempt was made to determine "*hot-spots"* for that property in the cutinase stereochemical pocket. We designed our first mutants based on the results obtained by molecular modelling. We also realized that temperature could be a useful parameter to affect E. However, the modelling studies could not progress to the point of highlighting the impact of substrate structure on enzyme enantioselectivity. A better mapping of the cutinase active site is needed to find the switches that are essential for cutinase enantiomer discrimination.

Effect of Immobilization Support, Water Activity, and Enzyme Ionization State on Cutinase Activity and Enantioselectivity in Organic Media.

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CONTENTS

ABSTRACT

We studied the reaction between vinyl butyrate and 2-phenyl-1-propanol in acetonitrile catalyzed by *Fusarium solani* pisi cutinase immobilized on zeolites NaA and NaY and on Accurel PA-6. The choice of 2-phenyl-1-propanol was based on modeling studies that suggested moderate cutinase enantioselectivity towards this substrate. With all the supports, initial rates of transesterification were higher at a water activity (aw) of 0.2 than at aw = 0.7, and the reverse was true for initial rates of hydrolysis. By providing acid–base control in the medium through the use of solid-state buffers that control the parameter pH-pNa, which we monitored using an organosoluble chromoionophoric indicator, we were able, in some cases, to completely eliminate dissolved butyric acid. However, none of the buffers used were able to improve the rates of transesterification relative to the blanks (no added buffer) when the enzyme was immobilized at an optimum pH of 8.5. When the enzyme was immobilized at pH 5 and exhibited only marginal activity, however, even a relatively acidic buffer with a pKa of 4.3 was able to restore catalytic activity to about 20% of that displayed for a pH of immobilization of 8.5, at otherwise identical conditions. As aw was increased from 0.2 to 0.7, rates of transesterification first increased slightly and then decreased. Rates of hydrolysis showed a steady increase in that aw range, and so did total initial reaction rates. The presence or absence of the buffers did not impact on the competition between transesterification and hydrolysis, regardless of whether the butyric acid formed remained as such in the reaction medium or was eliminated from the microenvironment of the enzyme through conversion into an insoluble salt. Cutinase enantioselectivity towards 2- phenyl-1-propanol was indeed low and was not affected by differences in immobilization support, enzyme protonation state, or aw.

INTRODUCTION

In nonaqueous solvents enzymes can catalyze reactions that are difficult or impossible to carry out in water, become more stable and can exhibit altered selectivity (Klibanov, 2001). Enzyme hydration is one of the fundamental parameters that affect enzymatic properties in nonaqueous media, and is now usually recognized as such. Another parameter that can have a strong impact on enzyme performance is the protonation state of the enzyme. Charged residues on the protein either form ion pairs with other residues or with counter-ions (Halling, 2000). Unlike in water, in low dielectric media counter-ions must be more closely associated with the protein. Therefore, for example,

deprotonation of a carboxyl group requires that a cation such as Na^+ available to form the ion-pair enzyme-$COO^- Na^+$ and allow the release of H+. In such media, an enzyme exhibits pH memory, i.e., its catalytic activity reflects the pH of the aqueous medium from which it was obtained (Xu and Klibanov, 1996). However, pH memory can be lost through changes in acid–base conditions caused by the production of acidic or basic species, or by direct addition of ion-exchangers such as zeolites (Fontes et al., 2002; Harper and Barreiros, 2002) or salt hydrates (Fontes et al., 2003a). The impact of such species may be particularly important when using forms of enzyme where buffer salts are present in relatively low concentrations, as is the case of immobilized preparations.

Optimization of the fundamental parameters that affect enzymatic properties requires the ability to standardize reaction conditions in terms of each of those parameters individually. The thermodynamic water activity (aw) is the parameter that best reflects enzyme hydration (Bell et al.,1995). aw may be fixed by pre-equilibrating reaction components with saturated salt solutions through the gas phase (Halling, 1994) a method that does not prevent aw from changing when water itself is a reactant or a product of the reaction, by using salt hydrate pairs in situ (Halling, 1992; Zacharis et al., 1997a), and very conveniently in the case of polar solvents, by direct addition of water to the reaction medium (Bell et al., 1997). The protonation state of the enzyme may be fixed with organosoluble buffers (Harper et al., 2000a) or with solid-state buffer pairs (Harper et al., 2000b; Partridge et al., 2000; Zacharis et al., 1997b). Remarkable enzyme activity enhancements have been obtained with buffers that control the protonation state of acidic residues by setting a fixed exchange potential of H^+ and Na^+ (i.e., buffers that fix the parameter pH-pNa) (Partridge et al., 2000), in particular those buffers that promote the formation of the enzyme–$COO^- Na^+$ ion pairs. The enzymes used in these studies are lipases and proteases, which require a formal negative charge on the active site for full activity and are usually impaired catalysts in acidic conditions. Promotion of a more basic and catalytically competent form of enzyme is also thought to explain the rate enhancements obtained when using zeolite NaA (Fontes et al., 2002; Harper and Barreiros, 2002). The exchange potential of H^+ and Na^+ can be measured with an organosoluble chromoionophoric indicator (Harper et al., 2000b). One of the great advantages of enzymatic catalysis is selectivity. With a growing demand for enantiopure pharmaceuticals, asymmetric conversions will likely be a major application of nonaqueous biocatalysis (Klibanov, 2001). It is impossible, at present, to predict the

effect of aw on enzyme enantioselectivity, which has been found to decrease, increase, or show no dependence on aw (Carrea et al., 1995; Fontes et al., 1998; Pepin and Lortie, 1999; Rariy and Klibanov, 2000; Persson et al., 2002). Counterion effects on both enzyme and substrate can have a significant impact on enzyme enantioselectivity in nonaqueous solvents (Ke and Klibanov, 1999; Okamoto and Ueji, 1999, 2000; Shin et al., 2000; Hsu and Clark, 2001; Quiro´s et al., 2001). However, there is only one report where enzyme enantioselectivity has been directly correlated with enzyme protonation states, fixed in situ with solid-state buffers (Quiro´s et al., 2001).

Cutinase is an extracellular enzyme excreted by the plant pathogen *Fusarium solani pisi* (Carvalho et al., 1999). It belongs to the class of serine hydrolases and is a very versatile enzyme that catalyzes synthetic and hydrolytic reactions on a wide range of substrates. The structure of cutinase is well known (Martinez et al., 1992) and that has helped the elucidation of its function (Carvalho et al., 1999). In the present study we report on the effects of aw and enzyme protonation state on the activity and enantioselectivity of cutinase immobilized on different supports. The organosoluble indicator (Harper et al., 2000b) was used to assess the efficiency of solid state buffers in controlling acid–base conditions and thus the protonation state of the enzyme. We also look at how the latter parameter affects the preference for the nucleophile in the deacylation step of the reaction mechanism, i.e., how it affects the competition between hydrolysis and transesterification.

MATERIALS & METHODS

Materials

Fusarium solani pisi cutinase was produced by an Escherichia coli WK-6, which was a gift from Corvas International (Gent, Belgium). The production, extraction, and purification of the enzyme were done following a protocol developed in one of our laboratories (Centro de Engenharia Biológica e Química, Instituto Superior Técnico), adapted from a published method (Lauwereys et al., 1990). The enzyme purity was controlled by electrophoresis and isoelectric focusing. The estereolytic activity of the enzyme (30 nM) was determined spectrophotometrically by following the hydrolysis of p-nitrophenyl butyrate (0.56 mM) at 400 nm in a 50 mM potassium phosphate buffer at pH = 8.5. (R, S)-2-phenyl-1-propanol (97% purity), (R)- and (S)-2- phenyl-1-propanol

(98% purity), zeolite molecular sieves 4A° (powder, zeolite NaA), zeolite Y molecular sieves (powder, zeolite NaY) were from Aldrich, Accurel EP 700 (PA-6 powder, particle size < 800 Am) was from Akzo Nobel, vinyl butyrate (99% purity) was from Fluka, sodium butyrate, tridecane, 3-(cyclohexylamino)-2-hydroxy-2-propanesulfonic acid (CAPSO), 3-[(1,1-dimethyl-2-hydroxyethyl) amino]-2-hydroxypropanesulfonic acid (AMPSO), 3-(N-morpholino) propanesulfonic acid (MOPS), L-glutamic acid (GLU) and their sodium salts (CAPSO-Na, AMPSO-Na, MOPS-Na, GLU-Na) were from Sigma, acetonitrile, potassium acetate, potassium carbonate, strontium chloride were from Merck, Hydranal Coulomat A and C Karl-Fischer reagents were from Riedel- de-Häens. The solvent, substrates, and tridecane were stored over molecular sieves 3A° (Merck). Preparation of (R,S)-2-phenyl-1-propyl butyrate: To a stirred solution of (R,S)-2-phenyl-l-propanol (0.503 g, 3.7 mmol) and triethylamine (0.62 mL, 1.2 eq) in anhydrous dichloromethane (20 mL), under argon atmosphere and at room temperature, was added dropwise butyryl chloride (0.42 mL, 1.1 eq). After completion of the reaction (TLC), the reaction mixture was partitioned between diethyl ether (100 mL) and hydrochloric acid (100 mL, pH = l), the organic phase was washed with a saturated solution of NaHC03 (100 mL), dried (MgSO4), evaporated under vacuum and purified by silica gel flash chromatography (eluent: 9.5:0.5 n-hexane/diethyl ether) to give the desired ester (0.704 g, 93 %) as a clear liquid. The indicator used was 5-(4-cyanophenyldiazo)-2-hydroxy-1,3-xylyl-18-crown-5. Its protonated form was synthesized according to a method given in the literature (Harper et al., 2000b).

Enzyme Immobilization

Cutinase was immobilized by deposition, according to the method developed by Serralha et al. (1998). The lyophilized enzyme was dissolved in a 50 mM sodium phosphate buffer solution (10 mg mL^{-1} or 6.3 mg mL^{-1} of enzyme in the case of zeolites and Accurel, respectively) at pH 8.5 unless stated otherwise. The support was added to the solution (25 mg of cutinase per g of support) and after vortex mixing for 1 min, the preparation was dried under vacuum for at least 24 h. The average yield of immobilization was (51 ± 8) % for zeolite NaA, (72 ± 12) % for zeolite NaY and (67 ± 12) % for Accurel, as determined by a modified Lowry method (Lowry et al., 1951) that involved a first step of enzyme desorption via alkaline hydrolysis. The latter method was also used to measure the average protein content of the preparations after 24 h of reaction at the three values of aw tested. Approximately 7% of the immobilized protein

was washed out from zeolite NaA and Accurel and this value did not depend on aw. In the case zeolite NaY, the amount of protein desorbed increased with increasing aw: approximately 1% at aw = 0.22, 2 % at aw = 0.43, 7% at aw = 0.71. These values were used in all calculations.

Enzyme Assays

The enzyme preparations were pre-equilibrated for about 2 days through the vapor phase with saturated salt solutions at 25 ºC, to achieve the values aw = 0.22 (potassium acetate), aw = 0.43 (potassium carbonate) and aw = 0.71 (strontium chloride), taken from the literature (Greenspan, 1977). When used, solid-state buffers were pre-equilibrated together with the enzyme preparations. Fifty milligrams of the pre-equilibrated enzyme preparation and the solid-state buffer when required for the experiment (except where otherwise stated, 128 mg of the sodium form of the salt and 32 mg of the more acidic form) were placed in a screwcapped polypropylene vial, to which 3.5 mL of acetonitrile were added, followed by 2-phenyl-1-propanol (100 mM), tridecane (14.5 mM, used as internal standard for GC analysis), water to give the required aw (amount of water derived from the data given by Bell et al., 1997) and the indicator (50 AM) when required. The mixture was stirred (with a stirring bar) for 1 h at 35 ºC before adding vinyl butyrate (300 mM) to start the reaction.

Analysis

Both the reaction conversion and the enantiomeric excess of the remaining alcohol substrate (ees) were measured by GC analysis performed with a Trace 2000 Series Unicam gas chromatograph. Column: 30 m - 0.32 mm I.D. homemade fused silica capillary column coated with a 0.25 Amthickness film of 15% heptakis-(2,3-di-O-methyl-6-O-tertbutyldimethylsilyl)-*h*-cyclodextrin in SE 52 (DiMe). Oven temperature program: 90 ºC for 5 min, 90–136 ºC ramp at 0.8ºC min 1, 200º C for 5 min. Injection temperature: 250 ºC. Flame ionization detection (FID) temperature: 250 ºC. Carrier gas: helium (2.0 cm3 min^{-1}). Split ratio:1:20. The retention times were 12.37 min (butyric acid), 36.48 min (tridecane), 42.22 min ((R)-2-phenyl-1-propanol), 43.42 min ((S)-2-phenyl-1-propanol), 58.48 min ((S)-2-phenyl-1-propyl butyrate), 58.81 min ((R)-2-phenyll- propyl butyrate). No products were detected in assays carried out without enzyme. The enantiomeric ratio, E, was calculated from the expression $E= \{\ln[(1-c)(1-ees)]\}/\{\ln[(1-c)(1+ees)]\}$, where c is the conversion (Straathof and

Jongejan, 1997) and ees is given for the (R)-enantiomer, by using data obtained for the racemic substrate along the time course of the reaction (whenpossible, up to a conversion of 0.5). We also calculated E from the ratio of the initial reaction rates for the single enantiomers ($v_{(R)\text{-enantiomer}}/v_{(S)\text{-enantiomer}}$), assuming these rates were proportional to the respective specificity constants (Straathof and Jongejan, 1997). The selectivity for the ester product was quantified by the ratio of the initial rates of formation of the ester and of the acid. When using the buffer CAPSO, butyric acid changed completely into sodium butyrate, causing the removal of butyrate from the solution. This also occurred with AMPS0 buffer, although to a lesser extent. Under these circumstances, routine GC analysis did not allow the measurement of the butyric acid effectively formed via hydrolysis. We thus quantified sodium butyrate by recovering the solids present in the reaction mixture at the end of reaction, filtering the solids and drying them in an oven at 70 ºC for at least 12 h. The resulting powder was resuspended in a solution of acetonitrile/HCl (60:40 v/v) that was shaken on a rotary shaker for 3 h, a period of time seen to be adequate for the recovery of all the sodium butyrate in the form of butyric acid. The latter was analyzed by GC.

To validate this method, we spiked the medium with different amounts of butyric acid in the presence of CAPSO or AMPSO buffers. Following the treatment indicated above, the amount of butyric acid recovered agreed with the amount added initially. To monitor the formation of butyric acid to calculate initial rates of hydrolysis for reactions done in the presence of the latter buffers, several reactions were run simultaneously, were stopped at given times during the period required for initial rate measurements, and subjected to the treatment described above.

The UV-visible spectra of the indicator were measured (blanked against acetonitrile or, in the case of reaction, blanked against a 100 mM solution of 2-phenyl-1-propanol in acetonitrile), using a Beckman Coulter DU 800 spectrophotometer. The spectra obtained were similar to those given by Harper et al. (2000b). The concentrations of the two forms of the indicator were calculated from the absorbance at the Emax for the two peaks taking into account the spectral overlap (Harper et al., 2000b). The Emax for the protonated and deprotonated indicator were 360 nm and 484 nm, respectively, in acetonitrile. The absorption coefficients for the protonated and deprotonated indicator were 0.0201 and 0.0004 $\mu M^{-1}cm^{-1}$ at 360 nm and 0.0024 and 0.0279 $\mu M^{-1}cm^{-1}$ at 484

nm. To test the sensitivity of the indicator, we analyzed its response to increasing concentrations of butyric acid (Fig. 1). Very small concentrations of butyric acid caused a very large increase in the protonated fraction of the indicator up to about 50%; from this value on, the indicator became less sensitive to changes in the acid–base conditions of the medium. Water concentration was measured by direct Karl-Fischer titration after water equilibration and at the end of the reaction.

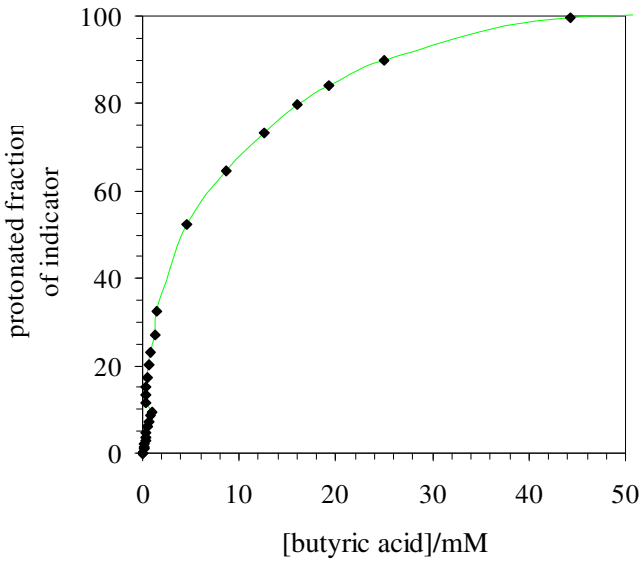

Figure 1. - Indicator response to butyric acid in acetonitrile. The indicator concentration was 50 μM.

RESULTS AND DISCUSSION

In an earlier study (Fontes et al., 1998), we showed that cutinase immobilized on zeolite NaA was virtually 100% selective towards the (R)-enantiomer for the resolution of (R,S)-1-phenylethanol and used computer modeling to elucidate this fact. To select a substrate that cutinase resolved less efficiently, we again focused on the deacylation step of the reaction mechanism, which involves the chiral agent, and modeled catalysis from differences in stabilization of the tetrahedral intermediate (Colombo et al., 1999, Warshel et al., 1989). In the case of 1-phenylethanol, this intermediate is well buried in the active site (Fig. 2A), which will result in large differences in substrate-enzyme

interactions between the two enantiomers. To make the enzyme less selective, the stereogenic center was moved towards the outside of the active site. 2-phenyl-1-propanol yields a tetrahedral intermediate that meets these requirements (Fig. 2C) and was the substrate selected for the present work.

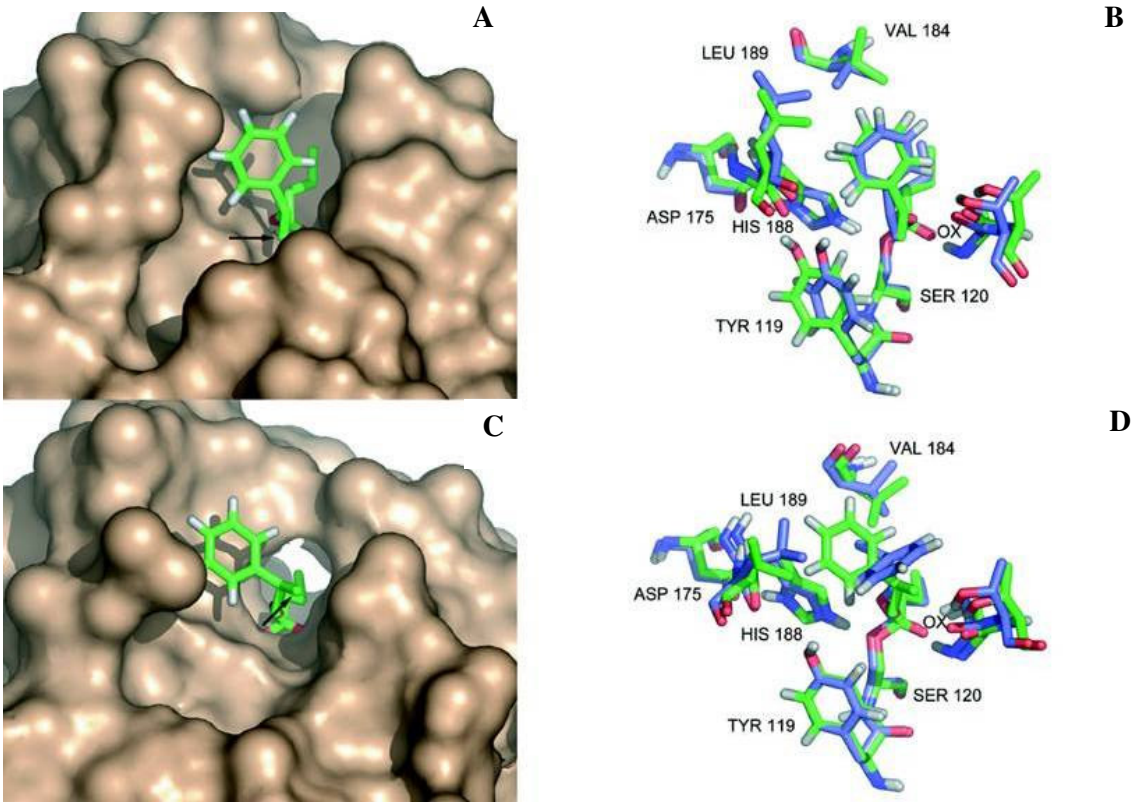

Figure 2. Modeling studies of tetrahedral intermediates in the active site of cutinase. The details of these studies (Micaelo et al., to be published) are similar to those in previous work (Soares et al., 2003). The molecular dynamics simulations of cutinase with the tetrahedral intermediate are made in n-hexane, with full neutralization of ionizable groups and considering a 5% (w/w) water content associated with the protein. GROMOS96 (van Gunsteren et al., 1996; Scott et al., 1999) was used in the calculations. The conformations used in (A) [(R)-1-phenylethanol] and (C) [(R)-2-phenyl-1-propanol] are average structures (1 ns) obtained after 2 ns of equilibration. The molecular surface of the protein is rendered without considering the tetrahedral intermediate; the latter is rendered using sticks. An arrow indicates the methyl group attached to the stereogenic centre. (B) and (D) contain the (R)-enantiomer conformation shown in (A) and (C), overlaid with an average structure of the corresponding (S)-enantiomer. Only important residues of the active site are rendered using sticks. Some relevant residues are labeled. The label ‘‘OX’’ indicates the oxyanion hole, found in all serine proteases. For clarity, the carbon atoms of the (R)-and (S)-enantiomers are colored green and blue,respectively. The figures were prepared using Pymol (DeLano, 2002).

Cutinase is known to perform better for protonation states initially set by a pH of immobilization between 7 and 8.5 (Serralha, 1999); we selected the value 8.5. At a_w = 0.2, the most hydrophobic support, Accurel, promoted transesterification to a higher extent than the two zeolites (Fig. 3). Under these conditions, moderate hydrolysis also took place, reaching higher and lower levels with zeolite NaA and Accurel, respectively. The formation of butyric acid changed the acid-base conditions of the medium markedly, as shown by the response of the indicator.

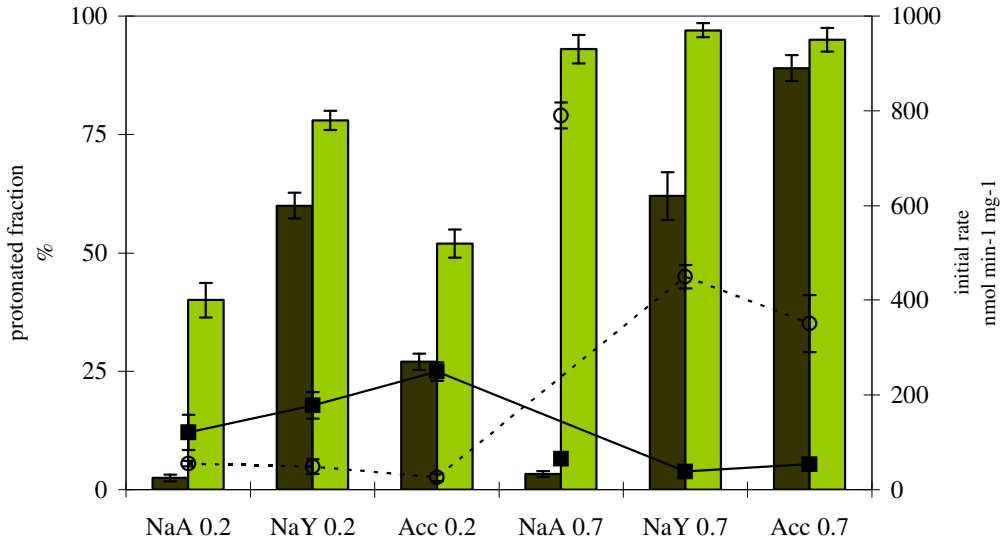

Figure 3. Effect of immobilization support (zeolite NaA, zeolite NaY, Accurel PA-6) and a_w on indicator response (bars), initial rates of transesterification (full squares), and initial rates of hydrolysis (open circles). Light green and dark green bars, protonated fraction of indicator at 0 h and at the end of the period used to measure initial rates, respectively. The enzyme was immobilized at pH 8.5.

The values indicated in the figure for the protonated fraction of the indicator at 0 h in the presence of cutinase immobilized on the zeolites reveal the relative acidity of the zeolite supports. Zeolite NaA is highly basic and deprotonated the indicator almost completely; zeolite NaY is more acidic. In this case, the response of the indicator at 0 h did not change with aw and was the same as that obtained with the supports alone, without enzyme. Accurel, on the other hand, does not have ionogenic groups. Thus, one might expect that the initial protonated fraction of the indicator in the presence of cutinase immobilized on this support would reflect the protonation state of the enzyme, and possibly be different from the protonated fraction of the indicator in the presence of Accurel alone. This was in fact observed, the two values being about 27% as shown in

the figure and about 85% in the absence of enzyme. Increasing aw eliminated this difference, possibly due to favoured deprotonation of the carboxyl groups on cutinase as a result of improved hydration of the enzyme-COO^-Na^+ ion pairs. In all three cases, transesterification rates were lower at aw = 0.7. The extent of hydrolysis increased substantially, as might be anticipated, and confirmed the trend observed at low aw. The larger amounts of acid formed protonated the indicator virtually completely.

The protonation state of cutinase at the start of reaction is set by the pH at which immobilization was carried out but may be subsequently altered by the formation of the acid by-product. One question that we sought to answer was whether rates of transesterification could be improved by providing acid–base control in the medium. In a recent study with subtilisin Carlsberg, we observed that the transesterification activity of the enzyme increased substantially with increasing aw in the presence of a relatively basic solid-state acid–base buffer (Fontes et al., 2003b).

Another issue that we sought to investigate was whether buffers that set a more basic protonation state of cutinase affected the competition between 2-phenyl-1-propanol and water for the acyl-enzyme. To standardize the acid-base conditions of the medium, we used buffers of different aqueous pKa. We restricted the a_w range of the experiments to values at which the buffers are not reported to form hydrates (Harper et al., 2000b), with the exception of CAPSO buffer at aw = 0.7. Nonetheless, in a hydrophilic solvent such as acetonitrile this should not impact on aw significantly, and CAPSO should still set a fixed pH-pNa value (Harper et al., 2000b). We selected cutinase immobilized on zeolite NaY for these studies. MOPS buffer set initial conditions that were sufficiently acidic to cause virtually complete protonation of the indicator; it was thus impossible to monitor possible changes in acid–base conditions brought about by the formation of the acid byproduct. The same applied to GLU buffer. In the presence of CAPSO buffer, the protonated fraction of the indicator remained approximately constant throughout the reaction: at aw = 0.2, 0.4, 0.7, it changed from (10.9 ± 3.1)% at 0 h to (14.3 ± 2.0) % at the end of the period used to measure initial rates. The same applied to AMPSO buffer: e.g., at aw = 0.2, 0.4, the protonated fraction of the indicator changed from (28.1 ± 2.1) % at 0 h to (35.5 ± 3.0) % at the end of the period used to measure initial rates. Thus, these buffers effectively controlled the acid-base conditions of the medium. It is reasonable to assume that the same applies to the other buffers tested.

At all the a_w values tested none of the buffers were able to improve the rates of transesterification obtained with the blanks (no added buffer) when the enzyme was immobilized at an optimum pH of 8.5 (Fig. 4). Although aqueous pKa alone does not fully account for the acid–base behavior of the solid buffers in nonaqueous media (Fontes et al., 2003a), the relatively low pKa of GLU might suggest a comparatively poorer performance of the enzyme in the presence of this buffer, unlike what has been observed. At a_w = 0.7, GLU even led to rates of transesterification that were very similar to those obtained with the blank, and much higher than those measured in the presence of AMPSO.

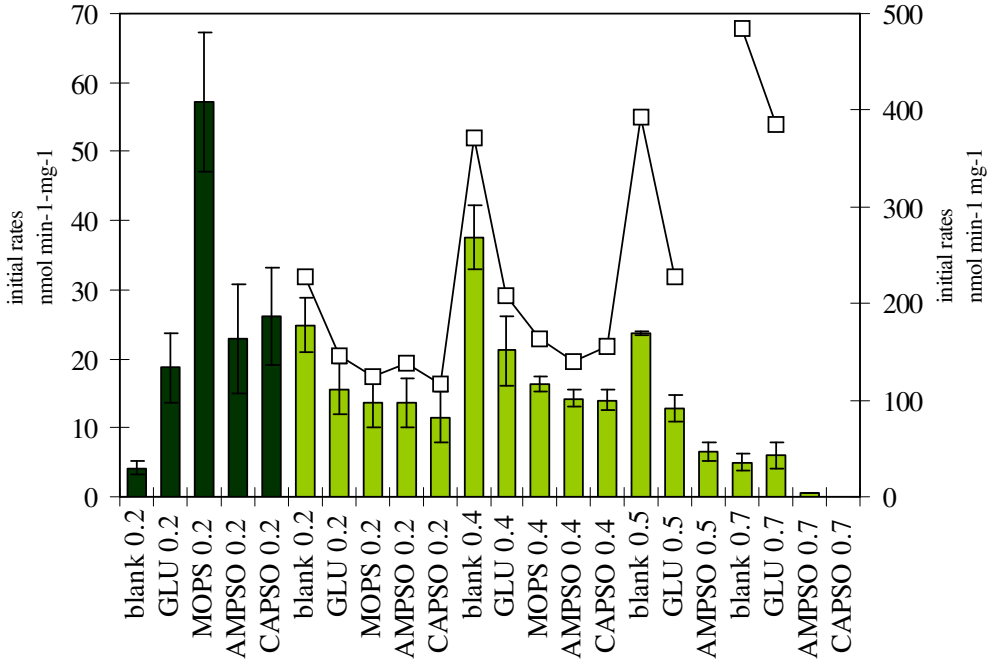

Figure 4. Effect of solid-state acid-base buffer and aw on initial rates of transesterification (bars) for cutinase immobilized on zeolite NaY at pH 5.0 (dark green bars, left axis) and at pH 8.5 (light green bars, right axis). Also shown are total initial reaction rates (line) for the enzyme immobilized at 8.5 (except for AMPSO buffer at aw= 0.5, 0.7, where the acid was not analyzed). Buffer aqueous pKa (at 25 ºC): 4.3 (GLU/GLU.Na), 7.2 (MOPS/MOPS.Na), 9.0 (AMPSO/ AMPSO.Na), 9.6 (CAPSO/CAPSO.Na).

No products were detected in experiments with CAPSO buffer. Cutinase thus behaves very differently from subtilisin Carlsberg (Fontes et al., 2002, 2003b; Harper and Barreiros, 2002). Although the two enzymes share a similar active site architecture and catalytic mechanism, they have substantially different structures, and this must account

for the observed differences in behaviour. An increase in a_w first promoted transesterification, but at aw = 0.7 the transesterification activity of the enzyme was significantly impaired. However, due to a continuous increase in the rates of hydrolysis, total enzyme activity showed a steady increase with increasing aw. As seen in Table I at constant aw changes in acid–base conditions of the medium did not impact on the selectivity for the ester product. The latter declined significantly as aw increased, as already observed in Figure 3.

Table I. Effect of solid-state acid– base buffer and aw on the selectivity for the ester product for cutinase immobilized on zeolite NaY at pH = 8.5. The blanks were without buffer.

	$a_W = 0.2$	$a_W = 0.4$	$a_W = 0.5$	$a_W = 0.7$
blank	3.6 ± 1.3	2.6 ± 0.5	0.75 ± 0.02	0.079 ± 0.020
GLU	3.2 ± 0.9	2.7 ± 1.0	0.67 ± 0.23	0.12 ± 0.04
MOPS	3.6 ± 1.3	2.4 ± 0.8	-	-
AMPSO	2.4 ± 0.8	2.7 ± 0.7	-	-
CAPSO	2.4 ± 1.0	1.8 ± 0.7		

The data obtained for the indicator in the blank experiment at aw = 0.2 suggests that at this a_w the enzyme in the blank is exposed to a less acidic medium than that imposed by GLU or MOPS, and more acidic than that imposed by AMPSO or CAPSO. At aw = 0.4, the indicator protonated completely in the blank and it is only possible to say that the enzyme was then exposed to a more acidic medium than that set by AMPSO or CAPSO.

Why were none of the buffers able to at least match the performance of the blanks up to aw = 0.5? Experiments done with twice as much buffer (320 mg) reproduced the results obtained when using the standard amount of buffer. On the other hand, when we replaced the buffer with an equal amount of zeolite NaY support (160 mg), the transesterification activity measured at aw = 0.4 reproduced that displayed by the blank at the same a_w. Thus, our results suggest that when the enzyme is immobilized at an optimum pH of 8.5 none of the buffers are able to improve the protonation state of the enzyme from the standpoint of enzyme activity. The situation changed when the enzyme was immobilized at the sub-optimal pH of 5. This had a markedly negative impact on enzyme activity, which fell to about 2% of that exhibited by the enzyme

immobilized at pH 8.5. In this case, all the buffers tested enhanced enzyme activity relative to the blank (Fig. 4), MOPS buffer clearly leading to a better performance of the enzyme. Again, the result obtained for GLU buffer confirms the tolerance of cutinase to relatively acidic conditions. Cutinase was indeed less enantioselective towards 2-phenyl-1-propanol than 1-phenylethanol (Fig. 5).

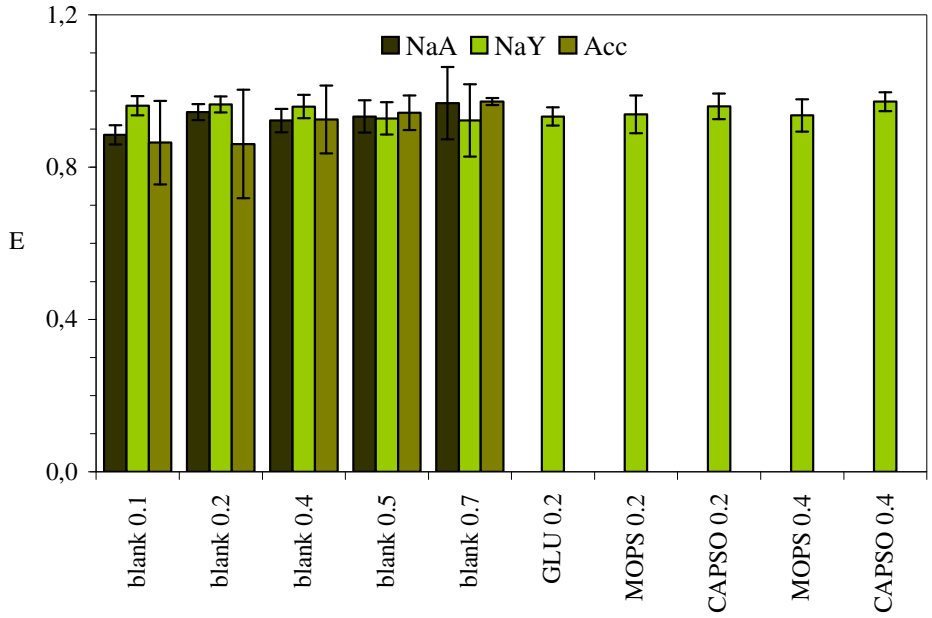

Figure 5. Effect of immobilization support, solid-state acid– base buffer and aw on the enantiomeric ratio, E. The enzyme was immobilized at pH 8.5.

The simulations presented in Figure 2B and 2D show that, while the two enantiomers of 1-phenylethanol are constrained in the same position of the active site, the corresponding enantiomers of 2-phenyl-1-propanol have a higher conformational space available, which evidences the lower discriminating power of cutinase towards the latter.

The E values given in Figure 5 were derived from measurements of reactions done with the racemic substrate. There was good agreement between E values at different reaction times, which we used to calculate an average at each a_w. There was also good agreement between the data in the figure and E values derived from measurements of initial reaction rates for the single enantiomers (E = 1.0 ± 0.3 at aw = 0.2 and E = 1.1 ± 0.2 at aw = 0.7). Quiro´s et al. (2001) were able to substantially improve both the activity and the enantioselectivity of *Candida antarctica* lipase B by using CAPSO buffer or

organo-soluble bases of high pKa to control the protonation state of one of the residues of the catalytic triad. The authors found that the accumulation of the acid by product in the microenvironment of the enzyme had a negative effect on enantioselectivity similar to that of increasing hydration. Cutinase enantioselectivity, however, was not affected by the absence or presence of acid–base buffers, by a_w or by the immobilization support.

CONCLUSIONS

Providing acid–base control has been shown in recent studies to be a powerful tool to modulate the activity and enantioselectivity of enzymes whose catalytic mechanism requires changes in the protonation states of residues at the active site (Quiro´s et al., 2001; Fontes et al., 2002). This is the case of cutinase. However, changes in the acid–base conditions of the medium directed towards changes in the protonation state of acidic residues on the enzyme had no impact on cutinase enantioselectivity. This approach was also not successful in improving the transesterification activity of the enzyme when it was immobilized at an optimum pH. Cutinase and the other serine hydrolases tested in these studies have a common catalytic triad but markedly different structures that might be the cause for the observed differences in enzyme behavior. In future studies we will try to elucidate these issues, and in particular, use protein engineering to improve cutinase enantioselectivity.

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Improving cutinase enantioselectivity using a combined rational design and medium engineering approach

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Keywords: Cutinase; Rational design, Medium engineering; Enantioselectivity; Organic Solvents, Low temperature

CONTENTS

ABSTRACT

We describe a concerted approach towards the rationalization of the enantioselectivity (E) of cutinase from *Fusarium solani pisi* in nonaqueous media. Simulations of wild-type cutinase with the tetrahedral intermediate of the substrate 1-phenylethanol built in the active site provided the basis for a protein engineering approach focused on flanking active site residues and aimed at decreasing the marked preference of the enzyme for the *(R)*-enantiomer of that substrate. Potentially interesting mutants were constructed *in silico* and the differences in the free energies of activation for the two enantiomers were calculated, confirming the initial choice of target residues. Two mutants bearing single-point mutations were constructed by site-directed mutagenesis. Despite the similar relative free energy difference between (*R*)- and (*S*)-tetrahedral intermediates for the two mutants selected, the L189A mutant exhibited an enantioselectivity towards 1-phenylethanol that was experimentally indistinguishable from that of the wild-type enzyme, whereas the Y119A mutant was much less selective towards the (*R*)-enantiomer of the alcohol. This was possibly due to the absence of the favorable van der Waals interactions between this enantiomer and Tyr 119, which were found to strongly stabilize the transition state for the wild-type enzyme. To expand the body of data on E for cutinase for testing the existing model, experimental data was generated for transesterification reactions between secondary alcohols and vinyl esters of varying chain-length. The accommodation of acyl donor alkyl chains up to butyl in the enzyme active site did not have any significant impact on enzyme enantioselectivity, which in that case depended solely on the alcohol. The size of 2-butanol afforded only marginal discrimination that increased and yielded similar values for 2-pentanol and 2-octanol, although far from matching the extremely high enantioselectivity observed for 1-phenylethanol. By using vinyl laurate as the acyl donor, the discrimination ability of cutinase towards 2-octanol increased substantially, suggesting a packing arrangement in the active site that stabilizes the *-(R)*-enantiomer relative to the *-(S)*-enantiomer. The results obtained at lower temperatures confirm the role of this methodology as a second order approach towards the fine tuning of enzyme enantioselectivity. In fact, only in the cases where cutinase already showed a more marked preference for one of the enantiomers, did this preference become more pronounced as temperature was lowered.

INTRODUCTION

Selectivity is considered the key enzymatic property. More than any other property, selectivity has been studied, rationalized, improved and used to illustrate several engineering concepts related with reaction/separations techniques. Most of the interest in this property stems from the tremendous potential that enzymes have in the resolution of racemic mixtures, and the utility of this strategy in the pharmaceutical industry (Krishna, 2002, Turner, 2003, Schoemaker, 2003, Chikusa et al., 2003, Ghanem and Abul-Enein, 2005]. Any factor that can enhance the enantiomeric ratio (E) has been the target of many studies. In the last decade, high-tech approaches such as *in vitro* evolution (Reetz, 2003) and *in silico* rational design (Hult and Berglund, 2003) have been successfully explored. In spite of the remarkable results obtained with the latter approaches, less resource demanding ones such as medium engineering keep being useful (Bornscheuer and Kazlauskas, 1999). The enhancement of E using temperature falls in that category.

The impact of temperature on E was first reported by Keinan *et al.* (1986) in the reduction of ketones by an alcohol dehydrogenase in a temperature range between 7 and 50 ºC, and also by Lam *et al.* (1986) in the hydrolysis of esters by pig liver esterase between -10 and 20 ºC. Later this fact was rationalized by Pham *et al.* (1989) and Pham and Phillips (1990) who used a thermophilic alcohol dehydrogenase in the kinetic resolution of secondary alcohols between 15 and 65 ºC. These authors introduced the concept of racemic temperature (T_r). In the light of the transition state theory, T_r is the temperature at which the enzyme does not discriminate the two enantiomers, i.e. E = 1. E is related to the difference in Gibbs activation energy for both enantiomers, $\Delta\Delta G^{\#}$, through $\Delta\Delta G^{\#} = -RT \ln E = \Delta\Delta H^{\#} - T\,\Delta\Delta S^{\#}$, where $\Delta\Delta H^{\#}$ e $\Delta\Delta S^{\#}$ are the differences in activation enthalpy and entropy, respectively, for both enantiomers. In the case of 2-butanol, Pham and Phillips observed an inversion in the enantioselectivity of the enzyme by decreasing temperature from 65 to 26 ºC. This example shows that temperature can be a critical parameter for enzymatic kinetic resolutions.

Holmberg and Hult (1991) have also considered temperature an important tool to tune the enantioselectivity of *Candida rugosa* lipase. They found that in both aqueous and nonaqueous media the E of this lipase could be enhanced between 6 and 37 ºC, a

temperature range where enzyme activity did not change substantially. Based on his previous results, Phillips (1996) suggested that the increase in E caused by temperature could be related with small variations in reaction stereochemistry. This author also distinguished the different thermodynamic contributions that were responsible for the enantioselectivity enhancement below and above the racemic temperature: for $T < T_c$, the dominant contribution is $\Delta\Delta H^{\#}$ and E should decrease with the increase in T until E equals 1 and $T = T_r$, whereas when $T > T_r$, $T\Delta\Delta S^{\#}$ dominates and E should increase together with T. The $\Delta\Delta H^{\#}$ term reflects the binding modes of both enantiomers in the enzyme stereochemical pocket, whereas $\Delta\Delta S^{\#}$ is related with the hydrophobic interactions between the enantiomers and the solvent and also with the loss of their rotational and translational mobility in the transition state.

The effect of temperature on enzyme enantiomeric discrimination has been explored since these early papers. Nevertheless its applicability increased a lot with the advent of nonaqueous biocatalysis (Zaks and Klibanov, 1984). In these media, enzymes are more stable than in water and can catalyze reactions that are thermodynamically difficult to carry out in water. In nonaqueous media enzymes can also exhibit a completely different selectivity than in water, and more importantly, this property can be tuned by the nature of the solvent (Klibanov, 2001, Krishna, 2002). Organic media became very useful to study the temperature effect on enzymes that exhibit a low enantioselectivity at room temperature, since many remain in the liquid state at the low temperatures required to obtain practically useful E values (Ljubovic et al., 1999). We can find in the literature many examples of the application of the so-called low temperature method to increase enantioselectivity (Sakai et al., 2003 b, Miyazawa et al., 1997, Wegman et al., 1999, Ljubovic et al., 1999, Aoyagu et al., 2003). Sakai et al. (1997) were the first to present and elucidate this concept. These authors reported that *Burkolderia cepacia* catalysed the kinetic resolution of 3-phenyl-2H-azirine-2-methanol at -40ºC, with a 6-fold improvement in E relative to room temperature. These authors implemented immobilization strategies in order to reduce the negative impact of temperature on reaction rate (Sakai et al., 2003 a, 2003 b, 2005).

In the present work we try to elucidate the enantiomeric discrimination ability of cutinase using both rational design and a medium engineering approach based on temperature. From a previous study (Fontes, *et* al., 1998) we knew that cutinase was

100 % selective towards the *(R)*-enantiomer of 1-phenylethanol in the transesterification of this alcohol with vinyl butyrate. Based on the model for cutinase developed at that time, we designed mutants that would be less enantioselective towards 1-phenylethanol by way of a reduction in the sterical hindrance of the methyl group attached to the chiral center. When this approach met with success, we tried to recover the loss of discrimination ability of the enzyme by decreasing the reaction temperature. We extended this approach to other secondary alcohols, namely 2-butanol, 2-pentanol and 2-octanol, via transesterification with vinyl acetate, vinyl butyrate and vinyl laurate.

MATERIALS & METHODS

Materials

Fusarium solani pisi cutinase was produced by an Escherichia coli WK-6, which was a gift from Corvas International (Gent, Belgium). (R, S)-1-phenylethanol, (98% purity), (R,S)-2-butanol (98% purity), (R,S)-2-pentanol, (98% purity) (R,S)-2-octanol (98% purity) (R)- and (S)-1-phenyletanol (98% purity), Hydranal Coulomat A and C Karl-Fischer reagents were from Riedel-de-Häens. Vinyl acetate (98% purity); vinyl acetate (98% purity); vinyl butyrate (98% purity); vinyl laurate (98% purity) were from Fluka. The solvent, substrates, and tridecane or decane were stored over molecular sieves 3A° (Merck). Preparation of (R,S)-2-phenyl-1-ethyl butyrate: To a stirred solution of (R,S)-1-phenylethanol (0.503 g, 3.7 mmol) and triethylamine (0.62 mL, 1.2 eq) in anhydrous dichloromethane (20 mL), under argon atmosphere and at room temperature, was added dropwise butyryl chloride (0.42 mL, 1.1 eq). After completion of the reaction (TLC), the reaction mixture was partitioned between diethyl ether (100 mL) and hydrochloric acid (100 mL, pH = 1), the organic phase was washed with a saturated solution of NaHC03 (100 mL), dried (MgSO4), evaporated under vacuum and purified by silica gel flash chromatography (eluent: 9.5:0.5 n-hexane/diethyl ether) to give the desired ester (0.704 g, 93 %) as a clear liquid.

Site-directed mutagenesis of cutinase aminoacid residues

Based on the existing model for cutinase, mutagenic oligonucleotides were designed that carry the modified codon near the center, in order to yield the mutations Y119A and L189A. Amino acid substitutions were made by the QuikChange (Stratagene) site-directed method using plasmid pMa/c5-CUF (Lauwereys, M. *et* al , 1991) as template

and *E. coli* strain XL1-blue as host. The mutations Y119A (TAC to GCC) and L189A (TGG to GCG) in the resulting plasmids, pMa/c5-CUF Y119A, and pMa/c5-CUF L189A, were confirmed by DNA sequencing. All plasmids carrying the different cutinase alleles were transferred to the *E. coli* strain WK6 (Zell, R., Fritz, H.J., 1987, EMBO J. 6:1809-1815) for protein expression analysis.

Protein expression

To confirm the production, estimate the yield and verify the solubility of the different target proteins, we performed small-scale analysis of total cell protein in the soluble and insoluble cell free extracts. For small-scale over-expression of the wild-type cutinase and mutant proteins, *E. coli* WK6 cells harboring plasmids pMa/c5-CUF WT (wild-type), pMa/c5-CUF WT woMS (wild-type without silent mutation), pMa/c5-CUF DEL (negative control) and different cutinase variants were grown at 37 ºC and 160 rpm in 10 mL of LB with appropriate antibiotic selection. When the OD 600 nm reached 0.6, protein expression was induced by the addition of 1 mM IPTG and the culture was further incubated 3 h at 37 ºC and 160 rpm. Cells were then harvested by centrifugation (13 000 g; 5 min). All subsequent steps were carried out at 4 ºC. The soluble and insoluble fractions were prepared as follows. Cells were resuspended in French Press buffer (20 mM Na-phosphate buffer, pH 7.4, 500 mM NaCl, 100 mM imidazole, glycerol 10%) and disrupted in the presence of lysozyme (1mg ml^{-1}) by three cycles of freezing in liquid nitrogen and thawing 5 min at 37 ºC, followed by incubation with benzonase (Merck) to destroy nucleic acids and PMSF (10 mg ml^{-1}), a protease inhibitor. After 15 min of centrifugation at 16 000 *g* and 4 ºC the soluble and insoluble fractions of the crude extract were obtained. The proteins were analysed by SDS–PAGE (12.5%). *E. coli* WK6 cells harboring plasmids, pMa/c5-CUF Y119A woMS, and pMa/c5-CUF L189A woMS were further used to large-scale production and purification of mutants 119A and L189A. The procedure for large scale-prodution is described elsewhere (Carvalho, *et al*, 1999).

Rational design

The computer-assisted generation of the transition states for both enantiomers was made on the basis of the structure of a complex of cutinase with the inhibitor N-hexylphosphonate ethyl ester30 (PDB accession code: 1XZL) using the programs Sybyl 6.2 from TRIPOS and Turbo-Frodo.47 The structures obtained in this way were

subjected to local energy minimization (residues around the transition state) in Sybyl using the TRIPOS force field plus Kolmann united atom charges (united atoms were used). A distance-dependent dielectric was used for electrostatics and interactions were truncated at 10 Å. The same procedure was followed for Y119A and L189A.

Enzyme Assays

All reactions were transesterifications of one of the secondary alcohols with one of the vinyl esters. The solvent was either *n*-hexane which was pre-equilibrated at a_w =0.22, as described (Vidinha et al., 2004). Reactions were performed in glass vials (reaction volume of 2 mL) placed in a constant temperature bath at 35 ºC, 0 ºC and -20 ºC. 50 mg of pre-equilibrated immobilized enzyme were placed in the vial and 2 mL of solvent were added, followed by the alcohol (100 mM). The system was left to equilibrate for 2 hours before the reaction was started by adding the vinyl ester (300 mM).

Analysis

Both the reaction conversion and the enantiomeric excess of the remaining ester product substrate (eep) were measured by GC analysis performed with a Trace 2000 Series Unicam gas chromatograph. Column: 30 m - 0.25 mm I.D. BGB-76SE fused silica capillary column coated with a 0.25 μm thickness film of 20% heptakis-(2,3-di-O-methyl-6-O-tertbutyldimethylsilyl)-*h*-cyclodextrin in SE 52 (5% phenyl-, 95% methylpolysiloxane). Three different programs were made. For acetate with 2-butanol and butyrate with 2-butanol: Oven temperature program: 40 ºC for 20 min, 85 ºC ramp at 1 ºC min^{-1}, 200º C for 5 min. Injection temperature: 250 ºC. Flame ionization detection (FID) temperature: 250 ºC. Carrier gas: helium (1.0 cm3 min^{-1}). Split ratio:1:20. The retention times for acetate with 2-butanol were 20,82 min ((*R*)-2-butanol), 21,21 min ((*S*)-2-butanol), 33,26 min (vinyl butyrate), 52.68 ((*S*)-2-butyl butyrate), 54.3 min ((*R*-2-butyl butyrate), 60.15 min (decane internal standard). Retention times when acyl donor was vinyl acetate: 42.53 min ((*S*)-2-butyl acetate), 44.32 min ((*R*-2-butyl acetate). For acetate with 2-pentanol and butyrate with 2-pentanol: Oven temperature program: 40 ºC for 17 min, 104 ºC ramp at 2 0 ºC min^{-1}, 200º C for 5 min. Injection temperature: 250 ºC. Flame ionization detection (FID) temperature: 250 ºC. Carrier gas: helium (1.0 cm3 min^{-1}). Split ratio:1:20. The retention times for butyrate with 2-pentanol were 32,21 min ((*R*)-2-pentanol), 32,40 min ((*S*)-2-pentanol), 43,55 min (decane internal standard), 45,45 min ((*S*)-2-pentyl butyrate), 46,45 min

((*R*)-2-pentyl butyrate), Retention times when acyl donor was vinyl acetate: 33,69 min ((*S*)-2-pentyl acetate), 41,23 min ((*R*-2-pentyl acetate). For acetate with 2-octanol and butyrate with 2-octanol: Oven temperature program: 40 ºC for 25 min, 113 ºC ramp at 1ºC min^{-1}, 200º C for 5 min. Injection temperature: 250 ºC. Flame ionization detection (FID) temperature: 250 ºC. Carrier gas: helium (1.0 cm3 min-1). Split ratio:1:20. The retention times for butyrate with 2-octanol were 18,18 min (decane internal standard) 34,53 min ((*R*)-2-octanol), 34,82 min ((*S*)-2-octanol), 65,12 min ((*S*)-2-octyl butyrate) and 66,18min ((*R*)-2-octyl butyrate), Retention times when acyl donor was vinyl acetate: 34,21 min ((*S)*-2-octyl acetate), 38,93 min ((*R*)-2-octyl acetate). No products were detected in assays carried out without enzyme. The enantiomeric ratio, E, was calculated from the expression E= {ln[(1-c)(1-ees)]}/{ln[(1-c)(1+ees)]}, where c is the conversion (Straathof and Jongejan, 1997) and ee_s is given for the (R)-enantiomer, by using data obtained for the racemic substrate along the time course of the reaction (when possible, up to a conversion of 0.5). The results reported are the average of least replicate measurements.

RESULTS AND DISCUSSION

In our earlier study (Fontes et al., 1998), we were able to rationalize the preference of cutinase towards the *(R)*-enantiomer of 1-phenyethanol. In the present study we used a similar approach to decrease the enantioselectivity of cutinase towards the *(R)*-enantiomer of 1-phenylethanol. Our assumption was, as before, that the cutinase active site was available to both enantiomers, and that the enantiomeric discrimination was related with the stabilization of the tetrahedral transition state for deacylation in both cases. Our previous study had allowed us to identify residues involved in the stabilization of the transition state. We found that a hydrophobic pocket defined by leucine 187, valine 184 and tyrosine 119 was responsible for the accommodation of the phenyl group of the alcohol. On the other hand, we also found that tyrosine 119 was responsible for establishing a hydrogen bond with the backbone oxygen of histine 188, and that this interaction was maintained during the transition states of both enantiomers. The enzyme preference for the *(R)*-enantiomer was found to be related with the role of tyrosine 119 on the immobilization and stabilization of the transition state for that enantiomer. The role played by those three residues made them suitable candidates for

our rational design strategy. The mutants selected were L189A, V184A, Y119A and V184A/L189A. The rationale was to mutate a larger side chain into a smaller one (alanine) in order to decrease the sterical hindrance of the substrate. The mutants were constructed *in silico* and simulations were performed for each one of them, followed by free energy studies where the differential binding of the *(R)*- and the *(S)*-enantiomers of 1-phenylethanol was evaluated. The results are presented in Table 1.

Table 1 – Relative free energy difference $\Delta\Delta G_{R\rightarrow S}$ between (*R*)- and (*S*)-tetrahedral intermediates for wild-type cutinase and mutants.

	Sequence after mutation	ΔΔG(R->S)* (kJ/mol)	error (kJ/mol)
Wild-type		21.0286	3.74752
L189A	AAPHAAYGP	12.6713	4.03798
V184A	GSLIAAAPH	20.6515	2.72374
Y119A	IAGGASQGA	10.5685	3.24193
V184A/L189A		14.5218	1.91398

The results show that three of the mutants decrease significantly the binding free energy difference between the (*R)*- and the (*S)*-enantiomers of the substrate, when compared with the wild-type enzyme, and especially the single point mutations L189A and Y119A (Figure 1). These two mutants were selected for large scale-prodution.

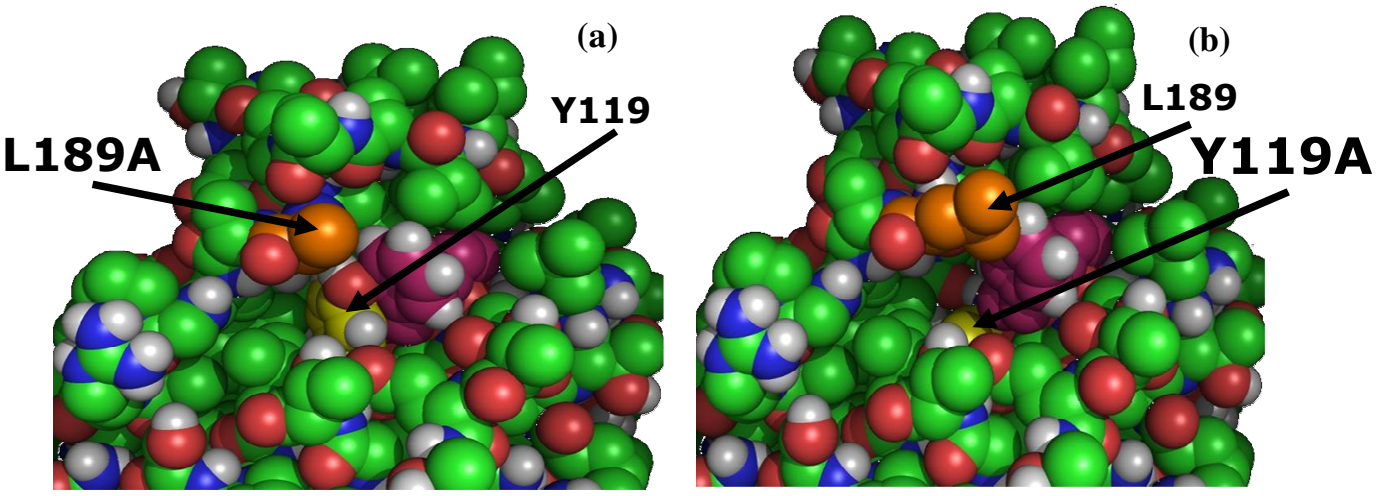

Figure 1 – Cutinase mutants. Orange on (a) - L189A mutation. Orange on (b) – non mutated Leu 189. Yellow on (a) – non mutated Tyr 119. Yellow on (b) – Y119A mutation.

Despite the similar $\Delta\Delta G_{R\rightarrow S}$ values for the two mutants selected, the L189A mutant exhibited an enantioselectivity towards 1-phenylethanol that was experimentally indistinguishable from that of the wild-type enzyme (Table 2). However, the Y119A mutant was much less selective towards the (*R*)-enantiomer of the alcohol. A possible explanation for this fact is the absence of the favourable van der Waals interactions between this enantiomer and Tyr 119, since the transition state is strongly stabilized by this type of interaction. The apparent failure of the L189A mutation, which also brings about easier access of the phenyl group to the active site, seems to confirm the role of Tyr 119 in the stabilization of the transition state for the *(R)*-isomer of 1-phenylethanol in the case of wild-type cutinase.

Table 2 – Cutinase enantioselectivity in the kinetic resolution of 1-phenylethanol, at 35 ºC and a_w = 0.2.

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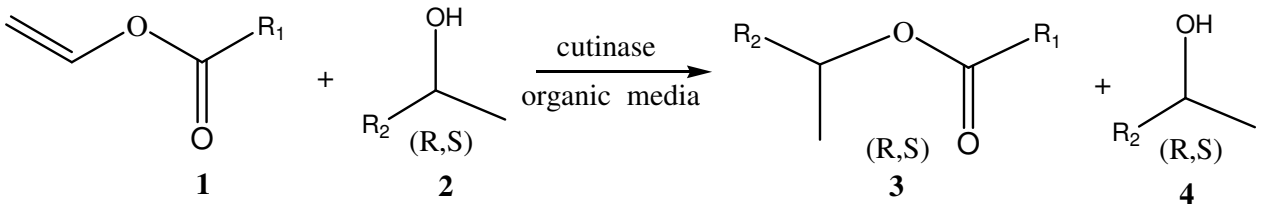
	% Conversion	ee_s	ee_p	E
Wild-type	43	0,87	1,00	∞
L189A	39	0,78	1,00	∞
Y119A	30	0,10	0,56	3,5

By lowering temperature, it was possible to partially recover the discrimination ability of cutinase upon removal of Tyr 119: at -20ºC, we obtained E ≈ 20 for the Y119A mutant. This confirms the utility of temperature in fine tuning the stereochemistry of enzyme catalyzed reactions (Phillips, 1996). However, temperature alone could not match the more pronounced effect of a structural change imposed by the Y119A mutation. To try and throw more light on the discrimination ability of cutinase, we extended our medium engineering approach by studying the transesterification of a homologous series of secondary alcohols, using also a homologous series of acyl donors, in a temperature range from -20 to 35 ºC (scheme 1).



	R_1		**R_2**
1a	CH_3, vinyl acetate	**2a**	$(CH_2)_2CH_3$, 2-butanol
1b	$(CH_2)_2CH_3$, butyl acetate	**2b**	$(CH_2)_3CH_3$, 2-pentanol
1c	$(CH_2)_9CH_3$, lauryl acetate	**2c**	$(CH_2)_6CH_3$, 2-octanol

Scheme 1.

Published structures of cutinase bound to ester inhibitors (Longhi S., *et* al., 1996) indicate that the acyl chain of the substrate is preferentially oriented towards a tunnel region defined by Val 184 and Leu 81, in a hydrophobic cavity limited by Leu 182. If we assume that the -R_2 substituent of the alcohol substrate is accommodated in the same hydrophobic pocket as the phenyl group of 1-phenylethanol, then it is likely that an increase in the alkyl chain length of the ester may affect the packing of the alcohol and that the alkyl chain length of the latter may also influence the stability of the transition state, and hence the enantiomeric discrimination of cutinase. Indeed, Kawasaki et al. (2002) looked at the impact of the acyl donor in transesterication reactions performed by several lipases, and found that the structure of the acyl donor required for the maximization of enantioselectivity was dependent on the type of alcohol.

Our results (Table 3) suggest that the accommodation of short acyl donor alkyl chains, up to butyl, in the active site of cutinase does not have any significant impact on enzyme enantioselectivity, which in that case depends solely on the alcohol. The size of 2-butanol affords only marginal discrimination that increases for 2-pentanol and hardly changes for 2-octanol, although far from matching the extremely high enantioselectivity observed when -R_2 is a phenyl group. By making the alkyl chain length of the acyl donor larger, as in vinyl laurate, the discrimination ability of cutinase towards 2-octanol increased substantially, suggesting a packing arrangement in the active site that stabilizes the *-(R)*-enantiomer relative to the *-(S)*-enantiomer.

Table 3 – Cutinase-catalyzed transesterification of secondary alcohols with vinyl esters, at 35 ºC and a_w = 0.2.

Acyl donor	Alcohol	% Conversion	ee_p	E	Stdev
1a	**2a**	45,3	0,24	2,2	0,4
1b	**2a**	43,4	0,25	1,8	0,1
1a	**2b**	48,6	0,69	9,8	1,8
1b	**2b**	46,2	0,68	8,9	0,8
1a	**2c**	48,2	0,66	6,8	0,8
1b	**2c**	48,6	0,68	8,3	0,9
1c	**2c**	44,1	0,90	40,7	2,7

The results obtained at lower temperatures (Figure 2) confirm the role of this methodology as a second order approach towards the fine tuning of enzyme enantioselectivity. In fact, in the case of 2-butanol that cutinase resolved with poor E at 35 ºC, lowering temperature hardly affected the discrimination ability of the enzyme. On the other hand, in the cases where cutinase already showed a more marked preference for one of the enantiomers, this preference became more pronounced as temperature was lowered.

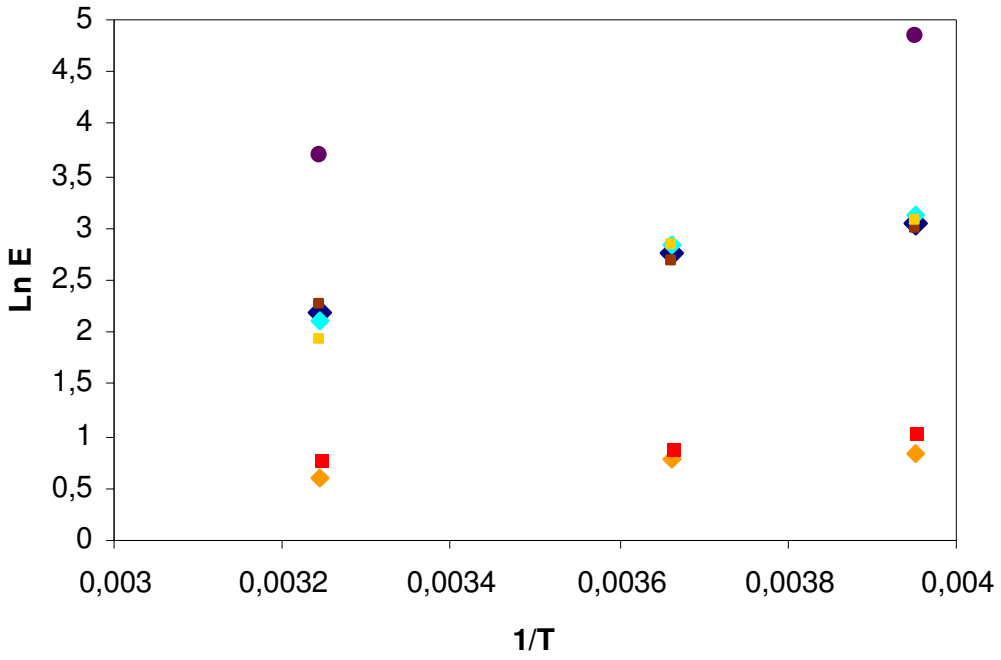


Figure 2 – Cutinase enantioselectivity at 35 ºC, 0 ºC and -20 ºC. Red squares: **1a** with **2a**; orange diamonds: **1a** with **2a**; Yellow squares: **1a** with **2c**; Light blue diamonds: **1b** with **2c**; Dark blue diamonds: **1b** with **2b**; Brown squares: **1a** with **2b**; Purple circles: **1c** with **2c**.

CONCLUSIONS

We went one step further in the rationalization of the enantioselectivity of cutinase from *Fusarium solani pisi* in nonaqueous media. The utility of temperature in fine tuning the stereochemistry of cutinase catalyzed reactions was confirmed, although temperature alone could not match the more pronounced effect of a structural change imposed by a single point mutation close to the active site of cutinase. The results obtained for the

homologous series of secondary alcohols should be an important contribution to the development of the existing model for cutinase.

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CHAPTER III

Enzyme immobilization
Confining biocatalysis.

Since the second half of the 20th century a tremendous amount of studies have been done to develop immobilized enzyme preparations for a myriad of different applications. The reason for this intensive research in related with the positive return of using enzymes in such form. Immobilized enzymes can be recycled, which by itself is an important factor for biocatalytic applications, since the cost associated to their use can be considerably reduced. In the previous chapter we saw that new enzymes are being developed for many industrial processes, which represents a large investment of resources whose return can be greatly increased through immobilization.

On the other hand enzymes are known to loose their activity during the reaction process due to their natural lack of adaptability to the novel environments forced upon them. Enzymes were not designed to serve all the chemical engineering intents, they are still biomolecules. Immobilization provides a simple way to protect enzymes from the harshness of reaction environments, and with the advent of molecular rationalization it became possible to alter the molecular environment of enzymes even in immobilized form. This last issue is particularly important and has been one on the areas of major interest within enzyme immobilization.

Today the aim of immobilization is to develop interactive matrices that not only have to be able to retain the biocatalyst, but also provide it with the most suitable environment for its specific function. The idea of developing a generic immobilized preparation that suits large number of enzymes in different applications is out of the question. Enzymes are not generic, which means that every enzyme is a particular case. Obviously enzyme stability and activity are related with a considerably number of factors that change with the different environments and with the different tasks that are requested of the enzyme. Rationalization is again the key. Understanding enzyme dynamics is crucial to the development of better enzyme preparations, and for that purpose we need to consider the molecular level at which all the interactions take place.

The aim of this chapter is to give an idea of the current state-of-the-art of enzyme immobilization, and analyze the developments that resulted from rational design and from the combination of different research areas.

Defining the function is the essential issue for developing the desired solution. One basic question is where and when are we going to use the enzyme? And what is more important? A high volumetric activity? A high stability? A high specific activity or an improvement in selectivity? Even with today's technology it is difficult to provide solutions to every demand.

For instance a high volumetric activity (HVA) is essential when a higher productivity and a space-time yield are process determinants. It is usually considered that only 10 % of the total mass of the immobilized preparation accounts for the enzyme, and it is also acceptable that the immobilized preparation occupies 10-20 % of the total volume of the reactor. In this case enzyme preparations with a high volumetric activity are an essential requirement[1-3]. To this end two major solutions have been presented. The first is one of the most common ways to immobilize enzymes and is based on the use of supports with a high payload[5-14], which is a high mass of enzyme per mass of immobilization material. The second one and altogether different solution is CLEA technology (Cross-Linked Enzyme Aggregates), which is one of the most interesting and efficient ways to immobilize enzymes today[2,3, 14-18].

For a carrier to be able to immobilize considerably amounts of enzyme while maintaining its activity and integrity, two factors are important: the size of the pore of the matrix and the accessible surface[3,5,10,12]. This type of strategy involves enzyme immobilization onto organic or inorganic porous particles. In general it is considered that a support that allows a payload ratio ($m_{enzyme}/(m_{support}+m_{enzyme})$) between 0,1 and 0,2 with a specific area of about 200 m^2/g and a pore size greater than 100 nm is perfectly suitable for this type of application[12]. In theory, any material that can meet these conditions can indeed be considered suitable for enzyme immobilization. Nevertheless the most common materials reported in the literature are organic polymers[19-23], which includes vinyl polymers, such as acrylates or polyvinylacetate (PVA), activated charcoals[24-26], and silica based materials[5-9;11-15;27-42].

In fact mesoporous silica is the material most used for enzyme immobilization. The reason for this is related with the enormous variety of solutions that this material offers to immobilize biocatalysts. This is not only due to the structural versatility of silica, but also and particularly the chemical versatility of silica, which allows its modification in

order to fix the enzyme onto it[35,39]. The binding of the enzyme to this material can follow some of the main routes to the immobilization of biomolecules: physical adsorption[5,7-9,12,14,15], which is the classical process for enzyme immobilization, covalent attachment[8,10-13] and entrapment[27-39], which confines the enzyme into the structure of the immobilization material. All these strategies have been shown to give good results, but again the choice of immobilization route has to take into account all the aspects that are crucial for the desired application[1-3]. One technique that has been widely exploited is that of sol-gel. There are numerous examples in the literature that demonstrate the efficiency and the success of this technique for obtaining particles to produce immobilized preparations with a high volumetric activity[27-42].

This process represents the most versatile way to obtain silica in different forms, since we can not only control the way in which silica is synthesized, thus obtaining materials with different design structures, but we can also introduce a range of different functionalities in the silica precursors that give the final material different chemical characteristics[35-39]. This makes sol-gel processing a very powerful immobilization technique. Special attention will be paid to sol-gel entrapment in the present chapter.

CLEAs have been attracting a lot of attention due to the simplicity of their concept, broad applicability and high stability associated to a high volumetric activity[3, 16-18.]. The simplicity of the approach is one its great advantages, as illustrated in figure 3.1. In this case no external support is needed since the aggregates are prepared by the chemical cross-linking of the enzyme molecules. The enzyme is precipitated by using agents such as inorganic salts or organic solvents, without undergoing denaturation. The immobilization via cross-linking is attractive because the final preparation is basically pure protein, with a high concentration of enzyme per unit volume[16-18].

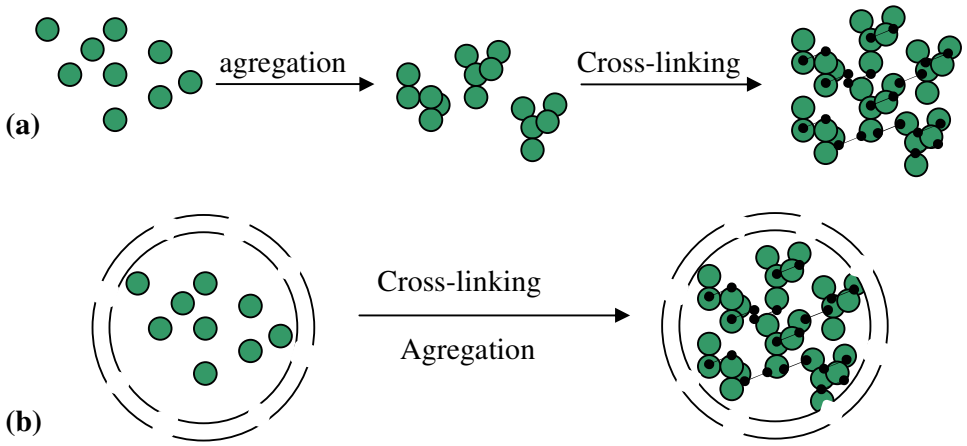

Figure 3.1 - CLEAs processing. (adapted from www.cleatechnologies.com). (a) – solution process; (b) – CLEAs associated to porous materials.

More recently Sheldon and his co-workers[18] have shown that immobilization via CLEAs could also be combined with immobilization onto a mesopourous support, combining the advantages of both techniques. In this case a porous membrane of controled pore size was impregenated with CLEAs. Instead of for instance attaching the enzyme to the surface of the support through covalent interactions that usually have a negative impact on catalytic performace, the authors filled the porous of the matrix with an enzyme solution and then made the CLEAs inside the pore cavities. They thus obtained a high density of immobilized enzyme inside the pores. The results that these authors obtained for *Candida antartica* lipase B were quite promising in terms of enzyme activity and stability, but also in terms of the mechanichal stability of the immobilized preparation which is one of the drawbacks of the CLEA immobilization strategy.

Stability is a major goal of all immobilization procedures, and every paper on enzyme immobilization considers that parameter. Stability can be quantified by the loss of activity upon recycling, through the impact of a given medium parameter, such as temperature or solvent polarity, or by long term enzymatic activity. As we saw in the first chapter of the present thesis, molecular biology has given a very good insight towards the improvement of enzyme stability. Nevertheless, these improvements can and should be complemented by enzyme immobilization, since the enzyme structure is not the only factor that contributes to an enhancement of this property. Factors such as

the number of bonds that are formed between the enzyme and the support, the degree of confinement of the enzyme molecules, the immobilization conditions, and of course the enzyme micro/molecular environment[1-3;35-39,43-45,55] are all relevant to achieve good enzyme stability in an immobilized preparation. Therefore, both approaches should be combined in order to obtain even more powerful and stable biocatalysts.

One of the first and simpler approaches to change the enzyme microenvironment consists on the addition of doping molecules during the enzyme immobilization procedure[3,35-39,42,43,47]. This strategy aims at the reduction of undesirable interactions between the enzyme and the chemical agents, as epoxy rings or aldehyde groups, which are used or formed during the immobilization procedure. The doping molecules act as blocking agents and can be simple molecules like aminoacids and small peptides, or macromolecules like biopolymers, surfactants, or even other proteins such as albumin.

A good example of this procedure is the immobilization of penicillin V acylase from *Streptomyces lavendulae* on Eupergit C. The immobilization of this enzyme together with albumin not only increased the enzyme activity 1.5-fold, but also produced a remarkable increase in the stability of the biocatalyst, which was used for at least 50 consecutive batch reactions without any loss of activity. The rationalization of this improvement was related with the creation of a more hydrophilic environment in the neighbourhood of the enzyme, to resemble the natural environment of this enzyme where hydrogen bonding plays an import role on its stabilization[43].

The modification of the microenvironment hydrophilic character has become a recurrent strategy and has led to other ways to achieve that effect, such as the binding of specific chemical molecules either to the enzyme or to the support, thus creating a spacer effect that mediates the direct interaction between both. A good example of this strategy was given by Hwang *et* al.[58] who investigated the effects of surface properties on the stability of *Bacillus stearothermophilus* lipase (BSL). For that purpose the authors used silica gel as carrier material, coated with polyethyleneimine (PEI) or silanized with hydrophobic molecules, to obtain supports with different hydrophilic characters. These authors observed an increased in enzyme stability in the supports with a higher hydrophilic character. The presence of PEI created a favorable microenvironment in which the BSL was more stable.

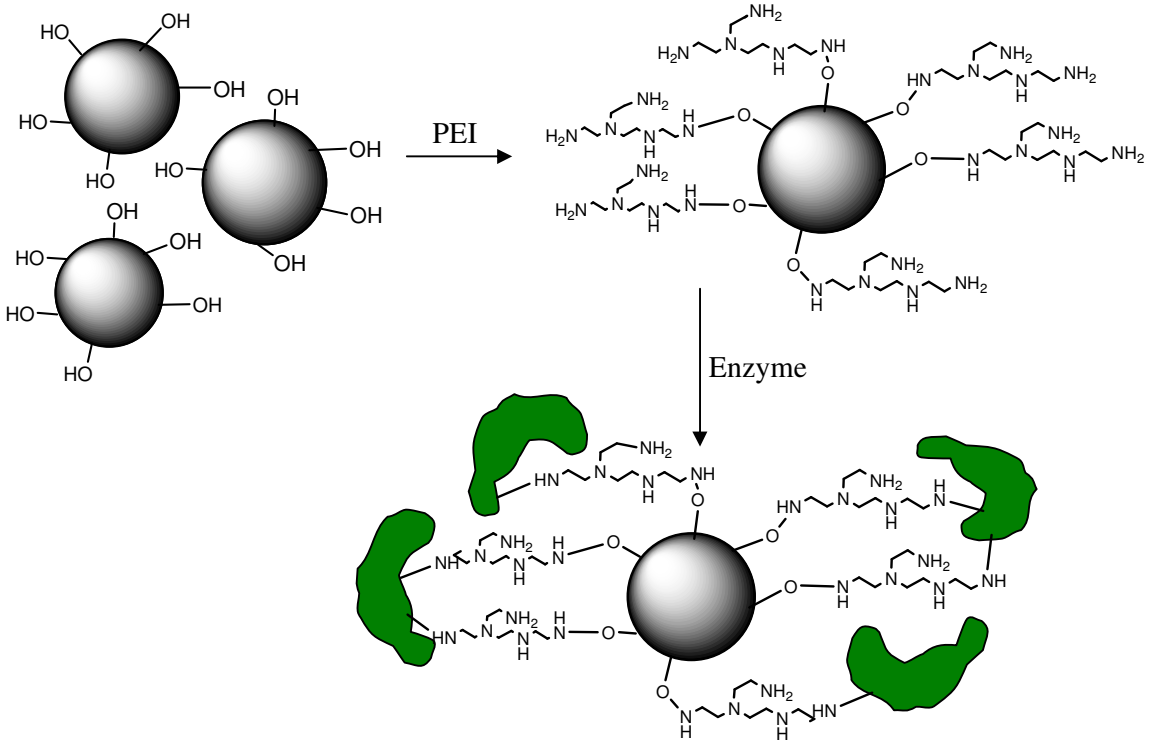

Figure 3.2 – Example of an immobilization strategy that involves the functionalization of the support with polyethyleneimine.

The modification of the enzyme microenvironment has been shown to be a very efficient way to improve enzymatic properties, and has triggered numerous publications in the field[35-39, 42, 43, 51, 53-55, 58]. With the advent of novel molecules and materials the combination of this strategy with other types of immobilization procedures has resulted in simpler and better ways to achieve the modification of the enzyme microenvironment.

One technique that the approach was successfully combined with is that of sol-gel [30, 31, 35-39]. In this case the enzyme is encapsulated inside the silica matrix rather than on the porous surface like in the Hwang approach. The entrapment of the enzyme in a sol-gel matrix affords not only an increase in enzyme stability but also provides a simple way to modify the enzyme microenvironment. This is possible since the sol-gel process is based on the use of different precursors, i.e., orthosilicates that can be synthesized in order to have attached different functionalities (figure 3.3). The success of the sol-gel

technique can also be seen by the huge number of precursors that are available in the market. Using one or combining several it is possible, at least in theory, to obtain the "perfect" microenvironment for a given enzyme.

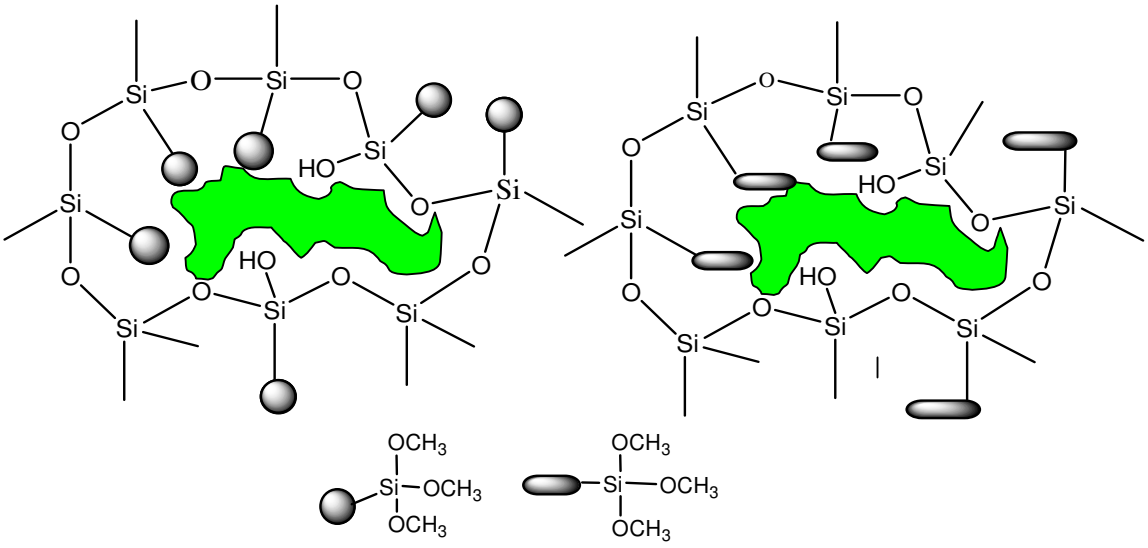

Figure 3.3 – Modification of enzyme microenvironment via a sol-gel approach.

As we saw in chapter II ionic liquids can affect enzymatic properties. Since it was shown that enzyme stability could be improved in some ionic liquids, the idea of using them as additives to change the enzyme microenvironment became the aim of several research groups that developed several ways to immobilize them together with enzymes[59-63].

One good example of this strategy was presented by Koo and colleagues[60] who immobilized *Candida antarctica* lipase B together with different ionic liquids. The aim of this work was to avoid enzyme inactivation caused by the release of alcohol during the immobilization procedure. On the other hand since the ionic liquid remains in the material, the authors evaluated its impact on enzyme stability. Their results showed that some of the tested ionic liquids acted as protecting agents, since enzyme activity increased in their presence. From the standpoint of stability these authors evaluate the impact that long term incubation (5 days in n-hexane) could had on enzyme activity. Their results revealed that best ionic liquid could exibit an activity 3.5 times higher when compared with the preparation without ionic liquid. Lozano et al.[59] obtained

similar results for the co-immobilization of CALB with ionic liquids onto silica membranes.

In addition to the modification of the enzyme microenvironment, the modification of the enzyme before or after the immobilization procedure is also an important tool to enhance enzyme stability.

The pre-immobilization procedures for stabilization aim at improving enzyme performance before the immobilization procedure takes place. In this case enzymes are chemically modified by conjugating reactions with polymers or even with cross-linking agents, yielding stabilized enzyme complexes[64-71]. The most classical way to accomplish this is to use polyethyleneglycol (PEG). The PEGylation of enzymes[67-71] is one of the most studied ways to alter enzymatic properties, with broad applications, as excellently reviewed by Veronese[71]. A good example of this approach was reported by Hsu and co-workers[66] who evaluated the impact of PEG modification of the stability of α-chymotrypsin. Their results showed that the PEGlyation of α-chymotrypsin before the encapsulation in calcium alginate beads increased both its thermal and its operational stability.

When the immobilization procedure does not produce the stability initially envisaged, it is possible to use the methodologies above in a post modification step. The enzyme can be subjected to further chemical modification like cross-linking, or a physical treatment such as pH enhancement, even lyophilization.

Post-modification can have a great importance in the case of multimeric enzymes. In this case, during or after the immobilization procedure, and due to ionic, acidic or even temperature stress, the enzyme subunits may dissociate and in this way the catalytic performance of the immobilization preparation can be significantly reduced.

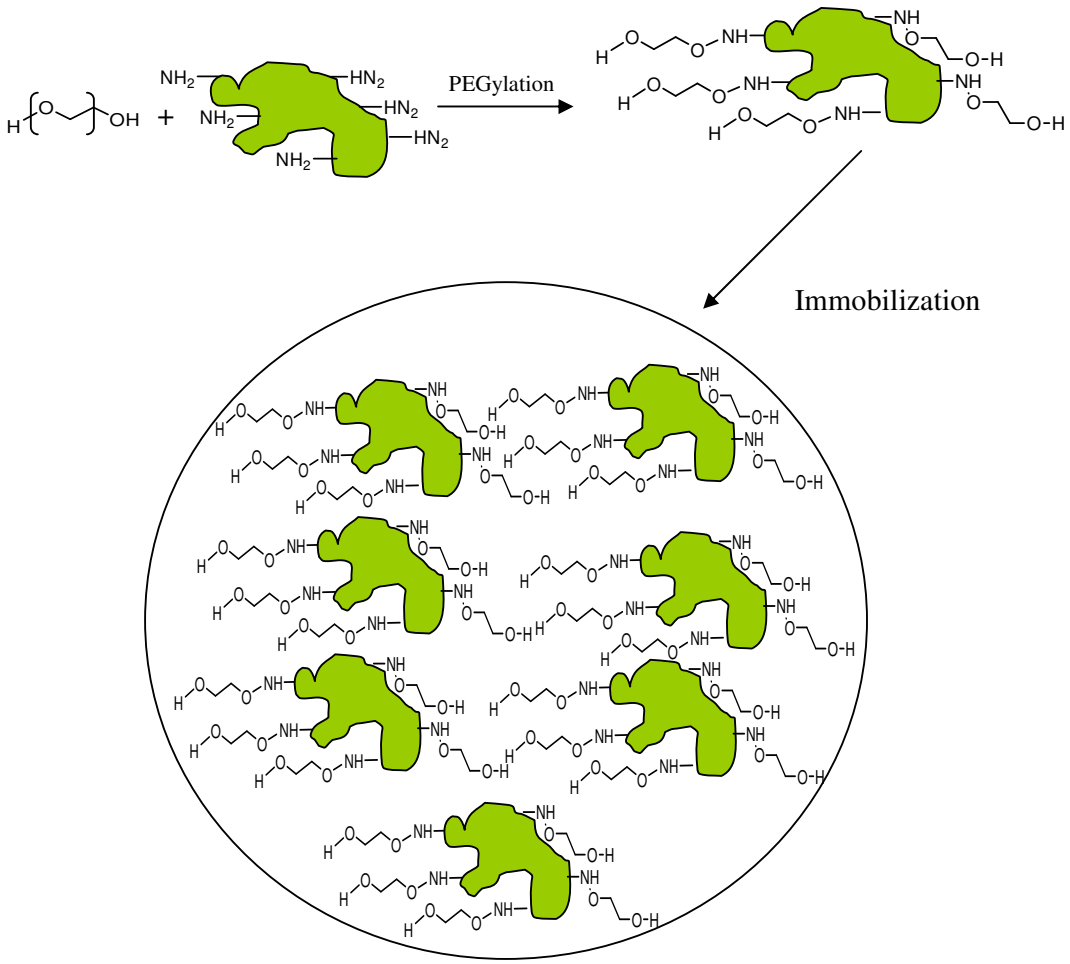

Figure 3.4 – Pre-immobilization strategy to enhance enzyme catalytic performance.

A good way to minimize this effect is to use a cross-linker agent. As we saw earlier these agents promote intermolecular interactions between enzyme molecules. However, they can also be used to promote intramolecular cross-linking, thus stabilizing the subunits of a multimeric enzyme. An excellent example of this approach was presented by Guisan and co-workers[49,50] on the immobilization of tetrameric bovine liver catalase (BLC) on highly activated glyoxyl agarose. This enzyme is easily dissociated in solution and it cannot be efficiently stabilized by covalent immobilization due to the limited surface available to form multipoint attachments. To overcome this problem these authors used a cross-linker with dextran-aldehyde, a partially oxidized dextran, to increase the stability of this enzyme on the surface of the support. This strategy was

applied to several other enzymes with excellent results. Actually this group is known for presenting very interesting and creative solutions for enzyme immobilization[45-57].

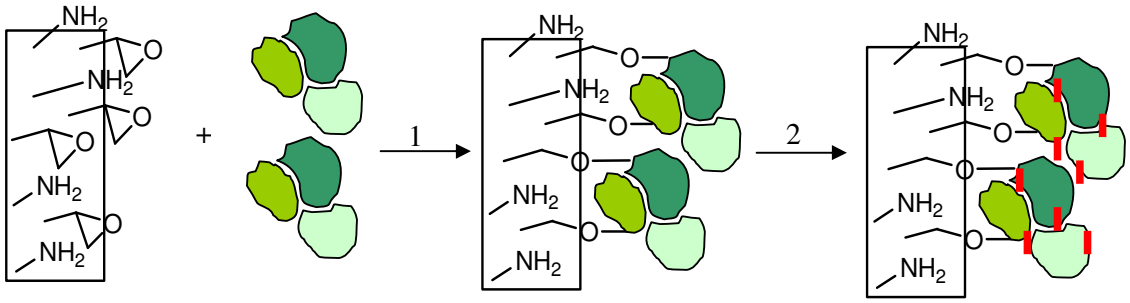

Figure 3.5 – Post modification strategy to enhance the catalytic performance of a multimeric enzyme. (1) covalent immobilization; (2) chemical modification with a cross-linking agent. Adapted from Guisan *et al*. [49]

The dichotomy between enzyme activity and stability is very important for process design. Equilibrium between these two parameters is usually a requirement for most immobilized preparations to have commercial applicability. If for a specific application it is desirable to have an immobilized enzyme with a high specific activity (U/mg), the viability of the process is time dependent and a fast conversion is critical. For this reason many authors have put a lot of effort into developing immobilization procedures centered on the improvement of catalytic activity. Most of the strategies follow the same concepts that the ones presented for stability improvement. Microenvironment, chemical and physical modifications, pre- and post-immobilization are also good strategies to improve enzyme activity, changes in enzyme microenvironment playing an essential role in this respect.

The sol-gel process has been applied successfully to modify the microenvironment of lipases for activity enhancements[29-32, 36, 41]. One characteristic of these enzymes is the presence of a "lid" covering their active site. This lid is formed by a sequence of hydrophobic residues that have to undergo a structural modification in the presence of hydrophobic surfaces to give access to the active site. This phenomenon is called interfacial activation[72] and is observed with many lipases. Actually it is one of the main differences between lipases and esterases. Usually this "lid" works as a switch that activates the lipase only when the substrates are present in critical concentrations. The

substrates must have a hydrophobic character in order to activate the enzyme catalytic function. This fact explains why in nature lipases are mainly involved in the hydrolysis of fat acids and triglycerides. Nevertheless in biocatalysis lipases are used to catalyze every possible trans- or esterification reactions, which means that not all the substrates possess the necessary hydrophobic character to activate them. This results in poor access to the active site, which is responsible for low enzymatic activity. Immobilization can be used to overcome this problem by providing the necessary hydrophobic interactions that are responsible for the activation of the enzyme. Since lipases are one of the most studied types of enzymes in biocatalysis, the number of publications on strategies to solve the interfacial activation problem is very large[27,29,30,31,36,41,45-47,57,60].

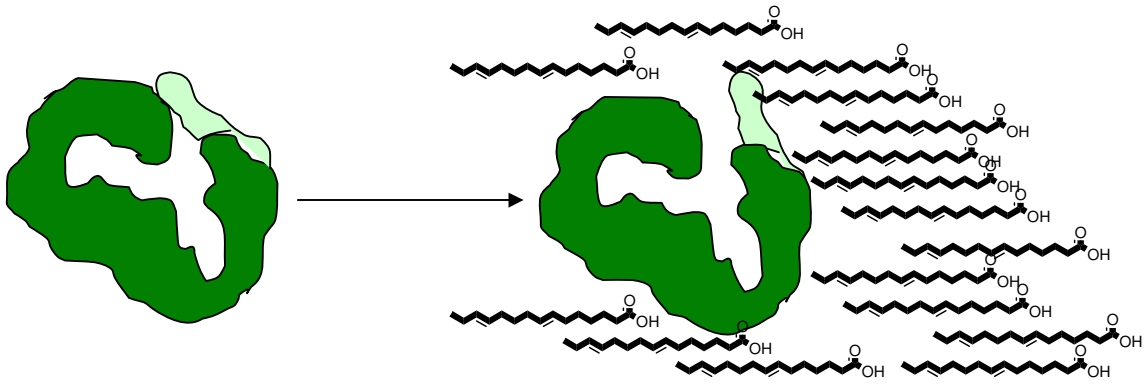

Figure 3.6 – A very simple representation of lipase interfacial activation.

Reetz and co-workers[30, 31, 73] have shown that the catalytic activity of several lipases could be enhanced with the increase in hydrophobic character of the immobilization matrix. This strategy is the same that was illustrated in figure 3.3, but in this case the substituents are hydrophobic alkyl chains. Those authors saw that the activity of *Pseudomonas fluorescens* lipase increased with the increase in the alkyl chain length of the precursors used in the sol-gel immobilization process.

Another strategy to obtain an activity enhancement using the microenvironment approach is related with imprinting-immobilization technology. In this case substrates, inhibitors or other additives are used during the enzyme immobilization step to prevent

the loss of activity. Their specify function is not always clear but in the case of the inhibitors their function is thought to be related with the preservation of active site integrity. This approach was tested with several immobilization techniques, including the sol-gel immobilization process, and proved to be a valid solution to tune enzyme activity[30,47,74-77]

Another interesting strategy that has shown good results on the activation of lipases was presented again by Guisan et *al.*[45, 47] The strategy followed by these authors is very similar to the one already illustrated on figure 3.5. But instead of using a support that only contained functional groups to establish ionic and covalent interactions, the authors used one with hydrophobic moieties on its surface. The increase in hydrophobic character of the support favored the interactions between the lipases and the support surface, promoting the immobilization of the lipases in the open-lid form. Further cross-linking stabilized the open-lid form of the enzyme at the surface of the support. These results are very interesting since using the same immobilization procedure, the authors were able to enhance, simultaneously, enzyme activity and stability.

Every time an enzyme is immobilized, one important factor to consider is its accessibility to reacting species. Obviously this is not an issue when the enzyme is immobilized at the surface of a support, but such type of immobilization is not always possible, especially for applications that involve continuous set-ups where shear stress can cause enzyme inactivation and leaching. The latter can be circumvented by entrapping enzymes in solid materials, such as a sol-gel. In a sol-gel matrix, the enzyme is inside a complex silica network and may not be directly exposed to the medium. Diffusion and partition effects for substrates and products between the reaction medium and the active site of the enzyme may then be a problem. If chemical or physical hindrances are at play, catalytic activity can be strongly affected. However, the sol-gel process allows the modulation of matrix pore size through the choice of precursors and reaction conditions. It is thus possible to generate immobilized preparations with a high volumetric activity from which enzyme leaching is minimized.

As we saw in chapter II of this thesis, enzyme selectivity is a top enzymatic property. Many creative approaches have been developed to produce improvements in this property, and enzyme immobilization is no exception. A recent study by Guisan and co-

workers[46] shows that by changing some parameters like temperature during the immobilization procedure, enzyme enantioselectivity could be improved. The authors used *Candida antarctica* lipase B adsorbed of PEI-Agarose and observed that when the immobilization was done at pH 5 and 4 ºC , the enzyme exhibited a 1 fold (E>100) increase in enantioselectivity compared with the procedure done at pH 5 and 25 ºC. The authors correlated this improvement with the different conformations that the enzyme could assume at the different immobilization conditions.

A very elegant approach was described by Faber and co-workers[80], who demonstrated that the selection of an appropriate immobilization strategy could impact on enzyme selectivity. The authors used the immobilization of alkylsulfatase on different anionic exchangers to illustrate this concept. They found that the enzyme enantioselectivity towards the kinetic resolution of *sec*-alkyl sulfate esters could be improved by a careful choice of the charged groups present on the anionic resin. This enhancement was attributed to the fact that those groups might be able to approach certain protein domains or sites, and in this way influence the geometry of the active site. Other authors have also demonstrated that enzyme enantioselectivity could be improved by microenvironment modification, using different approaches that include the sol-gel process, or the use of chemical additives. In some cases, in just one step it has been possible to increase the stability and selectivity of a given enzyme, with the extra advantage of reusability[30,46,47,70,77-80].

From what has been discussed above, it is clear that today enzyme immobilization is not regarded as a mere way to fix the biocatalyst for further reuse, but rather as a powerful technique that allows the fine tuning of several enzymatic properties. Finding a good immobilization procedure for a particular enzyme is still not trivial and requires experimentation. The same immobilization technique can sometimes be applied successfully to improve different enzymatic properties. And as more in known on enzyme structure and dynamics, which allows for a better understanding of why enzymes behave in a certain way when they are immobilized, immobilization strategies are becoming more focused on modulating the enzyme microenvironment.

One technique that has greatly expanded in the last 10 years of biocatalysis is sol-gel processing. Versatility in one of the keys to its success. With the sol-gel process it is

possible to obtain particles, nanoparticles and films with controlled porosity, and provide a particular microenvironment. The remainder of this chapter is going to be dedicated to this technology.

The sol-gel perspective that is about to be introduced is basically a bio-perspective since sol-gel is a vast area with applications disseminated in almost all scientific fields. The bio-applications of sol-gel materials have gained a lot of importance in the last decade and enzymes were just the first biomolecules to be used. Today we have living organisms that actually maintain their biological activity inside a sol-gel matrix. Biology was not only a source of interesting and profitable applications but also inspired the process itself. In the literature we can find many papers on sol-gel that include the word "bio-inspired" in their title[81-87,105]. Actually nature has created the most beautiful and functional silica structures and we are just beginning to learn from it. Most of the future trends point towards "bio-inspired materials" as the way to obtain better functional materials.

Inorganic gels are known since the middle of the 19th century, basically since the first developments in biocatalysis, as we saw in the first chapter of the thesis. Thus more than a century was needed to put these two areas in contact[89]. Nevertheless one area will only develop when there is potential applicability for its underlying science, and this was the case with sol-gel. The process was almost forgotten until the 60s, where the first coatings of flat-glass were reported, but the real development of sol-gel only occurred in the early 80s. The resurgence of this area was related with a growing interest in ceramic materials, and the sol-gel process offered a unique solution for ceramic fabrication at relatively low temperatures. Since then sol-gel methods have been used for the processing of a wide variety of materials: monolithic ceramic and glasses, fine powders, thin films, ceramic fibers, microporous inorganic membranes, and extremely porous aerogel materials, among others[36,40,89,90].

In the sol-gel process, one or more liquid inorganic silica precurors react, often in the presence of a catalyst and of additives such as stabilizers, to produce a gel consisting in a solid silica skeleton filled with a colloid, the sol. The sol contains unreacted species and additives dissolved in a water/alcohol mixture, and solid silica clusters[89,90]. As the process progresses, the solid skeleton expands, and through the so-called ageing, the

clusters connect to the main solid structure, forming a rigid three dimensional silica network containing remnants of the fluid phase in its pores. As shown in figure 3.7, the chemistry of sol-gel is quite simple and basically involves a two-step reaction, with a first step in which the hydrolysis of sol-gel precursors take place, and a condensation step which is responsible for the silica network growth. In this last step, condensation can occur either between two silanol groups that are formed in the first step of the process, or between one of these groups and a non hydrolyzed precursor molecule.

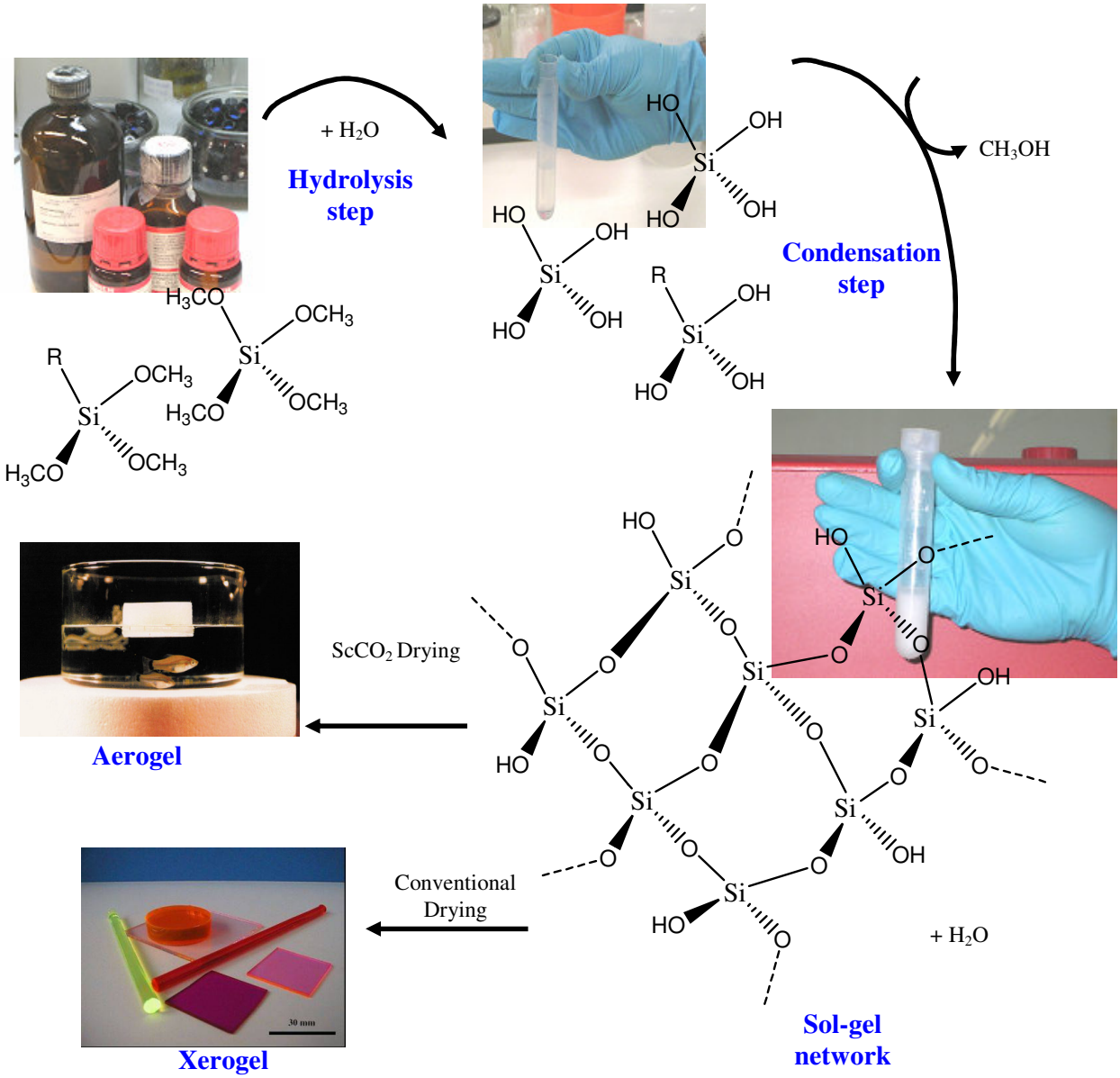

Figure 3.7 – Schematic representation of the sol-gel processg, using tetramethoxysilanes.

The evaporation of the solvent leads to the formation of a xerogel. The drying is more efficient if the material is spread over a solid surface. In fact, surface drying was one of the reasons why the technique became so popular and forms the basis of surface coating. In this respect, homogeneity is very important, and methods such as spin coating or spray coating allow for the formation of homogeneous and very thin films (100nm)[89,90].

Drying is a critical step of sol-gel processing since it can have drastic effects on the porous structure of the final material [91,92,93]. This is due to a phenomenon of surface tension. To maintain its structure during drying, the solid network has to behave as an elastic sheet. If the matrix cannot withstand capillary stresses, it collapses and its pore structure is destroyed. The best way to avoid this effect is to change the drying procedure[93]. Alternative drying methods are freeze-drying followed by sublimation, and solvent extraction. Extraction, especially if conducted with supercritical carbon dioxide ($scCO_2$), will produce highly porous materials called aerogels[93]. This type of material has been applied in several high-tech solutions, of which the most famous was STARDUST, a seven year NASA mission that successfully recovered samples from the tail of a comet. The sample collector was an aerogel plate which trapped the comet's cosmic dust inside its porous structure. Since areogels are extremely resistant materials, especially to high temperatures, it was possible to send these samples back to earth (figure 3.8).

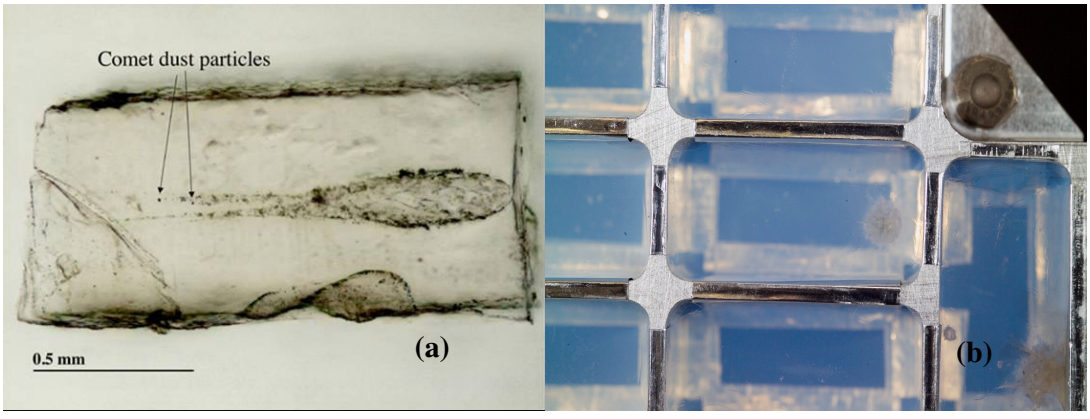

Figure 3.8 – (a) Comet dust particles inside the aerogel trap; (b) – Sol-gel trap. These two pictures are available to the public on the NASA website (www.nasa.gov).

Although the chemistry of sol-gel is simple, a good control of the experimental conditions such as pH, the type of catalyst, ionic strength, is essential to obtain materials with the desired characteristics. For instance, low pH values promote fast hydrolysis and slow condensation, leading to small silica particles that form a three dimensional gel. High pH promotes slow hydrolysis rates and rapid condensation, resulting in a smaller number of larger particles[89,90,93]. Usually the most commonly used catalysts are strong acids and strong bases, such as HCl or NaOH, but using organic acids such as acetic and formic acid, or weak bases such as sodium fluoride, can be quite effective for some solutions[89,90,]. However these reaction conditions are sometimes too aggressive for biological systems. This limitation has led to the development of more biocompatible solutions to make the sol-gel for bio-applications.

There are many ways to catalyze the formation of sol-gel networks, and pH is just one of them. Scientists have searched for solutions in nature, since a large number of biomineralized organisms inhabit our planet. Actually the evolution of biomineralization dates back to the Cambrian period (525-510 million years ago), where a great number of biomineralization organisms suffered an exponential increase. In fact it was during this period that the most important skeletal materials were formed[94,]. With this in mind, research focused on the living organisms that were capable of depositing silica in their biological structures. Species belonging to bacteria, algae protozoa or even higher plants use silica that is involved in different roles related with the cell type and with the surrounding environment[94-99]. Understanding the biochemistry behind this process was definitely one breakthrough, not only to this area, but especially to general science. The first studies were made in algae and sponges and led to the identification of peptides (i.e silafins)[98,99,103-105] and proteins (silcateins)[101,102,105] involved in the biomineralization processes. One of those proteins was silicatein α[101,102], which was found to catalyze the polymerization of silicas and organosilicones from silicon alkoxide precursors at neutral pH. This was an extremely important achievement since it became possible to biocatalyze the sol-gel process. The solving of the structures of some silicateins structures and the understanding of their catalytic mechanism revealed a high complementarity between these proteins and serine hydrolases[106-108,] whose catalytic mechanism was presented in chapter II. A very interesting fact is that silicateins cannot not catalyze proteolytic reactions, but serine hydrolases can catalyze the polymerization of silica, although the cleavage of peptides and the condensation

reactions involved in the polymerization process proceeded through the same hydrolytic reaction[101,102].

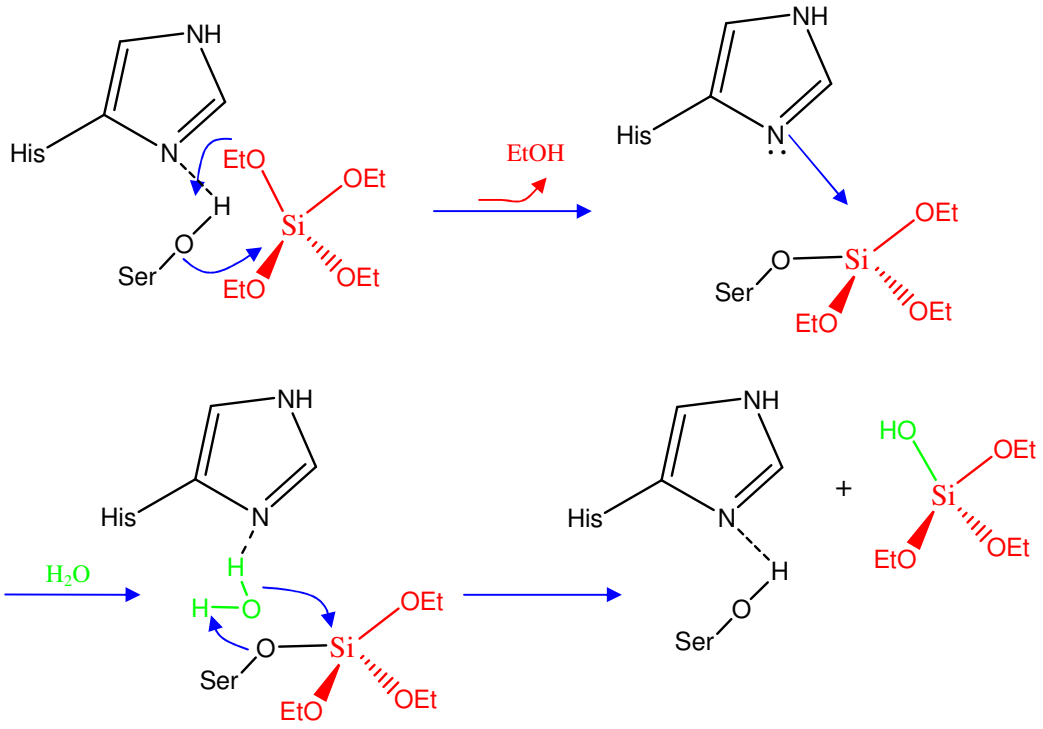

Figure 3.9 – Schematic representation of the biocatalytic hydrolysis of TEOS (tetraethylmethoxysilane). This example can be applied either to silicateins or to serine hydrolases[101,102, 106-108].

A very interesting study was performed by Brandstadt and his co-workers[106,107] who demonstrated that several proteases were able to catalyze the hydrolysis of tetraethylmethoxysilane (TEOS). Actually trypsin led to yields of almost 90 % in the hydrolysis of TEOS. Later these authors also showed that trypsin could catalyze the condensation reaction[106]. In this second study the authors used a 1,1-dimethyl-1-sila-2-oxacyclohexane as alkoxysilane. They observed that after 3 hours, the alkoxysilane was almost completely hydrolyzed and had already begun to condense into hydroxybutlydimethylsilanol. In this reaction trypsin yielded 81 % for the hydrolysis and 71 % for the condensation reaction. These results are another good example of the application of serine hydrolases in the development of biocatalytic solutions.

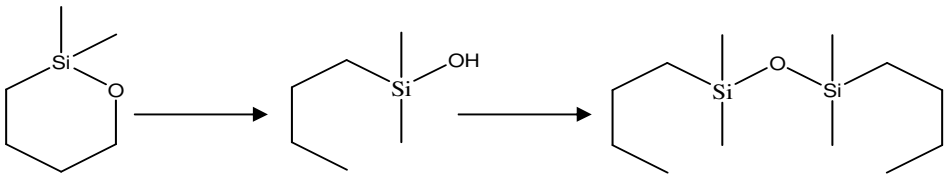

Figure 3.10 – Schematic representation of hydrolysis and condensation of a 1,1-dimethyl-1-sila-2-oxacyclohexane catalyse by a serine hydrolase[107,].

The hydrolysis and condensation of sol-gel precursors can also be achieved with the same polypeptides mentioned earlier. One of the most studied polypeptides is silafin extracted from the diatom *Cylindrotecha fusiformis*[98,99,103-105].

This peptide was found to have an important role on silica deposition and on diatoms cell wall shaping, acting as a stabilizer for Si-O-Si bonding[99]. The amine groups present on the silafin chain could hydrogen bond to with silicic acid and in this way facilitate the condensation between two adjacent silica molecules. It was found that when this polypeptide was added to silicic acid solutions, it led to rapid precipitation of silica and was simultaneously incorporated into the formed biomineral[99-100]. The shape of silica that precipitates from these solutions could be changed by the addition of poly-lysines, which were also found in the cell wall of these organisms[99]. This elegant lesson of biomineralization inspired the research into bio- and nanotecnological silica formation.

Silafin A1 was used to catalyze the hydrolysis of a mixture of two sol-gel precursors for the immobilization of butyrylcholinesterase[88]. The authors achieved 20 % of enzyme loading and found that the butyrylcholinesterase entrapped during the precipitation of the silica nanospheres retained all of its activity. The stability of this immobilized preparation was reasonably good, especially when the reaction was accomplished in a flow-trough reactor.

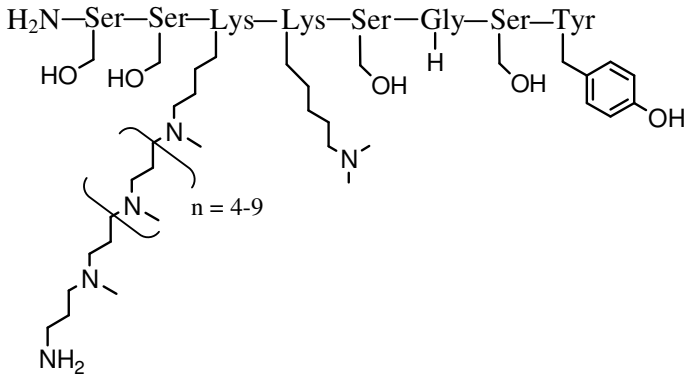

Figure 3.11 – Schematic representation of a silafin.[98,99,103-105.]

Perhaps the most interesting examples of sol-gel bioprocessing come from the immobilization of living organisms. The aim of entrapping life inside sol-gel structures is related with the advantages of using living organisms for bioprocessing. This can provide solutions for old problems, but especially presents new research lines to explore. Using sol-gel to immobilize enzymes, even the more complex and sensible enzymes, is a very simple task when compared to the immobilization of living organisms. The problem is not with the process itself, since it is always possible to play with experimental conditions, avoiding in this way the use or the formation of undesirable compounds. The question is rather how to maintain the organism viable for the desirable application. This is a difficult issue even for bacteria, which are typically considered the most resistant living organisms. Cells have their proper dynamics and small changes in their environment can have a dramatic impact on their viability. Nevertheless it has been possible to create microenvironments based on sol-gel that were capable of maintaining the viability of those organisms.

The pioneers of whole cell immobilization were Carturan and co-workers[110-115], who first immobilized *Saccharomyces cerevisiae* into sol-gel silicates. This microorganism is capable of performing other type of conversions, but a most interesting one is the millenary conversion of sugars to alcohols and CO_2. The high tolerance of this microorganism towards alcohols made it the perfect candidate for first organism to be immobilized in sol-gel. Main issues in this study were maintaining the organism viable during the process and, most importantly, after it. The most interesting and perhaps

surprising aspect of this work was that the immobilized yeast was not only viable, but also improved its specific activity when compared with free yeast. This was in fact even more remarkable since it remained so over time. This result was a very important achievement since yeast is a technological microorganism. For instance Moët et Chandon have several patents on yeast immobilization onto several different materials. Their interest is related with the fermentation process, which in the case of champagne occurs inside a bottle. After the fermentation, the yeast has to be separated from the liquid phase. With immobilization, the process keeps to its main and traditional concept, but the immobilized enzyme is much easier to separate.

The pioneering work of Carturan and co-workers led to the development of more compatible procedures for the encapsulation of cells into silicates. The fact that sol-gel is a low temperature process is not enough to obtain top quality preparations, since the processing conditions are far from the ideal ones for living organisms. The main problems associated with this technique are pH conditions, the alcohol toxicity, and the shrinkage during the drying stage which induces excessive stress on the immobilized cells[109,116]. Different approaches have been applied to deal with these problems. For instance to control the negative impact of the alcohol, many solutions were presented. Most of them were actually very simple since they consisted on the rapid elimination of the alcohol from the preparation. In this way Ferrer *et* al.[117] showed that controlled vacuum evaporation of the alcohol from the colloidal solution prior to encapsulation helped to preserve the *E.coli* cells integrity. Similar results were obtained by dipping the immobilized preparation into a buffer solution or even into a culture media. This strategy aimed at the dilution of the alcohol, which allowed cells to maintain a higher viability[118].

Changing the type of precursor also proved to be adequate to improve the stability of immobilized cells[119-121]. In this case, instead of using methoxysilanes as precursors, some authors replaced them with polyol-based silanes, whose hydrolyzable group contains a longer chain alcohol in place of the traditional methanol and ethanol. One of these types of precursors had a glycerol as substituent, which is more biocompatible. The results obtained with this precursor were quite interesting since cell viability was maintained during a considerable period[119-121]. An alternative approach was using sodium silicate or colloidal silica to form silica structured materials. This is a quite

popular approach in spite of the very low pH which obviously has to be neutralized before adding the cells[122-123].

One major issue related with cell long term stability is the stress that cells suffer inside silica matrices. A good solution to overcome this problem was combing silica matrices with organic polymers, creating in this way silicate-organic hybrids. A very good example of this approach is the work developed by Livage, Coradin and co-workers[109,124-126]. These authors have presented a strategy that involves a cell pre-immobilization step in perfectly biocompatible materials such as calcium alginate or gelatine. These immobilizates are then covered with a thin film of silica. This technique has the aim of combining the better of two worlds: on one side the excellent biocompatibility of organic polymers, and on the other the mechanical stability of silica materials. This immobilization strategy had a higher impact when animal cells were used. In this case the aim was related with the development of scaffolds for tissue engineering treatments. One of the best examples was provided by Ren and his co-workers[127] who used a gelatine-siloxane scaffold for encapsulating osteoblastic cells. These authors observed that cell proliferation occurred without any cytotoxic effects.

Carturan and co-workers[113-115] introduced a different concept for cell immobilization using the sol-gel process. In order to eliminate cellular stress during the encapsulation, the cells were deposited on a solid support which could be a flat glass, a membrane, etc. Then a stream of nitrogen containing a mixture of sol-gel precursors was sprayed over the film. The more interesting fact of this design is that the water required for the hydrolysis of the sol-gel precursors came from the humidity on the cell surface. This technique considerably reduces the stress usually associated to the ageing and drying of the material. In this case, and since there is no liquid in excess, the matrix will not shrink and the excess of pressure over the cell can be avoided. This process was then extended to hybrid organic-slicate immobilizates and collagen matrices containing viable cells, where similar positive results were obtained[115].

Other biotechnological applications of sol-gel processing include the immobilization of enzymes and whole cells for biosensing. Microorganisms are excellent tools for modern biosensing, not only because of the type of metabolic sensing mechanisms that are naturally present on them, but also because they can be engineered towards a specific

function. The combination of animal cells with sol-gel offers very reliable solutions for drug delivery which, together with tissue engineering and cell therapy, are mainstream applications of sol-gel materials. For instance artificial organs have been created, such as an artificial pancreas using immobilized islet cells[128-130]. Also using this type of cells, which are insulin producers, transplanted to glycemic rats, Carturan and co-workers[131] were able to control the levels of blood glucose. This was a remarkable result since the authors were able to maintain the rats alive for a period of 2 months. Many of these accomplishments should reach commercialization in the future. Only in the case of enzymes is the sol-gel process an already established technology, with commercial and industrial applications. For instance Sigma-Aldrich sells sol-gel immobilized preparations of several enzymes.

In the work that follows we focused our attention on the immobilization of cutinase in organically modified silicas, which are hybrid materials. Our aim was to develop microenvironments that were favorable to cutinase and potentiated its catalytic performance. In the first part of the work, several possibilities were considered to improve cutinase activity and stability, via changes in enzyme microenvironment. The latter was modulated by changing the type of sol-gel precursors and using additives. We found that cutinase activity was improved in the presence of several types of additives. The second and third parts of this work describe our attempts to rationalize these results: one is focused on the enzyme, and the other is focused on the material. Both approaches contributed to explain our results.

The next section will be divided in three parts. Before part II and part III, a small introduction to the spectroscopic techniques used in the characterization of both enzyme and material will be given.

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CHAPTER III

Experimental Section

PART I.

Cutinase immobilization

Sol-gel encapsulation: an efficient and versatile immobilization technique for cutinase in nonaqueous media

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ABSTRACT

Cutinase from *Fusarium solani pisi* was encapsulated in sol-gel matrices prepared with a combination of alkyl-alkoxysilane precursors of different chain-lengths. The specific activity of cutinase in a model transesterification reaction at fixed water activity in n-hexane was highest for the precursor combination tetramethoxysilane/n-butyltrimetoxysilane (TMOS/BTMS) in a 1:5 ratio, lower and higher chain lengths of the mono-alkylated precursor or decreasing proportions of the latter relative to TMOS leading to lower enzyme activity. Results obtained using combinations of three precursors confirmed the beneficial effect of the presence of BTMS in the preparations.

Scanning electron microscopy of the 1:5 TMOS/n-alkylTMS gels showed a direct correlation between the macropore dimensions and the alkyl chain length of the alkylated precursor and revealed that TMOS/n-octylTMS gels suffered extensive pore collapse during the drying process. The specific activity of TMOS/BTMS sol-gel entrapped cutinase was similar to that exhibited by the enzyme immobilized by adsorption on zeolite NaY. However, the incorporation of different additives (zeolites, silica, Biogel, grinded sol-gel, etc) having in common the capability to react with residual silanol groups of the sol-gel matrix brought about remarkable enhancements of cutinase activity, despite the fact that the global porosity of the gels did not change.

The behavior of the gels in supercritical CO_2 (sc-CO_2) paralleled that exhibited in n-hexane, although cutinase activity was c.a. one order of magnitude lower (i.e. sol-gel encapsulation did not prevent the deleterious effect of CO_2). The impact that functionalization of some of the additives had on cutinase activity indicates that the enzyme/matrix interactions must play an important role. Some of the best additives from the standpoint of enzyme activity were also the best from the standpoint of its operational stability (c.a. 80 % retention of enzyme activity at the tenth reutilization cycle). None of the additives that proved effective for cutinase could improve the catalytic activity of sol-gel encapsulated *Pseudomonas cepacia* lipase.

INTRODUCTION

Immobilization has long been used to improve enzyme activity and stability in aqueous media, and, more recently, also in nonaqueous media (Bickerstaff, 1997; Bornscheuer, 2003), once it became clear that in those media enzymes can catalyze reactions that are difficult to perform in water, become more stable and exhibit altered selectivity (Klibanov, 2001; Krishna, 2002). A wide variety of immobilization techniques can be used, including adsorption onto solid supports, covalent attachment or entrapment in silica matrices. Adsorption techniques are usually very easy to apply, but the bonding of the biocatalyst to the surface of the solid support is relatively weak. This usually leads to the leaching of the enzyme, even in nonaqueous media, in which the enzyme is not soluble. Covalent attachment normally leads to improved enzyme stability, often at the cost of partial deactivation due to the conformational restrictions imposed by the covalent bonding of enzyme residues to the support.

The incorporation of enzymes in silica matrices has proved to be a good strategy to improve the catalytic efficiency of enzymes. This is essentially accomplished by using the sol-gel technology (Avnir et al., 1994; Gill, 2001; Reetz et al., 2003). The aqueous character of sol-gel processing and the fact that the synthesis of the ceramic materials can be conducted at room temperature ensure that sufficiently mild conditions prevail, as required for biomolecules to retain their native structure, dynamics and function. The process allows for high yields of enzyme immobilization. The framework rigidity of sol-gel polymers prevents the leaching of the entrapped enzyme while also stabilizing its structure. The mesoporous structure and high pore volume of these gels allow the diffusion of low to medium molecular weight species and their free interaction with the enzyme (Gill, 2001).

A well-established sol-gel processing technique consists in hydrolyzing adequate precursors in aqueous solutions to produce soluble hydroxylated monomers, followed by polymerization and phase separation to produce a hydrated metal or semi-metal oxide hydrogel (Brinker and Scherer, 1990). Removal of water from the wet gel, which is usually accompanied by changes in the structure of the pores and of the gel's network, results in a porous xerogel. The most widely used precursors are alkyl-alkoxysilanes. These precursors were used already in the mid-1980s to prepare

organically modified silicates (Ormosils) for the successful encapsulation of antibodies and enzymes (Glad et al., 1985; Venton et al., 1984). Although the final structure of the material is basically determined by the differences in chain length, functionality and hydrophobic character of the precursors, it can be tailored via the addition of a wide range of molecules. Examples include surfactants (Chevalier, 2002; Coradin and Lopez, 2003; Reetz et al., 2003), room-temperature ionic liquids (Zhou et al., 2004), crown ethers, β-cyclodextrins or porous solid supports like Celite (Reetz et al., 2003). The commonly used catalysts are weak acids or bases (Brinker and Scherer, 1990; Reetz et al., 1996). Recent research describes the use of other species that include peptides like silaffins (Luckarift et al., 2004), polyamines (Sun et al., 2004) or enzymes such as hydrolases (Bassindale et al., 2003) and silicateins (Cha et al., 1999).

The versatility of the sol-gel process that makes possible the generation of materials with a wide range of inner surface chemistries is particularly suited to the immobilization of enzymes. In fact, enzymes differ in structure and function and it is not conceivable that a generic approach adequate for all enzymes may ever be established. Instead, the immobilization technique must be adjusted so as to offer the enzyme an optimum catalytic environment with favorable enzyme/matrix interactions, and an adequate balance between molecular restraint and conformational mobility, as required for efficient catalysis. The potential of the sol-gel technique for tuning the catalytic environment is well illustrated by the use of an enzyme transition-state analogue as a molecular imprint molecule to generate enantioselective silica particles (Markowitz et al., 2000).

Here we report on sol-gel preparations for the encapsulation of cutinase from *Fusarium solani pisi*. Cutinase is an extracellular enzyme involved in the degradation of cutin, the cuticular polymer of higher plants (Carvalho et al., 1999). It belongs to the group of hydrolases, the enzymes most employed for industrial biotransformations (Chikuza et al., 2003). In particular, it is a serine hydrolase, a relatively small globular protein of approximately 45x30x30 $Å^3$ size, with 197 residues and a molecular mass of 22 kD (Carvalho et al., 1999). Unlike most lipases, cutinase has no lid covering its active site that is thus accessible to the solvent. Also, the oxyanion hole that helps stabilize intermediates during the catalytic mechanism of lipases, and which is formed upon substrate binding, is preformed in cutinase. These two facts are thought to explain the

lack of interfacial activation observed with cutinase (Carvalho et al., 1999). When it exists, interfacial activation is manifested in a pronounced activity increase upon substrate aggregation (Mingarro et al., 1995). Enzyme interactions with these aggregates lead to conformational changes of the lid that result in an open, substrate-accessible form of the enzyme, and to the formation of the oxyanion hole. This phenomenon is found with most lipases.

Cutinase is a versatile enzyme that catalyzes synthetic and hydrolytic reactions on a wide range of substrates in aqueous and nonaqueous media (Carvalho et al., 1999). The latter include supercritical fluids (sc-fluids, any substance above its critical temperature and pressure) (Garcia et al., 2004a; Garcia et al., 2004b). The use of sc-fluids that can be easily eliminated through venting and leave no residues can be a good strategy to meet the requirements of environmentally friendly processes (DeSimone, 2002). Although sc-fluids are still regarded as unconventional, their unique characteristics make for a steadily increasing number of applications in many areas, including biocatalysis (Mesiano et al., 1999). Sc-CO_2 is the most popular sc-fluid but it has the ability to acidify the microenvironment of the enzyme (Beckman, 2004), even at very low water activity (a_W) values (Harper and Barreiros, 2002). Sc-CO_2 has also been reported to form carbamates with free amine groups of lysine residues on some enzymes, thereby leading to poor enzyme performance (Mesiano et al., 1999). Cutinase is one of the enzymes shown to perform markedly worse in sc-CO_2 than in other nonaqueous media (Garcia et al., 2004a; Garcia et al., 2004b).

In the present work we study the impact that encapsulation of cutinase in sol-gel matrices has on the enzyme activity and stability. We also look at the performance of the sol-gel preparations in sc-CO_2. The precursors used were alkyl-alkoxysilanes of different chain-lengths, the catalyst was a weak base, and several less-common additives were incorporated during the sol-gel process. Additionally, we look at how the same approach affects the catalytic activity of a different enzyme, *Pseudomonas cepacia* lipase (PCL).

MATERIALS & METHODS

Materials

Fusarium solani pisi cutinase was produced by an *Escherichia coli* WK-6 (a gift from Corvas International, Ghent, Belgium) and purified at Centro de Engenharia Biológica e Química, Instituto Superior Técnico (Carvalho et al., 1999; Lauwereys et al., 1990). The enzyme purity was controlled by electrophoresis and isoelectric focusing. The estereolytic activity of the enzyme (30 nM) was determined spectrophotometrically by following the hydrolysis of p-nitrophenyl butyrate (0.56 mM) at 400 nm in a 50 mM potassium phosphate buffer at pH = 7.0. *Pseudomonas cepacia* lipase (PCL) was from Amano (Nagoya, Japan). *(R,S)*-2-phenyl-1-propanol (97 % purity), *(R)*- and *(S)*-2-phenyl-1-propanol (98 % purity), tetramethoxysilane (TMOS), methyltrimetoxysilane (MTMS), propyltrimetoxysilane (PTMS), 3-aminopropyltrimetoxysilane (3APTMS), n-octyltrimetoxysilane (OCTMS), zeolite NaA and zeolite NaY molecular sieves (in powder form) were from Aldrich, n-butyltrimetoxysilane (BTMS) was from Polysciences Inc., vinyl butyrate (99 % purity) was from Fluka, Biogel PA6 was from BIORAD, silica (silica gel, 60 M) was from Macherey-Nagel, n-hexane, tridecane, potassium acetate, sodium fluoride and sodium chloride were from Merck, Hydranal Coulomat A and C Karl-Fischer reagents were from Riedel de Häen, polyvinyl alcohol (PVA; MW 15.000) was from Sigma, Krytox 157FSL (perfluoropolyether, PFPE, MW ≈ 2500) was from Dupont. *(R,S)*-2-phenyl-1-propyl butyrate was prepared as previously indicated (Vidinha et al., 2004). CO_2 and nitrogen were supplied by Air Liquide and guaranteed to have purities of over 99.995 mol %.

Additive modification

Zeolite NaA – 3APTMS and zeolite NaA – PTMS: 1 mL of sol-gel precursor was solubilized in 1.5 mL of dichloromethane. This solution was added to 50 mg of NaA zeolite powder, and the slurry obtained was stirred for 24 h at room temperature. The modified zeolite was washed with dichloromethane and dried under vacuum. This procedure was adapted from Mukhopadhyay et al. (2003). Krytox-NH_4: 5 g of Krytox were added to 25 mL of ammonia (24 % w/w) and the slurry obtained was stirred until a solid formed (after about 24 h). The solid (PFPE COO^- NH_4^+, henceforth referred to as

Krytox-NH_4) was then washed abundantly with water and dried under vacuum for 24 h. This procedure was adapted from Holmes et al. (1999).

Cutinase immobilization on zeolites

Cutinase was immobilized by deposition (Gonçalves et al., 1996; Serralha et al., 1998). The lyophilized enzyme was dissolved in a 50 mM sodium phosphate buffer solution (10 mg mL^{-1} of enzyme) at pH 8.5. The support was added to the solution (25 mg of cutinase per g of support) and after vortex mixing for 1 min, the preparation was dried under vacuum for at least 24 h. The average yield of immobilization was (51 ± 8) % for zeolite NaA and (72 ± 12) % for zeolite NaY, as determined by a modified Lowry method (Lowry et al., 1951).

Cutinase immobilization in sol-gel

This technique was adapted from that referred by Reetz et al. (1996). 1 mg of lyophilized cutinase was dissolved in an aqueous solution containing NaF (0.20 g L^{-1}) and PVA (4.64 g L^{-1}). The amount of water in this solution was fixed (6.86 mmol). The solution was vigorously shaken on a vortex mixer. The precursors were then added in amounts that yielded a water/silane molar ratio (R) of 8 irrespective of the type and number of precursors used (e.g. 0.142 mmol of TMOS and 0.712 mmol BTMS in 1:5 TMOS/BTMS gels). The mixture was again vigorously shaken on the vortex mixer, until it became homogeneous. It was then placed in an ice bath until gelation occurred (after a few seconds), and kept in the ice bath for an additional 10 minutes. The container with the obtained gel was kept at 4 ºC for 24 h, after which the gel was air-dried at 35 ºC for 24 h. The white gel obtained was crushed and washed (for about 10 min)/centrifuged (at 5400 rpm), first using phosphate buffer (50 mM, pH 7, 2 mL), then acetone and finally n-pentane (also 2 mL of each).

The gel was left at room temperature for 16 hours, after which it was equilibrated with a saturated salt solution at 25 ºC for about 3 days, to achieve the values a_W = 0.75 (sodium chloride) or a_W = 0.22 (potassium acetate), taken from the literature (Greenspan, 1977). When used, additives (4 mg additive/mg protein) were added to the NaF/PVA solution. The average yield of immobilization of cutinase in the sol-gel matrix was (91

± 8) %, as determined by the Lowry method (Lowry et al., 1951). This determination was based on the amount of enzyme found in the aqueous buffer used for washing the gel. Average enzyme loading in 1:5 TMOS/n-alkylTMS gels: 1.6 % for TMOS/MTMS, 1.4 % for TMOS/PTMS and TMOS/BTMS, 0.9 % for TMOS/OCTMS. Assays performed with crushed gels (average particle size of 1.5 mm; 2.5 mm maximum, 0.7 mm minimum) and grinded gels (average particle size of 0.3 mm; 0.8 mm maximum, 0.2 mm minimum) yielded comparable initial reaction rates (tests carried out with TMOS/BTMS gels without additive, with added zeolite NaY and with added Biogel, with TMOS/OCTMS gels without additive, and with TMOS/PTMS gels without additive).

PCL immobilization in sol-gel

This technique was also adapted from that referred by Reetz et al. (1996). The commercial powder enzyme was suspended in water and centrifuged (at 5400 rpm for 10 minutes). The supernatant was used for immobilization experiments and for the determination of protein content (0.085 ± 0.005 mg mL^{-1}, using the Lowry method (Lowry et al., 1951)). This solution was added to the NaF/PVA solution, and the following steps were the same as those referred for cutinase. The average yield of immobilization of PCL in the sol-gel was (92 ± 7) %. Average protein loading in 1:5 TMOS/n-alkylTMS gels: 0.25 % for TMOS/MTMS, 0.18 % for TMOS/PTMS, 0.14 % for TMOS/BTMS, 0.09 % for TMOS/OCTMS.

Enzyme activity assays

For reactions in sc-fluids, variable volume stainless steel cells (reaction mixture volume of about 12 mL for most experiments) equipped with a sapphire window, loading and sampling valves, were used. Details of the high pressure apparatus and experimental technique have been given elsewhere (Fontes et al., 2001). Reactions in n-hexane were performed in glass vials (reaction volume of 750 μL) placed in a constant temperature orbital shaker set for 450 rpm. With the exception of sc-CO_2, all the reaction mixture components were pre-equilibrated to a fixed a_W for about 3 days. The reaction studied was the transesterification of vinyl butyrate (300 mM in n-hexane; 170 mM in sc-CO_2) by *(R,S)*-2-phenyl-1-propanol (100 mM in n-hexane; 60 mM in sc-CO_2) (Scheme 1).

The concentration of sol-gel encapsulated enzyme was 6 g L^{-1} in n-hexane and 2 g L^{-1} in sc-CO_2. The concentration of cutinase adsorbed on zeolite NaY was 6 g L^{-1} in n-hexane and 4 g L^{-1} in sc-CO_2. Assays in sc-CO_2 were performed at 100 bar. Vinyl butyrate addition marked the start of the reaction. Tridecane (15.4 mM) was used as external standard for GC analysis. Water concentration was measured by Karl-Fisher titration. In sc-fluids, a_W values were calculated dividing the water concentration in the reaction mixture by the water concentration in the same mixture at saturation (Fontes et al., 2002).

Analysis

The reaction conversion was measured by GC analysis performed with a Trace 2000 Series Unicam gas chromatograph. Column: 30 m x 0.32 mm i.d. home-made fused silica capillary column coated with a 0.64 µm thickness film of 30 % heptakis-(2,3-di-O-methyl-6-O-*tert*-butyldimethylsilyl)-ß-cyclodextrin in SE 52 (*DiMe)*. Oven temperature program: 125 ºC for 2 min, 125-171 ºC ramp at 6 ºC min^{-1}, 200 ºC for 3 min. Injection temperature: 250 ºC. Flame ionization detection (FID) temperature: 250 ºC. Carrier gas: helium (2.0 cm^3 min^{-1}). Split ratio: 1:20. No products were detected in assays carried out without enzyme. The initial rates given (per mg of protein) are the average of at least two measurements.

Matrices characterization

The porosity of the dried gels (before grinding and washing) was assessed with a dry-powder pycnometer (GeoPyc 1360, Micromeritics), using a consolidation force of 50 N for 17 measurement cycles. The samples were outgassed at room temperature prior to weighing. The fractured surfaces of the gels were examined with a DSM962 Zyce scanning electron microscope (SEM), operating between 3 and 10 kV. To avoid charging effects during observation, the surfaces were previously sputter-coated with a gold layer.

RESULTS AND DISCUSSION

The type and combination of silica precursors had a great influence on cutinase activity (Figure 1). When only TMOS was used, no enzyme activity was observed. When TMOS was combined with a similar mono-alkylated precursor, the enzyme activity increased with increasing alkyl chain up to a certain point, decreasing from there on: for the TMOS/OCTMS combination, the cutinase activity is clearly reduced. There is also a clear increase in the enzyme activity by increasing the proportion of alkylated precursors relative to TMOS, as can be observed for the pairs TMOS/BTMS and TMOS/OCTMS containing different relative proportions of TMOS.

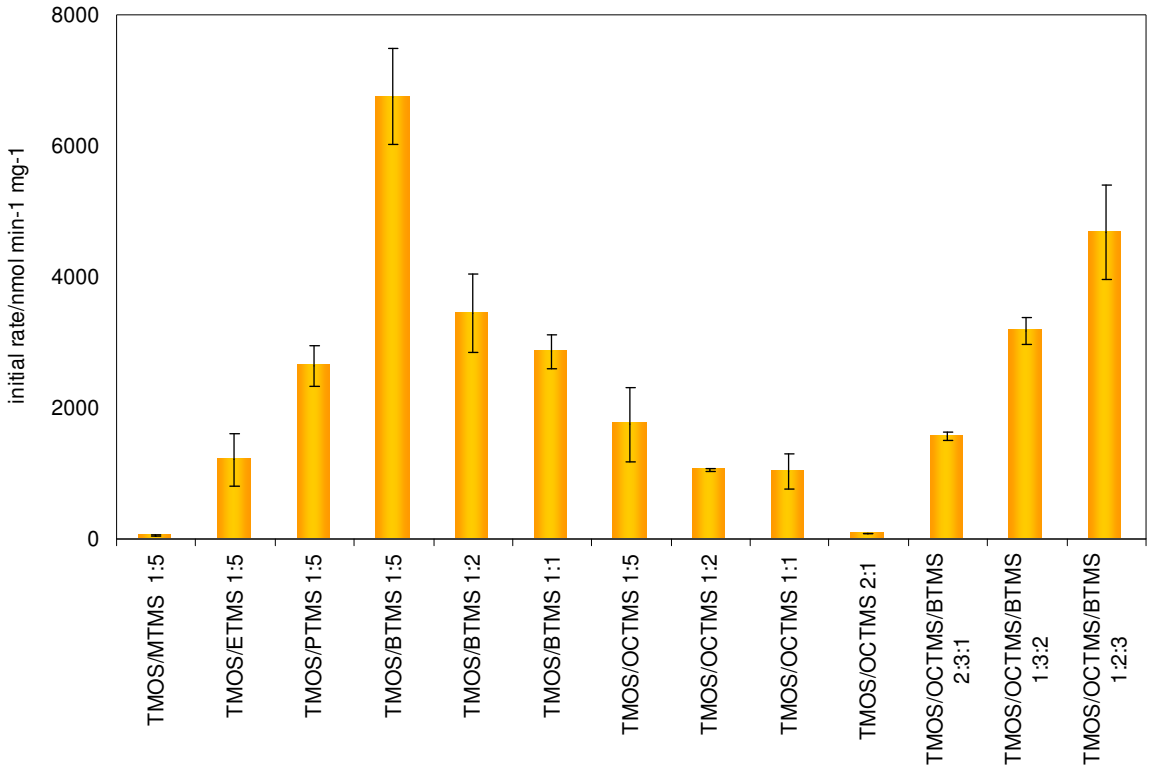

Figure 1 – Effect of precursor combination on the catalytic activity of cutinase in n-hexane at a_W = 0.7 and T = 35 ºC. Precursors used: tetramethoxysilane (TMOS) and n-alkyltrimetoxysilane (n-alkylTMS; M = methyl, P = propyl, B = n-butyl, OC = n-octyl). The reaction studied was the transesterification of vinyl butyrate by *(R,S)*-2-phenyl-1-propanol.

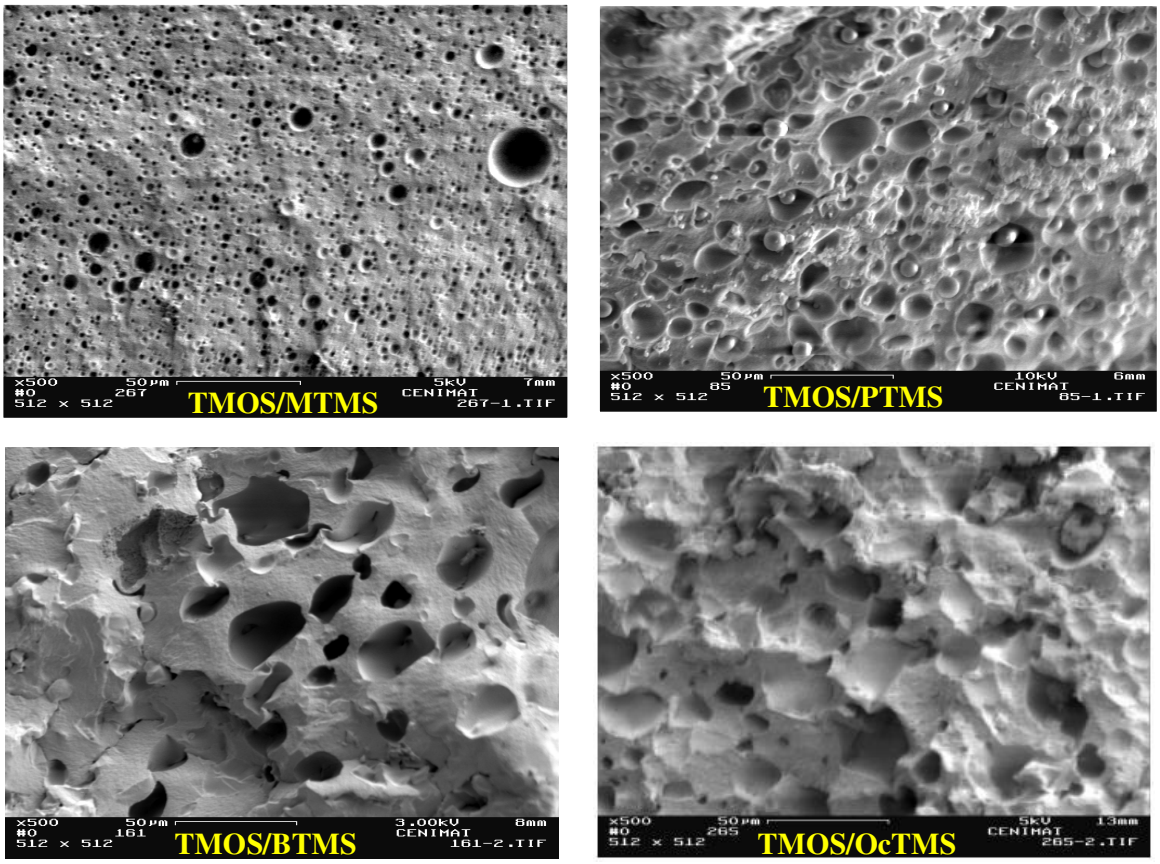

Figure 2 – SEM micrographs of the sol-gel matrices (1:5 TMOS/n-alkylTMS) with encapsulated cutinase. The bar represents 50 µm.

The SEM micrographs for the gels prepared with a TMOS/n-alkylTMS ratio of 1:5 (Figure 2) show a direct correlation between the macropore dimensions and the alkyl chain length of the alkylated precursor. The micrograph for TMOS/OCTMS evidences partial collapse of the pore structure during the drying process. The extent of shrinking during this process is determined by the bulk modulus of the matrix (Fidalgo et al., 2003). Combinations containing alkylated precursors with a higher alkyl chain length, such as OCTMS, are expected to yield more highly stretched matrices, with lower mechanical strength. Therefore, the critical drying point (which corresponds to the maximum capillary pressure) is reached after considerable shrinking, leading to extensive pore collapse. Thus, cutinase activity seems to increase with increasing alkyl chain length of the precursor up to the point where the matrix formed lacks the strength to withstand the drying process.

Reetz et al. (1995) have used the same precursors as in the present study to encapsulate a lipase from *Pseudomonas cepacia* (PCL). When using a TMOS/n-alkylTMS ratio of 1:1, they observed an increase in enzyme activity with the increase in alkyl chain length of the alkylated precursor added to TMOS, up to n = 18. The sharp increase in enzyme activity occurred up to n = 4, further increases in chain length bringing about relatively small enhancements in that property. The authors hypothesized that in more hydrophilic gels the lipase might still have to undergo the conformational changes associated with the movement of the lid covering the enzyme active site, as opposed to being entrapped in the active conformation (Reetz at al, 1996). Kunkova et al. (2003) also correlated increased lipase activity via the opening of the lid with hydrophobic interactions between the enzyme and the alkyl groups of the sol-gel matrix.

As mentioned earlier, cutinase has no lid covering its active site and this argument does not apply. However, the enzyme activity responds to its microenvironment and to the interactions with the solid matrix, which may explain the activity increase with the alkyl chain length up to n ≈ 4 observed in this work. Also shown in Figure 1 are the results obtained for combinations of three precursors. By comparison with those obtained by using two precursors in corresponding proportions, it seems clear that when the third one has a long alkyl chain (e.g. OCTMS) its effect on the enzyme activity is negligible. However, when it has a shorter alkyl chain, such as BTMS, it results in a significant enhancement of the cutinase activity. This suggests again that the presence of BTMS and the increase in its proportion relative to TMOS give the best compromise between structural integrity of the material and cutinase/matrix interactions, thus yielding a higher enzyme activity.

To try and improve cutinase activity, and also to gain a better understanding of the nature of the matrix/enzyme interactions, we incorporated additives in the 1:5 TMOS/n-alkylTMS matrices during the sol-gel process (Figure 3). Included in this figure are results for cutinase adsorbed on zeolite NaY. This immobilization technique constitutes an already existing efficient method for cutinase (Gonçalves et al., 1996; Serralha et al., 1998) and seems appropriate as a reference (Persson et al., 2002). To the best of our knowledge, none of the additives used in the present study have been used before to alter the catalytic activity of enzymes entrapped in sol-gel.

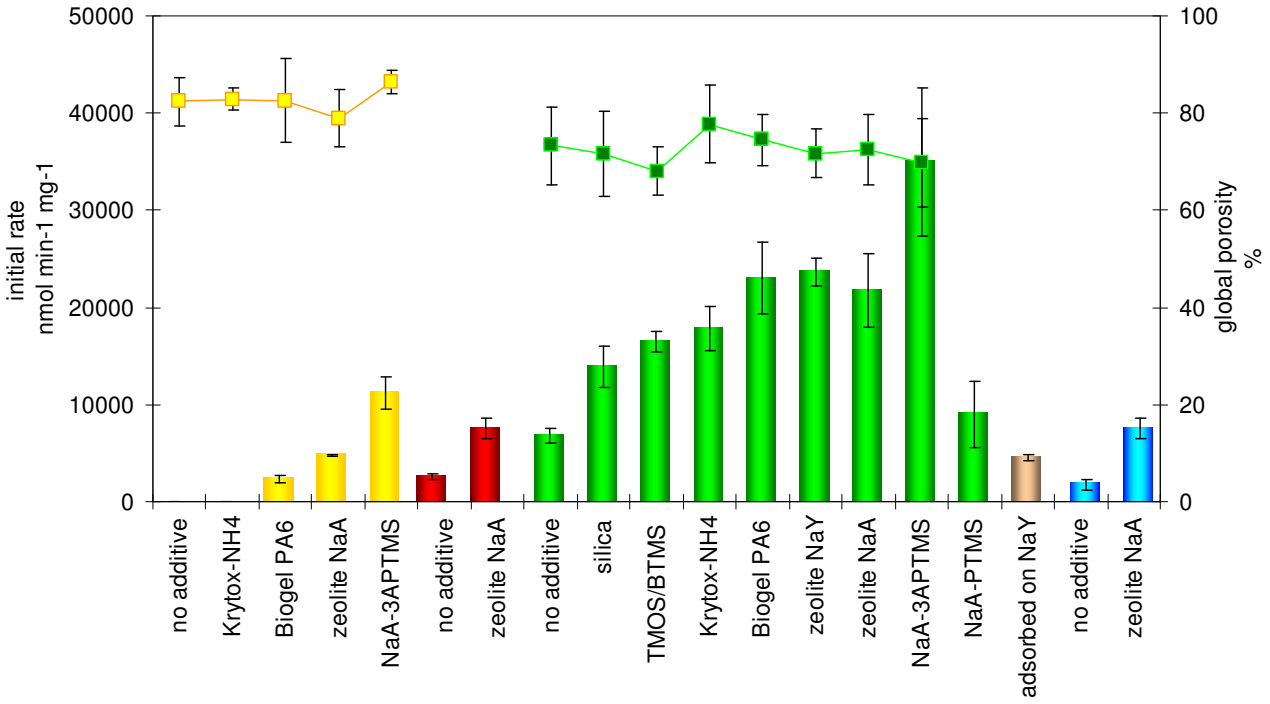

Figure 3 – Effect of additives on the catalytic activity of cutinase encapsulated in 1:5 TMOS/X sol-gel and adsorbed at the surface of zeolite NaY (bars, left axis) and on the global porosity of the dried gels (solid lines, right axis), in n-hexane at a_W = 0.7 and T = 35 ºC. X = MTMS (yellow bars), PTMS (red bars), BTMS (green bars), OCTMS (blue bars). The assays with TMOS/MTMS gels with no additive or with the additive Krytox-NH_4 yielded initial rates below 100 nmol min^{-1} mg^{-1}. The reaction studied was the transesterification of vinyl butyrate by *(R,S)*-2-phenyl-1-propanol.

The common characteristic to the additives used is their capability to react with residual silanol groups of the sol-gel matrix. The striking feature of Figure 3 is the positive impact that such different additives have on enzyme activity, although the global porosity is very similar for all the samples prepared. This suggests that the structural characteristics of the matrix cannot be the only reason for the observed differences in cutinase activity. Again, enzyme/matrix interactions probably play a major role. By reacting with silanol groups, the additives decrease the number of those available for binding to residues on the enzyme, thus reducing constraints on enzyme conformational mobility. This seems to be the primary effect of the additives, which explains why so many different additives had a positive impact on enzyme activity.

This argument also explains why the sol-gel matrix itself, when used as additive, can enhance enzyme activity (e.g using a TMOS/BTMS grinded gel as additive to a TMOS/BTMS sol). It further elucidates the results obtained when using zeolite NaA functionalized with the precursor PTMS (NaA-PTMS) as additive. This functionalization yields additives capable of reacting with silanol groups to a lesser extent than pure zeolite NaA, and enzyme activity in this case was very close to that exhibited by the blank. But the changes imposed on the microenvironment of the enzyme by the additive are also seen to be relevant. A good example is the case of zeolite NaA functionalized with APTMS (NaA-3APTMS). Compared to zeolite NaA, NaA-3APTMS differs mostly in the surface charge of the zeolite, and this has led to a significant increase in cutinase activity for both TMOS/MTMS and TMOS/BTMS. Overall, our results highlight the potential of additives for modulating the activity of enzymes encapsulated in sol-gel matrices.

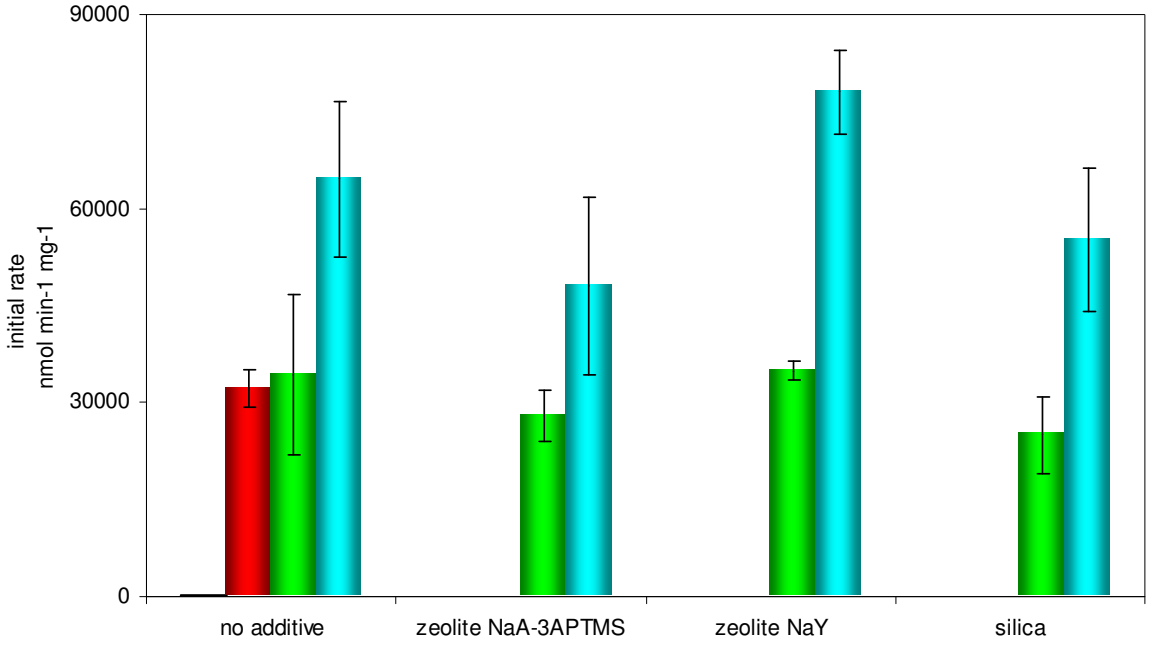

Figure 4 – Effect of precursor combination on the catalytic activity of *Pseudomonas cepacia* lipase (PCL) encapsulated in 1:5 TMOS/X sol-gel and effect of additives on TMOS/BTMS and TMOS/OCTMS gels, in n-hexane at a_W = 0.7 and T = 35 ºC. Code for bars as in Figure 3. The reaction studied was the transesterification of vinyl butyrate by *(R,S)*-2-phenyl-1-propanol.

To see how a different enzyme would respond to this approach, we used PCL (Figure 4). The results for the blanks do not exactly follow the pattern reported by Reetz et al. (1995) for this enzyme, probably because the precursor ratio used in this work was different (1:5 versus 1:1). In both studies the higher activity was observed for TMOS/OCTMS, the precursor combination with the highest hydrophobicity. However, in the present study, TMOS/PTMS and TMOS/BTMS yielded very similar results and the combination TMOS/OCTMS offered a significant improvement in enzyme activity relative to TMOS/BTMS. The use of additives was not successful in increasing the enzyme activity in this case. Since the dynamics required for efficient catalysis depend on the enzyme, all the factors that interfere with the conformational mobility of the enzyme can have impact on its catalytic ability. The fact that, contrarily to cutinase, PCL has a lid covering the active site may also contribute to the observed responses of the two enzymes to additives, and supports the notion that the choice of additive must be tailored to fit the enzyme (Reetz et al., 2003).

Table 1 - Stability of cutinase encapsulated in 1:5 TMOS/BTMS sol-gel preparations, as measured by the percentage of enzyme activity retention in n-hexane at the tenth reutilization cycle (a_W = 0.7; T = 35 ºC). The reaction studied was the transesterification of vinyl butyrate by *(R,S)*-2-phenyl-1-propanol.

additive	% activity
-	64
zeolite NaA	41
zeolite NaA-3APTMS	50
zeolite NaY	75
Biogel PA6	87

Enzyme stability is a key enzymatic property that can be improved using the sol-gel technology (Gill et al., 1998; Park and Clark, 2002; Reetz et al., 2003). In the present study, cutinase stability was assessed in the gels that yielded highest activity. The measure of stability was the retention of enzyme activity at the tenth reutilization cycle (Table 1). The harshness of the manipulations endured by the enzyme between the

moment it is recovered from the medium and the moment it begins the next cycle is considerable but should be about the same for all the preparations. Therefore, the results must reflect the interactions established between the enzyme and the sol-gel matrix.

Overall, the additives Biogel PA6 and zeolite NaY appear to be the best compromise for cutinase from the standpoint of enzyme activity and stability. We note that when cutinase was adsorbed on zeolite NaY, total loss of activity was observed after six cycles, a number that fell down to two when zeolite NaA was used instead. However, in this case the results mostly reflect the leaching of the protein, both during reaction and during the treatment involved in the reutilization process, since the enzyme is not caged as when it is encapsulated in the sol-gel. The extent of leaching during reaction in nonaqueous media is higher for zeolite NaA than for zeolite NaY, although values for the two supports become similar at higher a_W (Vidinha et al., 2004). This and the fact that the average yield of cutinase immobilization onto zeolite NaY is higher than on zeolite NaA (Vidinha et al., 2004; Gonçalves et al., 1996; Serralha et al., 1998) reflect a stronger interaction between the enzyme and zeolite NaY.

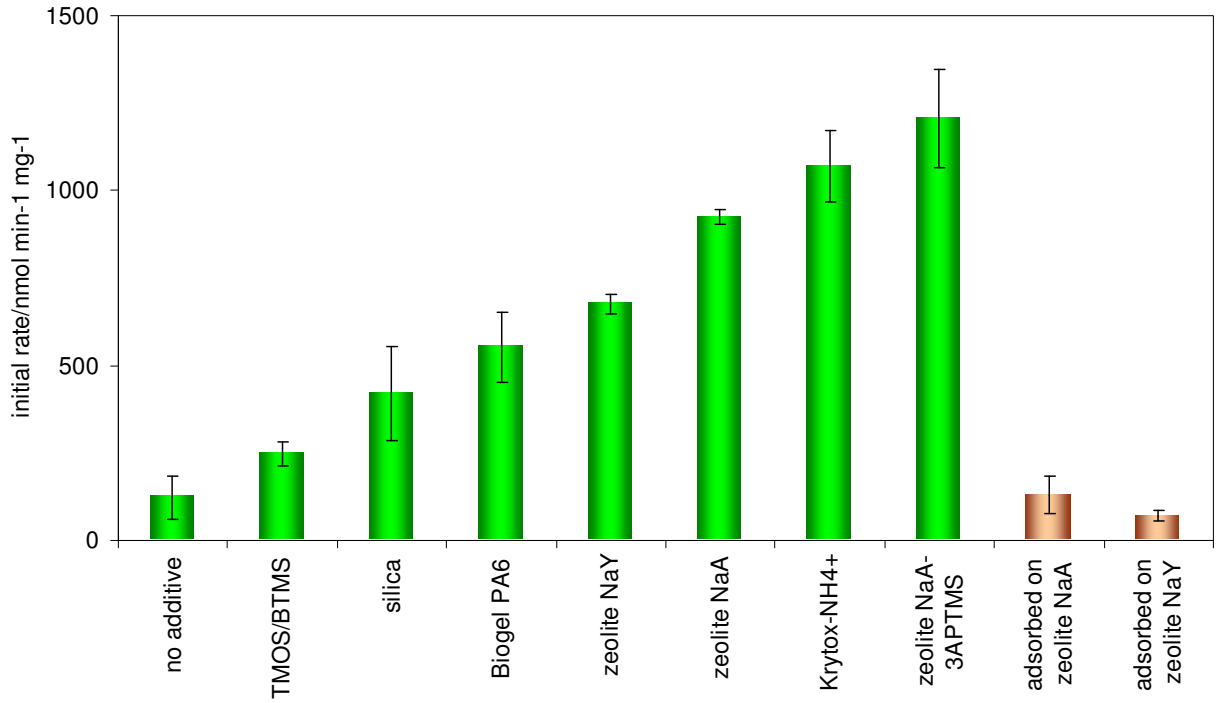

Figure 5 – Effect of additives on the catalytic activity of cutinase encapsulated in 1:5 TMOS/BTMS sol-gel and adsorbed at the surface of zeolite NaY, in sc-CO_2 at a_W = 0.2, T = 35 ºC and P = 100 bar. The reaction studied was the transesterification of vinyl butyrate by *(R,S)*-2-phenyl-1-propanol.

We are interested in implementing environmental friendly processes. Sc-CO_2 is a solvent of choice in this context but it is known that it can have a markedly negative effect on the catalytic activity of many hydrolases, and cutinase is no exception (Garcia et al., 2004a; Garcia et al., 2004b). Encapsulation of cutinase in sol-gel matrices in the presence of additives also led to remarkable improvements in enzyme activity in sc-CO_2 (Figure 5). Although the impact of the additives when using this solvent was not exactly the same as when using n-hexane, our results generally show that a gel matrix with good activity in n-hexane propitiates good activity in sc-CO_2 as well. Encapsulation in sol-gel has been found to provide protection against the deleterious effect of CO_2, as reported by Novak et al. (2003) for a lipase. This is not the case here. Sol-gel encapsulation yields a more competent enzyme, but it does not bridge the gap between cutinase activity in sc-CO_2 and in other nonaqueous media.

CONCLUSIONS

Encapsulation in a sol-gel matrix is a good strategy to improve cutinase activity and stability in nonaqueous media, as shown here for an organic solvent (n-hexane) and sc-CO_2. Although enzyme loading in some of the sol-gel matrices used in the present study was slightly lower than that normally used for adsorption on zeolites (Gonçalves et al., 1996; Serralha et al., 1998) (c.a. 1.4 % w/w for TMOS/BTMS versus 2.5 % w/w for adsorption on zeolite NaY), we have already verified that enzyme loading in the TMOS/BTMS sol-gel can be doubled without decreasing the enzyme specific activity. The impact of the additives on the properties of cutinase and the fact that these agents did not affect the structural characteristics of the sol-gel matrix suggest that enzyme/matrix interactions must play an important role in determining enzyme performance. The results achieved with some of the additives highlight the potential of this strategy for a finer tuning of the properties of enzymes encapsulated in sol-gel matrices. As might be anticipated, the additives most adequate for one enzyme may not necessarily be the best for another, as seen here for cutinase and PCL.

ACKNOWLEDGMENT

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PART II.

The cutinase point of view

In the second part of our study, our aim was to try and correlate differences in the activity of cutinase with the microenvironment that the enzyme experiences inside the different sol-gel matrices. As we saw in the introduction to the present chapter, the rationalization of the interaction between enzymes and solid surfaces can give valuable information for the development of new immobilization solutions.

The strategies that were presented in part I to enhance enzyme activity and stability were essentially related with the enzyme microenvironment, since they involved both changes in the hydrophobic character of the sol-gel matrices through the use of different sol-gel precursors, and the use of additives. The role of zeolite NaY, in particular, appeared to be worth pursuing. We knew from other authors that adsorption at the surface of zeolites was a good immobilization technique for cutinase, which led to the preservation of its three-dimensional structure, as observed with fluorescence spectroscopy. Fluorescence spectroscopy is one of the most sensitive techniques for studying protein conformation. Here we have used it to probe enzyme/matrix interactions.

The technique is based on the fact that all biological molecules carry specific spectroscopic signatures that define not only their identities, but also their physical states and environments. Thus if the optical signals transmitted by biomolecules were intense enough, they could reflect very small changes in their environment[1,2]. There are several species, namely aromatic aminoacids, which can act as fluorescence probes, and fortunately they are present in the majority of proteins or peptides. The aromatic amino acids are tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe), which have strong UV absorption bands corresponding to $S_0 - S_1$ transitions when exited between 260-280 nm, or $S_0 - S_2$ transitions when exited below 230 nm. Usually fluorescence experiements use the S_1 exited state in order to minimize photoreaction events and to enhance the quantum yield. At these longer wavelengths, Trp has the largest molar extinction coefficient (ε) and a better quantum yield (ϕ_f), compared to the other two aminoacids. On the other hand, Phe has the poorest ε and ϕ_f, and that makes Phe rarely useful for fluorescence studies. When subjected to UV irradiation, both Trp and Tyr show emission spectra similar to spectra of Trp alone. The reason for this is related with the transfer of nonradiative energy from Tyr to Trp. Although these two residues are not highly fluorescent, undergo many photoreactions with higher quantum yield and their

emission could be quenched by several factors, they are very useful probes to study protein structure[1,2]. .

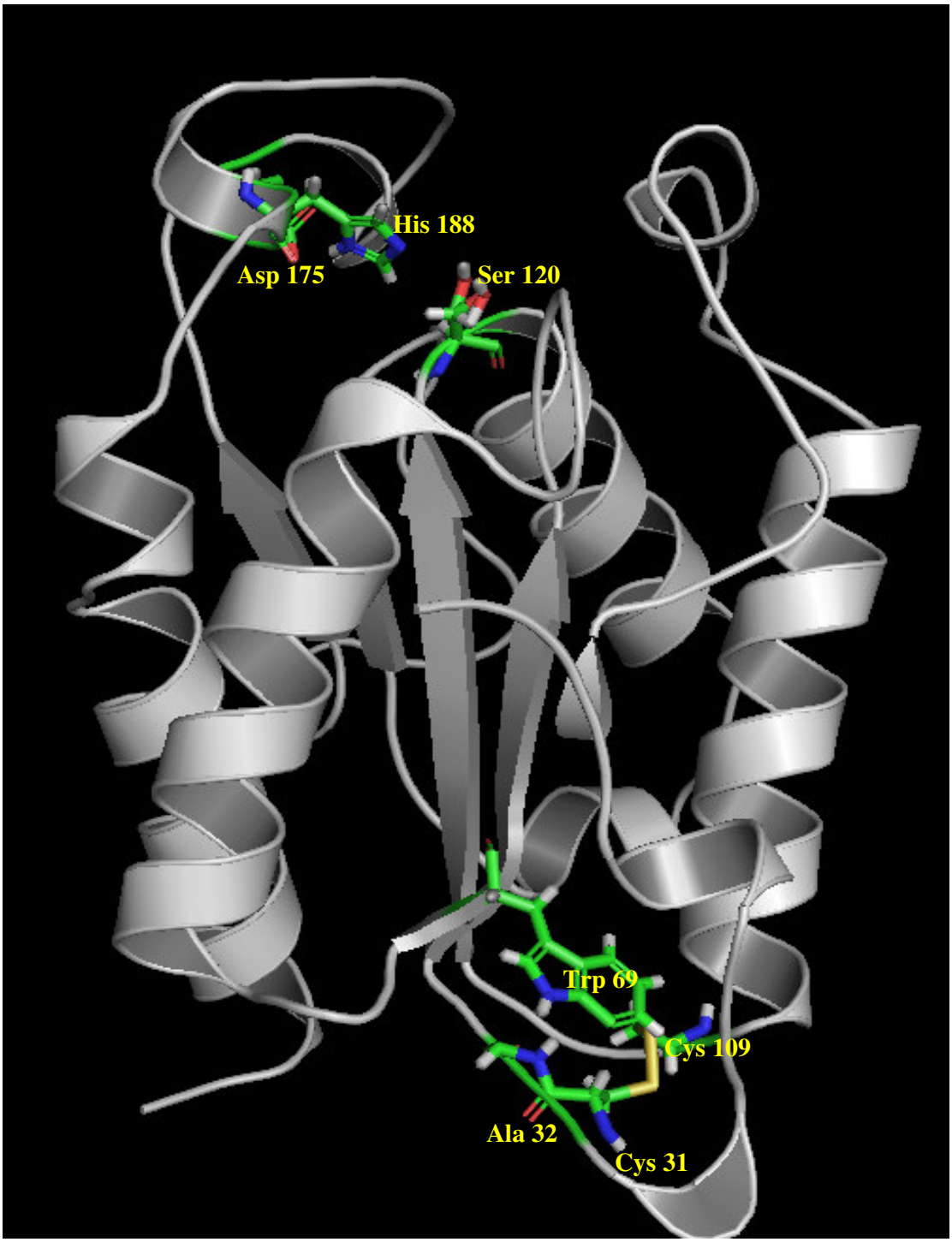

Figure II.I- *Fusarium solani pisi* cutinase structure (PDB 1CEX). At the top, the active center catalytic triad (Ser 120, His 188 and Asp 175). At the bottom, Trp 69, the disulfide bridge Cys31 – Cys109), and Ala 32.

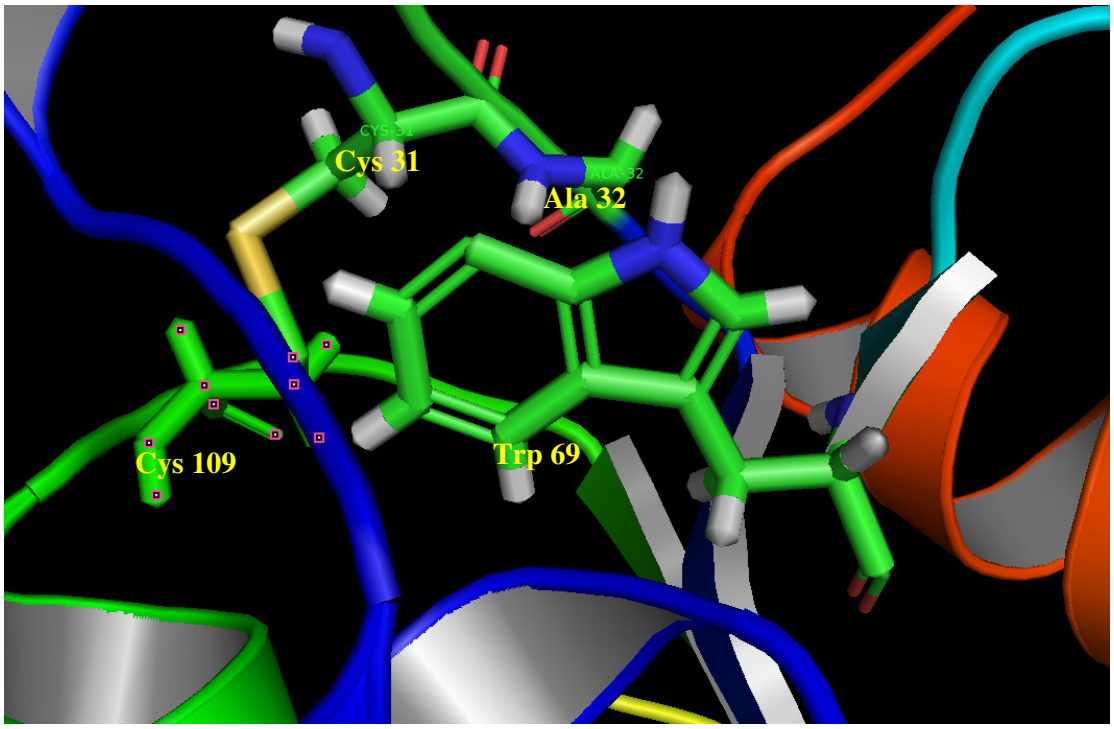

Figure II.II- Detail of the region in the vicinity of Trp 69.

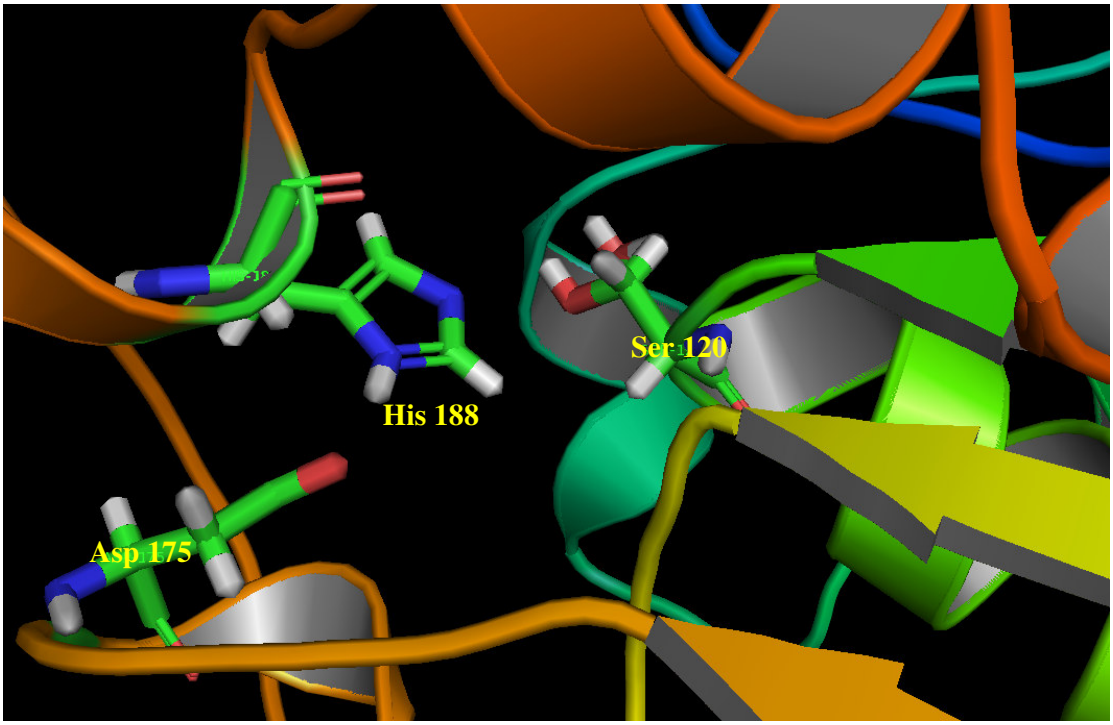

Figure II.III- Detailed catalytic triad.

Fusarium solani pisi cutinase is a serine hydrolase with a catalytic triad formed by serine 120, histine 188 and aspartic acid 175, as well as an oxyanion hole, like all cutinases. Their catalytic mechanism was described in Chapter II. One important aspect of the cutinase structure is the presence of only one tryptophan (Trp 69) that is located at the opposite pole of the active center. This residue is on a β-sheet (residues 68-79), close to one of the two disulfide bridges of this esterase (the one between Cys 31 and Cys 109), forming a hydrogen bond with an alanine (Ala 32) located on the loop (residues 27-33)[5-7].

The fluorescence studies of cutinase are based on the fluorescence of its single Trp that can be used as a polarity probe of the enzyme microenvironment. Trp can also provide very important information on the conformation of the enzyme, since when cutinase is in its native conformation, the fluorescence of Trp 69 is highly quenched[5-7]. This is due to the presence of the Cys 31-Cys 109 disulfide bridge. Disulfide bridges are known to be excellent quenchers of the excited-state of aromatic residues. The fluorescence quantum yield of the Trp residue increases with an increase in irradiation time, but in this case what is taking place is the photo-induced disruption of the disulfide bridge, which reduces its quenching ability[5-7]. Neves-Peterson and co-coworkers[5] have presented a correlation between the number of absorbed photons and the disruption of this disulfide bridge on cutinase. These authors verified that the overall structure of cutinase is damaged in the process. In fact, irradiation of cutinase at 295 nm causes 50% loss of the enzyme activity, confirming a negative impact on structure integrity since the active site is on the opposite pole of the Trp region (figure III.I).

A first question that one may ask is why is the exited state of Trp responsible for the rupture of the disulfide bridge? This is possibly related with the dipole moment of Trp inducing a dipole moment on the adjacent disulfide bridge. The resulting energy transfer would induce vibrational modes on the disulfide bridge, causing its disruption. This is more plausible than direct light absorption by the disulfide bridge, since at 295 nm its extintition coefficient is 300 M^{-1} cm^{-1}, whereas the extinction coefficient of Trp at the same wavelength is 5600 $M^{-1}cm^{-1}$, almost 19 times higher. Thus, an electronic transfer from Trp is the likely explanation for the disruption of the disulfide bridge[5,7].

Another question one may ask is why a disulfide bridge is so near an aromatic residue? This structural feature can be found in many other proteins. Apparently the answer is simple: when aromatic residues such as Trp or Tyr are exited by UV light, H or an electron are triggered from these residues and can lead to the formation of radicals. The latter can develop a cascade reaction involving several reactive groups of the protein, causing great damage to the protein structure. By placing a disulfide bridge in close proximity to Trp, nature and evolution made the intelligent choice of choosing the less harmful way[5].

Trp 69 and the Cys 31- Cys 109 disulfide bridge can work as an integrity probe for the structural integrity of cutinase. Serralha and co-workers[3] and Melo and co-workers[4] give very good examples on the utility of this approach to assess the impact of several immobilization techniques. E.g. the first authors show a very good correlation between the decrease in the activity of cutinase adsorbed at the surface of zeolites with the shift in the emission spectrum of Trp. They found that the reason for the loss of activity when cutinase was immobilized on the most dealuminated supports was essentially a strong denaturation caused by the type of interaction of cutinase with those supports.

In the study that follows, we have used fluorescence spectroscopy to clarify the impact of different sol-gel matrices, with or without added zeolite NaY, on cutinase activity.

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Probing the microenvironment of sol-gel entrapped cutinase with fluorescence spectroscopy: the role of added zeolite NaY

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CONTENTS

ABSTRACT

Cutinase, an esterase from *Fusarium solani pisi*, was immobilized in sol-gel matrices of composition 1:5 tetramethoxysilane(TMOS): n-alkyltrimethoxysilane (n-alkylTMS). Fluorescence spectroscopy using the single tryptophan (Trp-69) residue of cutinase as a probe revealed that the polarity of the matrices decreased as their hydrophobicity increased up to the TMOS/n-butylTMS pair, which correlates with an increase in cutinase activity. Fluorescence emission was suppressed (a higher than two orders of magnitude reduction) in the TMOS/n-octylTMS matrix, suggesting a greater proximity of the tryptophan to a nearby disulfide bridge. When zeolite NaY was used as additive, cutinase activity increased in all the matrices. The presence of the zeolite did not affect the fluorescence emission intensity maximum (λ_{max}) of the tryptophan. And although the addition of the zeolite led to the recovery of fluorescence emission from the TMOS/n-octylTMS matrix, the corresponding λ_{max} fell in line with the values obtained for the matrices with lower n-alkyl chain lengths. This indicates that tryptophan does not sense the proximity of the zeolite and suggests that the zeolite is in a position to affect the active site of the enzyme, located at the opposite pole of the enzyme molecule. Scanning electron microscopy and energy dispersive X-ray spectroscopy revealed that the zeolite particles were segregated to the pores of the matrices. Optical microscopy following the staining of the protein with a fluorescent dye showed that the enzyme was distributed throughout the material, and tended to accumulate around zeolite particles. Data obtained for sol-gel matrices with epoxy or SH groups provided further evidence that cutinase responded to changes in the chemical nature of the precursors.

INTRODUCTION

The sol-gel process allows the immobilization of biomolecules and microorganisms at sufficiently mild conditions to preserve the native structure, dynamics and function of the biomolecules as well as the activity and viability of the encapsulated whole cells[2,7,10]. The inorganic matrix or host provides an inert and mechanically strong framework or interface whose porosity, pore size and permeability to solutes such as substrates, products or nutrients can be controlled[1,20]. Applications of the resulting hybrid materials include sensors based on protein microarrays[12] or encapsulated bacteria[33], bioartificial blood vessels[35], bioartificial organs and cellular grafts[6,40], and bioreactors containing the enzymes(s) of interest for the screening of certain analytes[25] or living cells for the biodegradation of pollutants[17]. Additional fields of application of hybrid sol-gel materials include drug release systems[5], bioactive materials[16] and biomimetic systems[13].

The immobilization of enzymes using the sol-gel process can lead to increased enzyme stability, one of the key properties for industrial applications. The enzymes are held within a confined space, with restricted conformational mobility that contributes to the observed stability enhancement[14]. In fact, sol-gel encapsulation has been used to mimic the effects of confinement on the structure and stability of proteins, as occurs in living cells, in particular surface-induced water structure effects that are also thought to contribute to the observed stabilization[15]. One of the advantages of the sol-gel system is that it allows studies on the impact of agents that cause the protein to aggregate in solution, a situation that is precluded in the sol-gel matrix. It has also been shown that sol-gel entrapment can protect enzymes against exposure to extreme pH conditions[18]. Additionally the entrapment of enzymes in sol-gel matrices can prevent the leaching of the enzyme[8], which is equally important from an economic and environmental standpoint and has long been pointed out as one of the important advantages of the sol-gel process[1]. The effective prevention of enzyme leaching may sometimes require additional steps in the immobilization protocol[4].

The use of additives that remain entrapped in the material is a second-order approach towards impacting on enzyme activity and stability via the sol-gel process. Examples include polymers that reduce the extent of shrinking during the making and drying of

the matrix[47], sugars that act as stabilizers[8], and various species such as surfactants or cyclodextrin[38]. In line with the latter study, we have recently studied the immobilization of cutinase in sol-gel matrices formed via the hydrolysis and condensation of mixtures of a silicon alkoxide and a trimethoxysilane, in the presence of several additives[45]. We have shown that when cutinase is immobilized in 1:5 tetramethoxysilane(TMOS)/*n*-alkyltrimethoxysilane(*n*-alkylTMS) sol-gel matrices where the alkyl groups range from methyl to *n*-octyl, its activity increases up to *n*-butyl and then decreases. Studies with a lipase immobilized in similar sol-gel matrices[36.37] showed increased enzyme activity for longer alkyl chain lengths, a fact attributed to the entrapment of the enzyme in an active, open lid conformation. The fact that cutinase does not have a lid covering its active site and lacks interfacial activation[11] could explain the different correlation with the composition of the sol-gel matrix obtained for this enzyme.

In our earlier study[45] we have shown that when cutinase is immobilized in 1:5 TMOS/*n*-butylTMS) sol-gel matrices, enzyme loading and enzyme activity can match the levels reached with the technique of physical adsorption at the surface of zeolite NaY, a successful immobilization procedure for cutinase in nonaqueous media that is suitable for transesterification[19,41] as well as for hydrolysis reactions[21,22]. The advantages of the sol-gel process relative to the latter technique appeared to be twofold: an enhancement of enzyme stability, namely the operational stability of the enzyme, as assessed in a series of reutilization cycles, and an enhancement of specific activity when several additives were incorporated in the matrix. One of these was zeolite NaY. The purpose of the present work is then to elucidate the role that zeolite NaY plays when it is used as additive not only in the TMOS/*n*-alkylTMS matrices that we have used earlier, but also in sol-gel matrices bound to provide a different microenvironment for the enzyme. To build these we selected a trimethoxysilane bearing a thiol-substituent or an epoxy group that in principle could form covalent bonds with the enzyme.

Cutinase from *Fusarium solani pisi* is an extracellular enzyme involved in the degradation of cutin, the cuticular polymer of higher plants. It is a serine hydrolase with 197 residues, a molecular mass of ca. 22 kD, and a volume of ca. (45x30x30) $Å^3$. Fluorescence spectroscopy has been extensively used to provide information on the conformation and dynamics of cutinase dissolved in aqueous media[29,44], adsorbed on solid supports[3,42] encapsulated in reverse micelles[23,29,44]. The maximum fluorescence

intensity for cutinase is dominated by the contribution from its six tyrosines[27]. However, the fluorescence from the single tryptophan residue of cutinase (Trp-69) can be selectively measured by chosing an appropriate wavelength of excitation[31,34,46]. The fluorescence spectrum of tryptophan is very weak because tryptophan is highly quenched by a disulfide bridge between cysteines 31 and 109, whose distance from the indole ring of tryptophan is ca. 5 Å. The tryptophan fluorescence emission peak depends on the polarity of its surroundings, e.g. in more hydrophobic microenvironments the fluorescence emission intensity maximum of tryptophan will shift to smaller wavelength values.

In the present work we look at the fluorescence emission spectra of the tryptophan of cutinase when the enzyme is entrapped in sol-gel matrices of composition 1:5 TMOS:XTMS, in which X is an *n*-alkyl chain, a propyl chain modified with an SH group, or an epoxy, with or without co-entrapment of zeolite NaY. We also look at the impact that added zeolite NaY has on enzyme activity, and discuss the role of the zeolite in the sol-gel matrices, which we characterized using scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy, and optical microscopy after staining the protein with a fluorescent dye.

MATERIAL & METHODS

Materials

The cutinase producing strain Saccharomyces cerevisiae SU50-pUR7320 was constructed and provided by the Unilever Research Laboratory at Vlaardingen, The Netherlands. The production and purification of cutinase was performed at Centro de Engenharia Biológica e Química, Instituto Superior Técnico[9]. The enzyme purity was controlled by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The estereolytic activity of the enzyme was determined spectrophotometrically. A 20 μL sample from the culture medium was added to 980 μL of 11.3 mM sodium cholate, 0.43 M tetrahydrofuran, 50 mM potassium phosphate buffer (pH 7.0) solution, in which the hydrolysis of p-nitrophenyl butyrate (0.56 mM) was monitored at 400 nm. (R,S)-2-phenyl-1-propanol (97 % purity), tetramethoxysilane (TMOS), methyltrimetoxysilane (MTMS), ethyltrimethoxysilane (ETMS), propyltrimetoxysilane (PTMS), n-

octyltrimetoxysilane (OCTMS), zeolite NaY molecular sieves (in powder form) were from Aldrich, n-butyltrimetoxysilane (BTMS) was from Polysciences Inc., vinyl butyrate (99 % purity) was from Fluka, n-hexane, tridecane, sodium fluoride and sodium chloride were from Merck, Hydranal Coulomat A and C Karl-Fischer reagents were from Riedel de Häen, 2-(3,4-epoxycyclohexyl)ethyltrimethoxysilane (EXETMS), 3-mercaptopropyltrimethoxysilane (SHPTMS), and polyvinyl alcohol (PVA; MW 15.000) were from Sigma. Sypro Ruby red gel stain was from Molecular Probes. Technovit 2000 LC paste and light-curing resin were from Heraeus Kulzer.

Immobilization of cutinase in the sol-gel.

This technique was adapted from that referred by Reetz et al.[37], as indicated by Vidinha et al.[45]. 2 mg of lyophilized cutinase was dissolved in an aqueous solution containing NaF (0.20 g L^{-1}) and PVA (4.64 g L^{-1}). The amount of water in this solution was fixed (6.86 mmol). The solution was vigorously shaken on a vortex mixer. The precursors were then added in amounts that yielded a water/silane molar ratio (R) of 8 irrespective of the type and number of precursors used (e.g. 0.142 mmol of TMOS and 0.712 mmol BTMS in 1:5 TMOS/BTMS gels). The mixture was again vigorously shaken on the vortex mixer, until it became homogeneous. It was then placed in an ice bath until gelation occurred (after a few seconds), and kept in the ice bath for an additional 10 minutes. The container with the obtained gel was kept at 4 ºC for 24 h, after which the gel was air-dried at 35 ºC for 24 h. The white gel obtained was crushed and washed (for about 10 min)/centrifuged (at 5400 rpm), first using phosphate buffer (50 mM, pH 7, 2 mL), then acetone and finally n-pentane (also 2 mL of each). The gel was left at room temperature for 16 hours, after which it was equilibrated with a saturated salt solution at room temperature for about 3 days, to achieve the value a_W = 0.7 (sodium chloride), taken from the literature[24]. When used, zeolite NaY (4 mg) was added to the enzyme/NaF/PVA solution. The average yield of immobilization of cutinase in the sol-gel matrix was (91 ± 8) %, as determined by the Lowry method[26]. This determination was based on the amount of enzyme found in the aqueous buffer used for washing the gel. Average enzyme loading ranged from 3.2 % in TMOS/MTMS gels to 1.8 % in TMOS/OCTMS gels.

Matrices with acid-base indicators and no enzyme.

Matrices containing fluorescein were prepared as above, but with fluorescein (1 mg) replacing the enzyme. The reddish-orange dianionic form of fluorescein (λ_{abs} = 490 nm) protonates at mildly acidic pH values in water (pK_a = 6.43) to yield the yellowish-orange monoanionic form (λ_{abs} = 472 nm; λ_{abs} = 453 nm)[43]. The matrices containing an indicator (methyl orange, bromocresol green, phenol red or bromothymol blue) were prepared by replacing part of the water that was formerly added separately with an aqueous solution of the indicator, prepared according to standard protocols. The actual colours conferred by the indicators in the reaction mixture, in the acidic and basic pH ranges, were checked by adding concentrated HCl or NaOH solutions to the mixture immediately upon addition of the precursors.

Immobilization of cutinase on zeolite NaY.

Cutinase was immobilized by deposition[21,41]. The lyophilized enzyme was dissolved in a 50 mM sodium phosphate buffer solution (10 mg mL^{-1} of enzyme) at pH 8.5. The support was added to the solution (25 mg of cutinase per g of support) and after vortex mixing for 1 min, the preparation was dried under vacuum for at least 24 h. The average yield of immobilization was (72 ± 12) % zeolite NaY, as determined by a modified Lowry method[26].

Steady-state fluorescence spectroscopy.

The fluorescence emission and excitation spectra of the samples were recorded on a Jobin Yvon-SPEX Fluorolog 3.22 spectrofluorometer. The emission was measured with excitation at 290 nm, to avoid interference from other luminescent residues, in a 4 mm path fluorescence cell filled with the powder, in front face geometry, with 1.5 nm band path excitation and emission slits. The excitation spectra, collected at the maximum of the observed emission, were recorded for all samples in order to confirm the origin of the observed emission. In the cases where significant emission was observed but the excitation spectrum did not match the absorption spectrum of tryptophan, the sample was rejected and re-synthesized. The fluorescence emission intensity maximum (λ_{max})

values given are the average of at least six measurements performed on replicate sol-gel supports. The fluorescence intensity was quantified in arbitrary units. The specific fluorescence intensity is equal to the fluorescence intensity divided by the amount of enzyme (in mg).

Matrices characterization.

The fractured surfaces of the matrices were examined with a DSM962 Zyce scanning electron microscope (SEM), operating between 3 and 10 kV. To avoid charging effects during observation, the surfaces were previously sputter-coated with a gold layer. Some matrices were also examined with an Oxford Instruments SEM equipped with an INCAx-sight energy dispersive X-ray spectroscopy (EDS) detector, as well as with a Zeiss Axioplan-2 optical microscope. Prior to examination with the optical microscope, each sample was mounted on a plastic mould using a paste and a light-curing resin. Excess cured resin was removed by grinding. A few drops of the protein staining fluorescent dye solution were then deposited on the surface of the sample, which was kept in the dark for 10 min. Excess dye was then removed.

Enzyme activity assays.

Reactions were performed in glass vials (reaction volume of 750 μL) placed in a constant temperature orbital shaker set for 400 rpm. All the reaction mixture components were pre-equilibrated to a_W = 0.7 for about 3 days. The reaction studied was the transesterification of vinyl butyrate (300 mM) by (R,S)-2-phenyl-1-propanol (100 mM) in *n*-hexane. The concentration of sol-gel encapsulated enzyme or of cutinase adsorbed on zeolite NaY was 6 g L^{-1}. Vinyl butyrate addition marked the start of the reaction. Tridecane (15.4 mM) was used as external standard for GC analysis. Water concentration was measured by Karl-Fisher titration. Enzyme operational stability tests performed in *n*-hexane revealed very high retention of cutinase activity[45]. Thus the data in Figures 6 and 7 are not affected by enzyme leaching.

Analysis of reaction progress.

The reaction conversion was measured by GC analysis performed with a Trace 2000 Series Unicam gas chromatograph equipped with a 30 m x 0.32 mm i.d. fused silica capillary column coated with a 0.25 µm thickness film of 20 % 2,3-dimethyl-6-tert-butyldimethylsilyl)-ß-cyclodextrin dissolved in BGB-15, from BGB Analytik AG. Oven temperature program: 125 ºC for 2 min, 125-171 ºC ramp at 6 ºC min^{-1}, 200 ºC for 3 min. Injection temperature: 250 ºC. Flame ionization detection (FID) temperature: 250 ºC. Carrier gas: helium (2.0 cm^3 min^{-1}). Split ratio: 1:20. No products were detected in assays carried out without enzyme. The initial rates given (per mg of protein) are the average of at least two measurements.

RESULTS AND DISCUSSION

Steady-state fluorescence spectroscopy data

The fluorescence emission intensity maximum (λ_{max}) of the single tryptophan (Trp-69) residue of cutinase reflects the polarity of the microenvironment of that residue. Our first concern was to establish that the changes in λ_{max} upon changing the composition of the sol-gel matrix could not be attributed to different levels of exposure of tryptophan to the surroundings of the protein, as caused by its denaturation. If this were the case, the recorded emission spectra would be expected to vary among supports prepared with such different precursors as *n*-alkylTMS or EXETMS, thus reflecting the presence of many different microenvironments of the tryptophan residue upon protein denaturation and/or aggregation. Figure 1 shows emission spectra obtained for cutinase entrapped in three different sol-gel matrices. As seen in the figure, the spectra have similar band widths, which indicates that the enzyme populations sensed by the tryptophan in each case are mostly in the same conformational state. The average full width at half the maximum of the emission band for all the samples tested that emitted fluorescence, including all the different sol-gel matrices, was ca. 55 nm. Also when normalizing the signals obtained by dividing the intensity of the fluorescence emission by the amount of protein, the specific intensities obtained agreed to within one order of magnitude, with

no trend, which is another indication that the enzyme populations did not vary much among samples. In fact, when denaturation occurs, the quenching of the tryptophan fluorescence emission is less pronounced, fluorescence intensity increases and a broader emission band is obtained[23,27]. Evidence that the conformational states of cutinase adsorbed on zeolite NaY resemble those of the native enzyme has been provided by Serralha et al.[42].

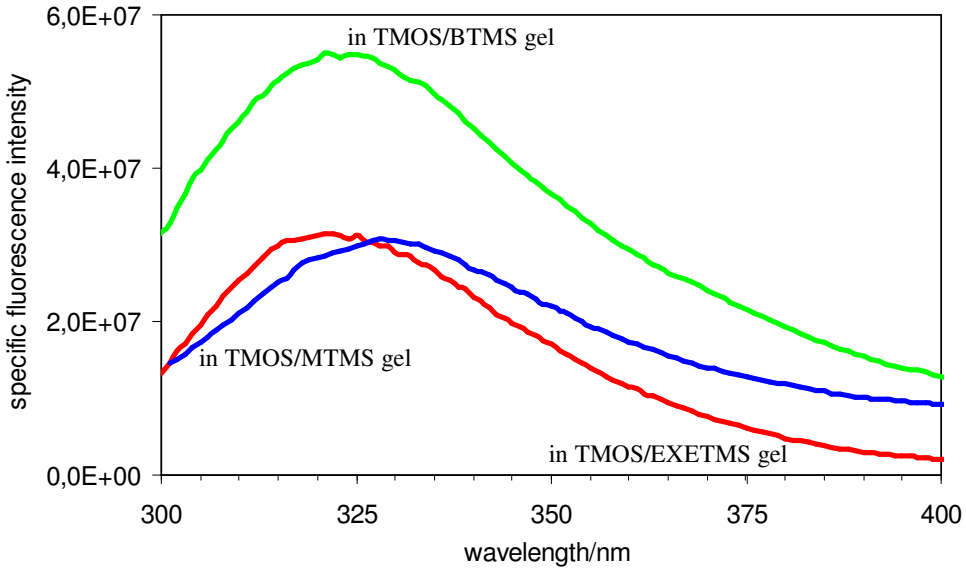

Figure 1 - Emission spectra of cutinase entrapped in sol-gel matrices of different compositions. Legend: (green) TMOS/BTMS; (blue) TMOS/MTMS; (red) TMOS/EXETMS.

Figure 2 provides further evidence that the tryptophan of cutinase responds to changes in the polarity of its microenvironment, as effected by permeating the sol-gel matrix with different solvents. λ_{max} was 319.0 nm in the case of the TMOS/BTMS matrix permeated with *n*-hexane, and shifted to 324.9 and 329.0 nm for the matrices permeated with acetonitrile and methanol, respectively. These two solvents have similar dielectric constants (35.94 and 32.66, respectively) but methanol has a higher value of the empirical parameter of solvent polarity E_T^N (0.762; 0.460 for acetonitrile)[39]. The emission spectra for cutinase dissolved in aqueous buffer were highly quenched and are not shown in the figures. In this case, λ_{max} was ca. 338 nm.

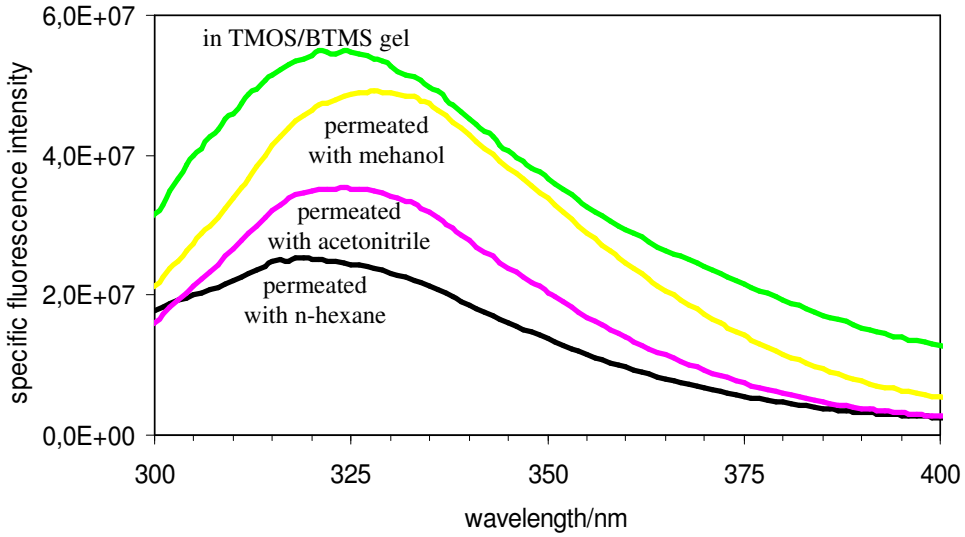

Figure 2 - Emission spectra of cutinase entrapped in TMOS/BTMS sol-gel permeated with different solvents. The top curve, taken from Figure 1, is shown for comparison. Legend: (green) TMOS/BTMS; (yellow) TMOS/BTMS permeated with methanol; (pink) TMOS/BTMS permeated with acetonitrile; (black) TMOS/BTMS permeated with n-hexane.

As referred in the Introduction, our main goal was to elucidate the role of zeolite NaY as additive in the sol-gel materials. To this end, we first looked at how the emission of the tryptophan responded to the composition of the sol-gel in the 1:5 TMOS/*n*-alkylTMS homologous series (Figure 3). The sequential decrease in λ_{max} registered in the gels up to TMOS/BTMS reveals that tryptophan is sensitive to the changes in polarity imparted by the increase in the chain length of the *n*-alkylated precursor and hence the increased hydrophobicity of its surroundings. However, in the case of the TMOS/OCTMS gel the specific fluorescence intensity recorded was significantly lower than that obtained with the other matrices (more than two orders of magnitude lower; lower curve in Figure 4). By comparison and for the purpose of the present discussion, this situation will be referred to as the 'suppression of fluorescence emission'.

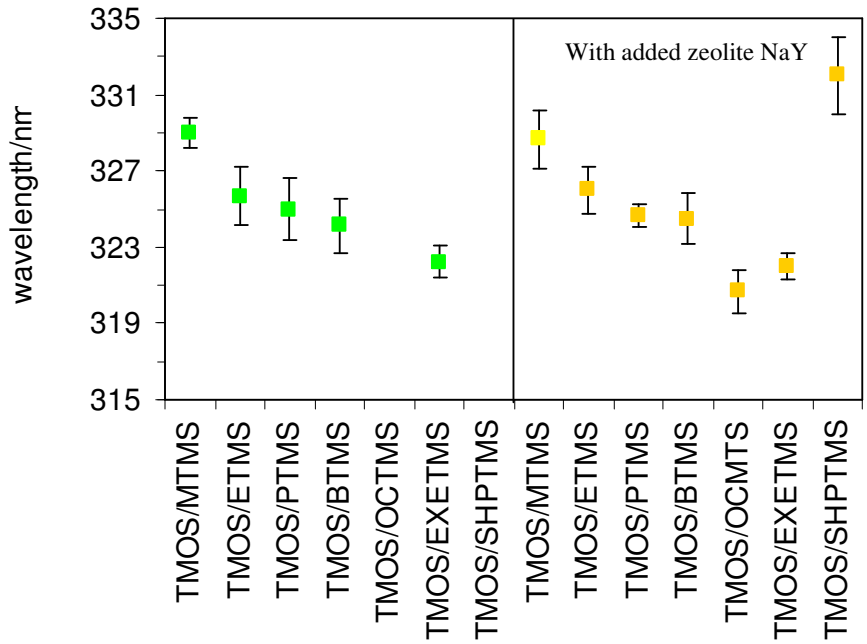

Figure 3 - Fluorescence emission intensity maximum (λ_{max}) for cutinase entrapped in sol-gel matrices prepared without or with added zeolite NaY. In the case of the TMOS/OCTMS and TMOS/SHPTMS matrices prepared without zeolite NaY, the specific fluorescence intensity recorded was more than two orders of magnitude lower than that obtained with the other matrices, and no λ_{max} values are given.

As seen in Figure 3, fluorescence emission was also suppressed when cutinase was entrapped in the TMOS/SHPTMS gels. It is possible that some of the SH groups in the TMOS/SHPTMS matrix reacted among themselves to form additional disulfide bridges to further quench the emission signal from the enzyme. This would involve the release of protons and possibly formation of SH_2 as well. There is evidence that both processes occurred to some extent, as shown by the colors of the preparations with different indicators that suggest a medium pH range of 6.0-6.5, and a clear smell of SH_2 while preparing this matrix. No comment can be made on the λ_{max} values in the TMOS/EXETMS matrices that were similar to those obtained with TMOS/BTMS, despite the dissimilarity of the compositions of the two matrices.

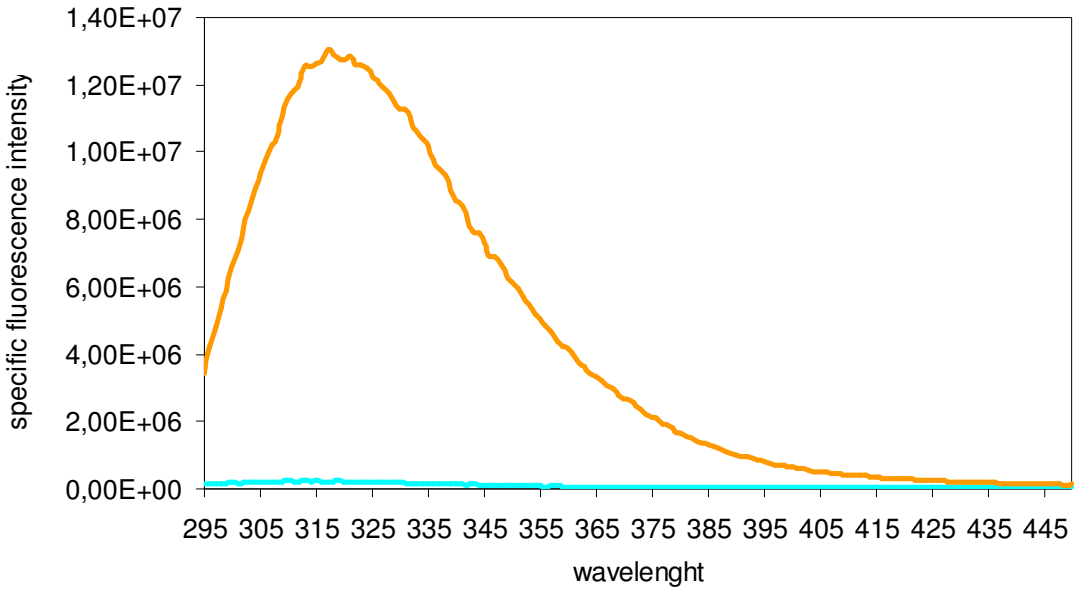

Figure 4 - Emission spectra of cutinase entrapped in the TMOS/OCTMS gel with added zeolite NaY (upper curve). The emission signals obtained from the TMOS/OCTMS matrix alone or with added zeolite NaY, and for cutinase entrapped in the TMOS/OCTMS gel without zeolite NaY are shown for comparison (lower curves). For the matrices that contain the enzyme, the Y-axis values represent the specific fluorescence emission of cutinase.

Included in Figure 3 are the data obtained when zeolite NaY was incorporated in the materials via its suspension in the enzyme solution before the addition of the sol-gel precursors. In those cases where cutinase already emitted fluorescence with a given λ_{max}, this parameter did not change upon the addition of zeolite NaY. This suggests that tryptophan cannot sense the zeolite. The specific fluorescence emission intensity of the preparations also did not change significantly with the addition of the zeolite. In those cases where the emission signal was suppressed in the absence of zeolite NaY, the presence of this additive was responsible for the recovery of the fluorescence emission, at normal levels of intensity, leading to λ_{max} values that fall in line with appropriate references when they exist to allow comparison. Such is the case of the TMOS/OCTMS gel (Figure 4), which in the presence of zeolite NaY yielded spectra with a λ_{max} of ca. 321 nm, a value close to/slightly lower than that recorded for the TMOS/BTMS gel with or without added zeolite. Indirect evidence that such is also the case of the TMOS/SHPTMS matrices is provided by experiments in which the five parts of SHPTMS in the above matrix were replaced by different combinations of SHPTMS and

BTMS (Figure 5). Interestingly, only a small amount of BTMS was required for the matrix to recover fluorescence emission. The data suggest that if the emission signal in the TMOS/SHPTMS gel were not suppressed, the corresponding λ_{max} should be ca. 333 nm, close to the values recorded for the matrices with a higher proportion of SHPTMS relative to BTMS, and also very similar to the λ_{max} obtained for TMOS/SHPTMS with added zeolite NaY (Figure 3). Slight changes in the degree of compactness of the enzyme structure might have important reflexes on the distance between the tryptophan and the disulfide bridge in its vicinity.

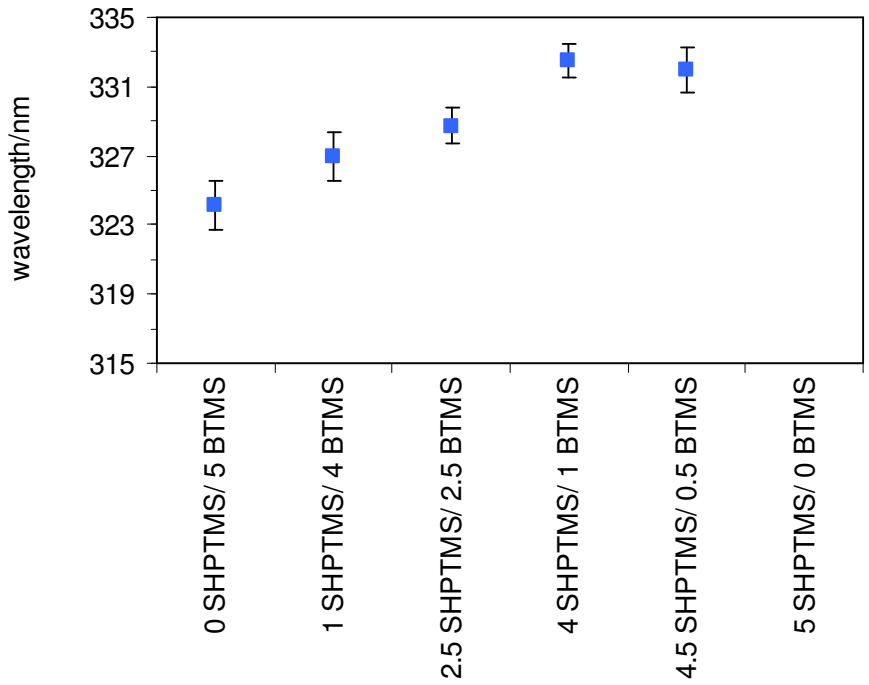

Figure 5 - Fluorescence emission intensity maximum (λ_{max}) for cutinase entrapped in sol-gel matrices of composition 1:5 TMOS:(SHPTMS+BTMS), in which the proportions of SHPTMS and BTMS precursors were varied from 100 % BTMS to 100 % SHPTMS.

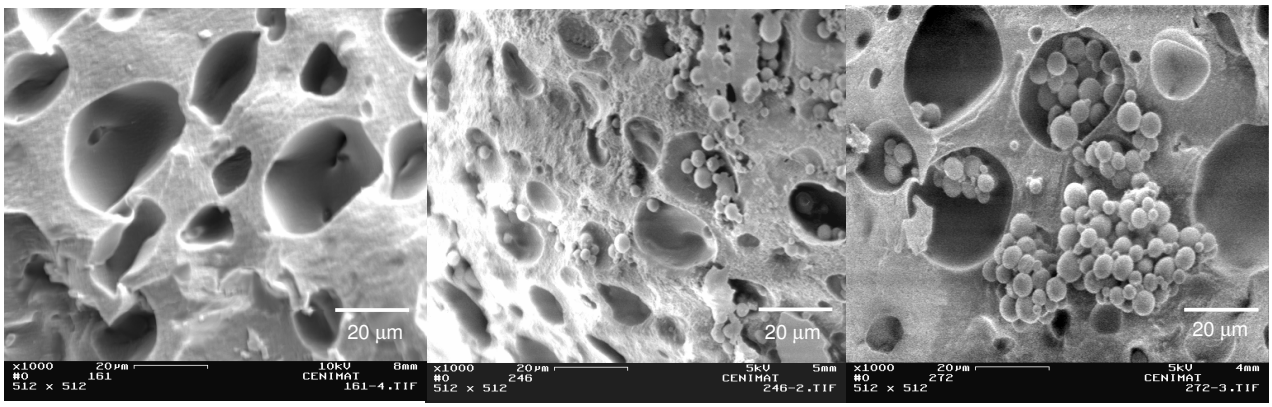

Figure 6 - SEM micrographs of 1:5 TMOS/BTMS matrices. Left, without additives. Centre, with added zeolite NaY. Right, with added zeolite NaA functionalized with 3-aminopropyltrimetoxysilane. The white bar represents 20 µm.

Confirmation that the granules that appear in Figure 8 are zeolite particles was provided by X-ray spectroscopy (Figure 7). As seen in this figure, carbon and silicon are spread all over the material, whereas aluminum and sodium, which only exist in the zeolite, appear in the regions where the granules are found. Indeed, X-ray emission spectra collected from a point on one of the granules (Figure 8, left) reveal the presence of those two metals that are almost absent from locations devoid of granules, as shown by spectra recorded at points on the wall of the pores or outside the pores (Figure 10, right; the detection of small levels of Al and Si can be explained by the penetration of the radiation below the plane of observation).

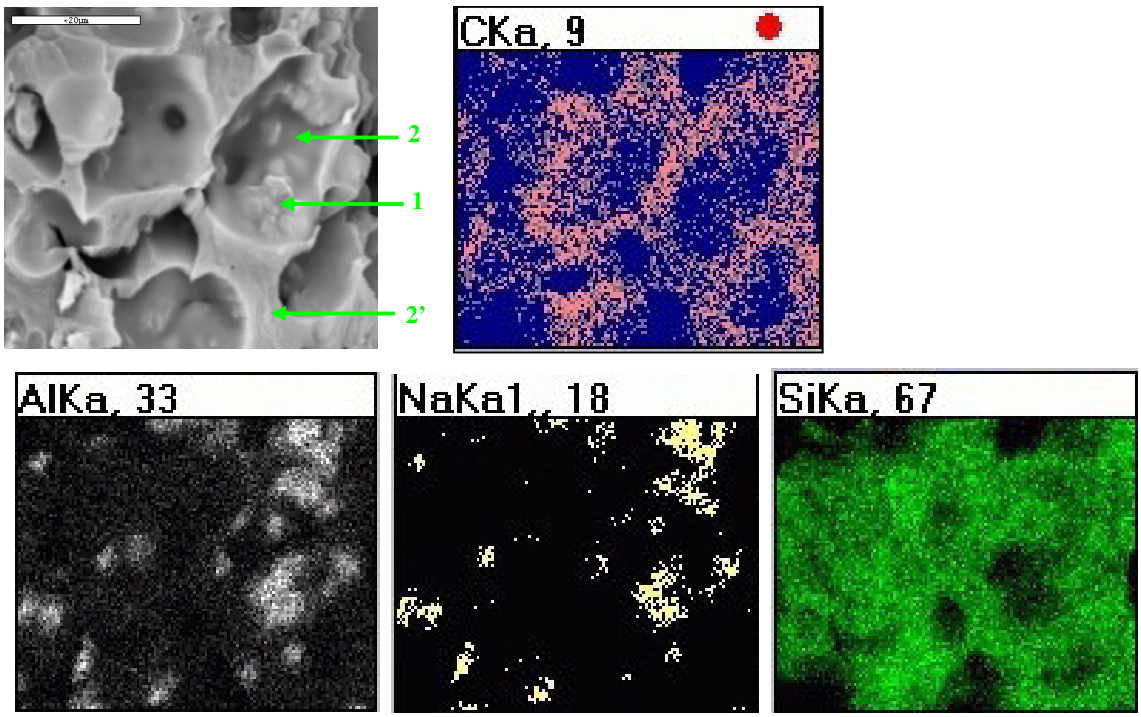

Figure 7 - X-ray microanalysis on a scanning electron microscope for the 1:5 TMOS:BTMS matrix with added zeolite NaY. From top to bottom: EDS micrograph of the surface of the sample followed by micrographs depicting the X-ray emission from carbon, silicon, aluminum and sodium. 1 and 2 are points at which the spectra shown in Figure 10 were recorded

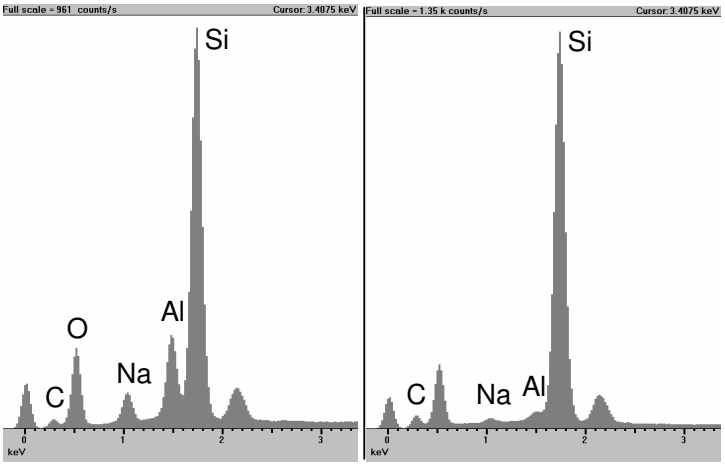

Figure 8 - X-ray emission spectra from points 1 (left) and 2 (right) in Figure 9. The data collected at points 2 and 2' are very similar.

Optical microscopy following the staining of the protein with a fluorescent dye (Figure 9) was used to characterize enzyme adsorbed at the surface of zeolite NaY particles, as well as entrapped in TMOS/BTMS matrices. The presence of the enzyme at the surface of the zeolite is indicated by yellow spots of fluorescence, as shown in image A. These spots are not found in image B, the control sol-gel matrix without enzyme. Image C shows that the enzyme is distributed throughout the matrix; matrices prepared with varying enzyme loads showed a correlation between the latter parameter and the intensity of the uniformly distributed fluorescence of the matrices. The presence of the zeolite induces the accumulation of protein in spots of more intense fluorescence at the pores of the matrices. These are regions of the materials where the enzyme is also found in the absence of the zeolite, as shown in image C, but in seemingly lower amounts (for a fixed quantity of enzyme in the sol-gel). As referred earlier, the specific fluorescence intensities of the materials prepared with or without added zeolite are very similar. This can explain the overall impression of image D of a less intense uniformly distributed fluorescence.

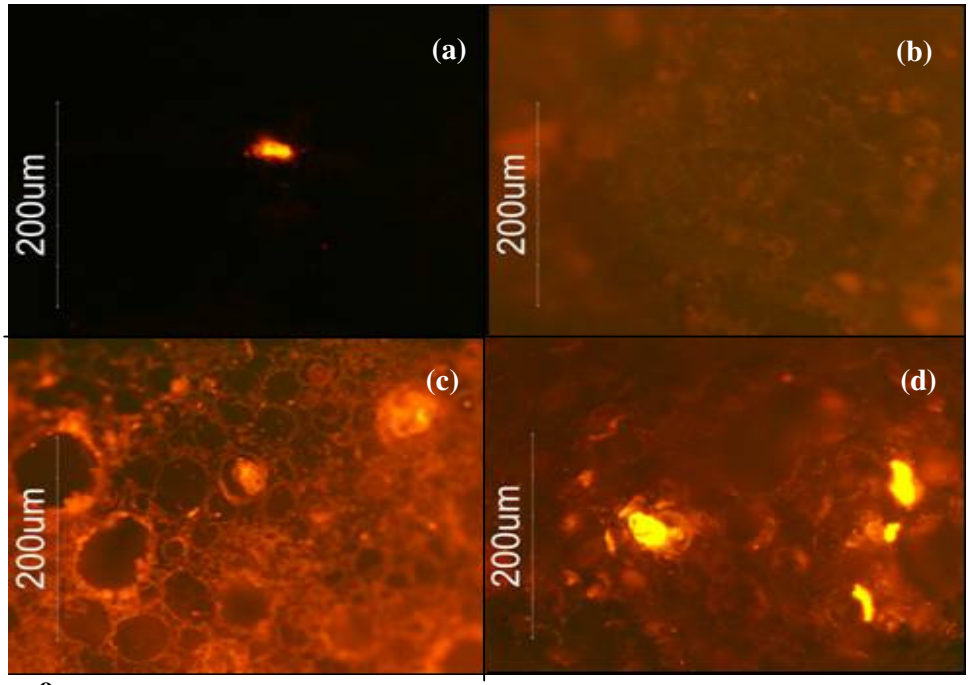

Figure 9 - Optical microscopy images obtained after applying a protein staining fluorescent dye solution to the materials. A – enzyme adsorbed at the surface of zeolite NaY particles. B – TMOS/BTMS matrix without enzyme (control). C – TMOS/BTMS matrix with enzyme but without zeolite. D – TMOS/BTMS matrix with both enzyme and zeolite. Visualization of the protein was done with a blue light transilluminator. All pictures were taken with an exposure of 25, except A, which was taken with an exposure of 50.

We have seen that the co-entrapment of zeolite in the matrices does not cause changes in the λ_{max} values obtained without this additive. And when the presence of the zeolite restores the fluorescence emission of the tryptophan, as seen for the TMOS/OCTMS and TMOS/SHPTMS matrices, the λ_{max} values obtained are what appropriate references suggest they should be: below the λ_{max} recorded for the TMOS/BTMS matrix, and very close to the λ_{max} measured for the TMOS/(4.5 SHPTMS/0.5 BTMS) matrix, respectively. This indicates that tryptophan does not sense the presence of the zeolite in its vicinity. On the other hand, the zeolite has a positive effect on enzyme activity. The tryptophan residue and the active site of cutinase are located at opposite poles of the enzyme molecule. One possibility to account for both the impact of the zeolite on cutinase activity and its lack of impact on λ_{max} is to hypothesize that the enzyme turns its active site towards the zeolite. We cannot at present confirm this hypothesis. But by simply promoting the accumulation of the enzyme at the pores of the material, the zeolite should improve the accessibility of the enzyme to the substrates and lead to a higher activity of the entrapped enzyme. The role of zeolite is thus similar to that described for certain osmolytes[8], although its mode of action is different: the zeolite does not appear to promote enhanced pore size, as shown in the latter study. The degree of activity of a sol-gel entrapped enzyme has long been found to correlate with its accessibility in the matrix[1].

CONCLUSIONS

The continuous advances in the combination of the sol-gel process with biomolecules or biological systems will surely lead to a greater number of bio-applications of sol-gel materials. The mechanical strength and chemical inertness of the sol-gel matrix, the protection it offers against several agents, its high retention ability and the possibility to control its properties, such as porosity and pore size are some of the aspects that make the sol-gel process a very powerful technique for the encapsulation of enzymes. The possibility to change the groups or functionalities of the sol-gel precursors or to use additives lends great versatility to the technique. However, relatively little is known on how a given matrix will interact with/affect/control the behavior of a sol-gel entrapped enzyme. We have used several techniques to characterize the microenvironment of

cutinase entrapped in sol-gel matrices. Our results indicate a type of organization in the material that places the zeolite in close proximity to the enzyme, as required to account for the beneficial effects that the co-entrapment of the zeolite can have on cutinase activity. We hope to be able to apply other experimental approaches for a deeper understanding of enzyme/matrix interactions, and thus contribute to the current developments of the sol-gel process for the encapsulation of biomolecules.

ACKNOWLEDGMENT.

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PART III.

The material's point of view

In part II we have tried a different approach to explain the differences in cutinase activity observed in part I. If in part I we considered that question from the point of view of the enzyme, here we look at it from the perspective of the material, and attempt to correlate molecular structural differences of the support where the enzyme is entrapped with differences in enzyme activity. The structure of the immobilized preparation was probed using two different spectroscopic techniques: Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectroscopy and Solid State ^{29}Si and ^{1}H NMR.

The aim of this brief introduction is to introduce these two spectroscopic techniques that are not very common in biology related studies, but are extremely powerful techniques to characterize solid materials, thus providing very valuable information on materials that are used to immobilize biomolecules.

DRIFT spectroscopy is used when conventional infrared spectroscopy cannot be used, for instance with solid powders or fibres. In DRIFT, the energy that penetrates the analyte particles is reflected in all the directions, making it necessary to use a different experimental set up (figure. III.I)[1,2].

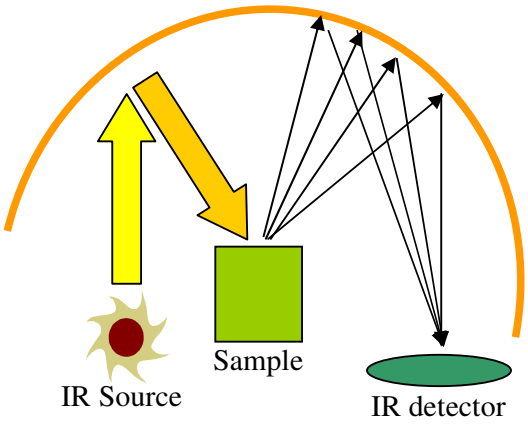

Figure III.I – Schematic representation of instrumentation for DRIFT analysis.

Kubelka and Munk[1] developed a theory that describes the diffuse reflectance process for powdered samples, which relates the sample concentration with the scattered radiation intensity:

$$(1-R)^2/2R = c/k$$

R is the absolute reflectance of the layer, c is the concentration of analyte, and k is its molar absorption coefficient.

This technique has been used to characterize the structure of several types of materials. For instance it is a recurrent technique to characterize silica based materials such as porous silica, zeolites, sol-gel matrices or even composite materials. In the case of hybrid siloxane organic networks, such as those that we used to immobilize cutinase, this technique can provide information not only about the siloxane backbone network, but also about the organic moieties that are introduced via the sol-gel process[3,4]. DRIFT allows the evaluation of the impact of different organic functionalities on the formation of a particular silica network.

The other technique that was used here to characterize sol-gel matrices was solid state NMR. This spectroscopy is similar in concept to the NMR of liquids, but is characterized by having an anisotropic component, i.e. directionally dependent interaction. Solid state NMR can also be considered a common technique in the materials science field[2]. Using solid state NMR, it is possible to obtain high-resolution spectra providing the same information that is available from the corresponding solution NMR spectra. Nonetheless a number of special techniques and equipment are needed to obtain the same accuracy and resolution. One of those techniques is regarded as one of the most remarkable discoveries in that area: magic-angle spinning (MAS)[2]. The success of this technique is related with the suppression of anisotropic dipolar interactions, which result from the interaction of one nuclear spin with a magnetic field generated by another nuclear spin; and vice versa. In a solid, every magnetic spin is coupled to every other magnetic spin, whereas in solution molecules reorient quite quickly, which makes the spin less dependent from the contribution of dipolar interactions. These dipolar couplings have a dramatic contribution to broaden the NMR spectra, but can be suppressed by MAS[2].

This can be accomplished by introducing artificial motions in the solid, through rotation of the sample around its axis, the latter making a specific angle (54,74º magic angle)

with the axis of the magnetic field. In a very simple way this technique mimics the NMR of liquids by artificially introducing the molecular motions that are usually associated to a liquid. Another important technique used in solid state NMR is cross polarization (CP), which allows the transfer of polarization from the abundant spins such as ^{1}H or ^{19}F to the most dilute spins, such as ^{13}C or ^{15}N. This technique requires that nuclei are dipolar coupled to one another, and it naturally occurs while samples are being spun rapidly at the magic angle. The fundamentals behind these techniques are far from being simple, but the advantages of the MAS with CP are easy to understand in Figure III.III[2], which shows that the spectrum obtained with CP MAS is far more informative than the static solid spectrum.

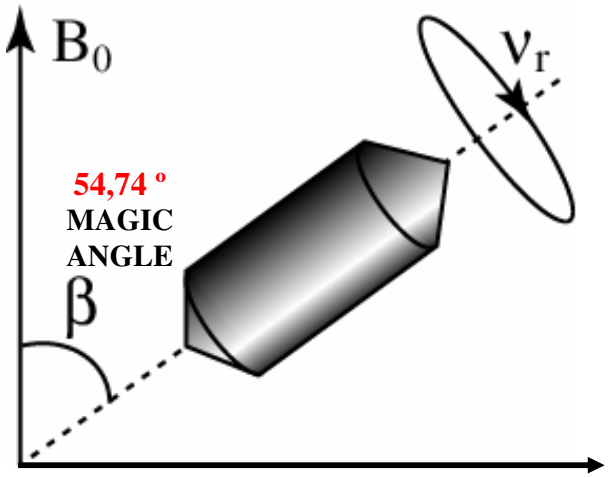

Figure III.II – Magic spin angle (MAS).

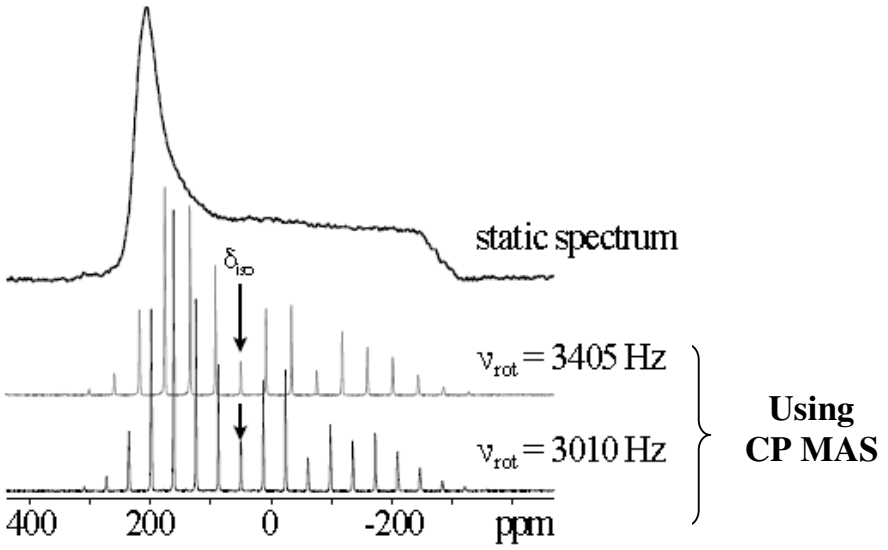

Figure III.III – Differences between a solid state NMR spectrum with or without CP MAS (adapted from reference 2).

^{29}Si was one of the nuclei analyzed in the present thesis, and it is important to have an idea of the information it provides[2,4,5]. ^{29}Si is a spin 1⁄2 species that is more sensitive than ^{13}C. The presence of ^{29}Si in siloxane-silica systems and organic modified silica makes it one of most studied elements is solid state NMR. The next figure helps understand the type of silica structures that can be present in a sol-gel material. The most important for our work are the Q^n and T^n chemical shifts, where Q designates the presence of 4 oxygens that are first neighbours about a Si atom, T the presence of 3 oxygen first neighbours about a Si atom, and the exponent *n* (from 0 to 4 for Q, and from 0 to 3 for T) gives the number of Si second neighbours. The silica chemical shifts are described in the next two figures[2,4,5].

In the study that follows, we have used DRIFT and solid state NMR to evaluate the impact of different sol-gel matrices on cutinase activity.

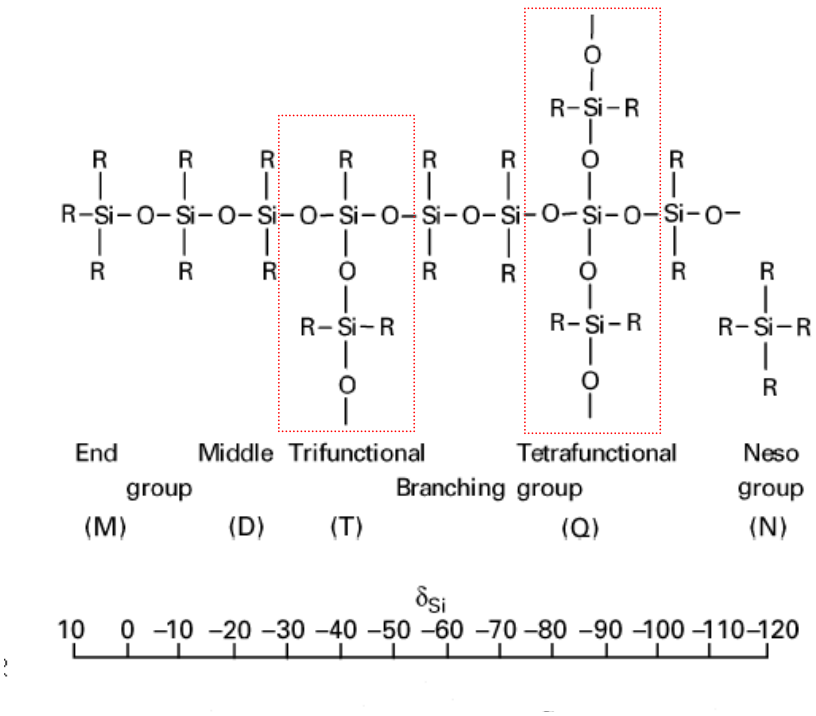

Figure III.IV – Building units of a hypothetical polysiloxane.

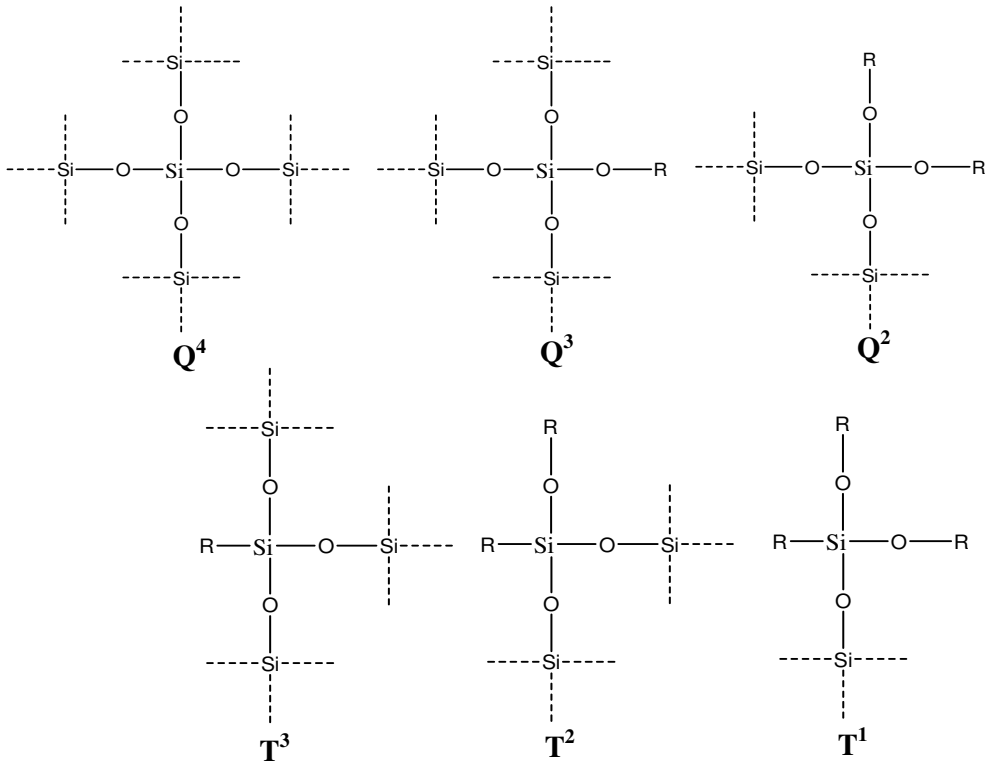

Figure III.V – Slica chemical shift structural representation.

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Structural Basis for the Enhanced Biocatalytic Activity of ORMOSIL Encapsulated Cutinase

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Keywords: cutinase, porous ORMOSIL; DRIFT; NMR.

CONTENTS

ABSTRACT

Cutinase from *Fusarium solani pisi* was encapsulated in sol-gel matrices prepared using combinations of tetramethoxysilane and monoalkyl-methoxysilane precursors with different chain-lengths (from methyltrimetoxysilane to *n*-octyltrimetoxysilane) in molar proportion of 1 to 5. The specific activity of the encapsulated cutinase in a model transesterification reaction in *n*-hexane has been correlated with the structure of the ORMOSIL support. The matrices were characterized by Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectroscopy and by Solid State ^{29}Si and ^{1}H Nuclear Magnetic Resonance (NMR). The enhancement of the bioactivity with increasing alkyl chain lengths up to C_4 was correlated with the nature of the ORMOSIL surface groups, namely with a decrease in the hydrophilic-lipophilic balance (HLB) of the matrix. Such trend suggests that the kinetics of the catalyzed transesterification in *n*-hexane is a diffusion-controlled process. For co-precursors with longer alkyl chains (C_6 and C_8), the activity of encapsulated cutinase suddenly falls, despite the continuous decrease in the matrix HLB. For the ORMOSIL with *n*-hexyltrimetoxysilane, in particular, the enzyme activity is zero, due to the alkyl chain acting as a pore blocker. This effect is more moderate for *n*-ocyltrimetoxysilane, because the alkyl chain has a higher mobility within the inorganic matrix.

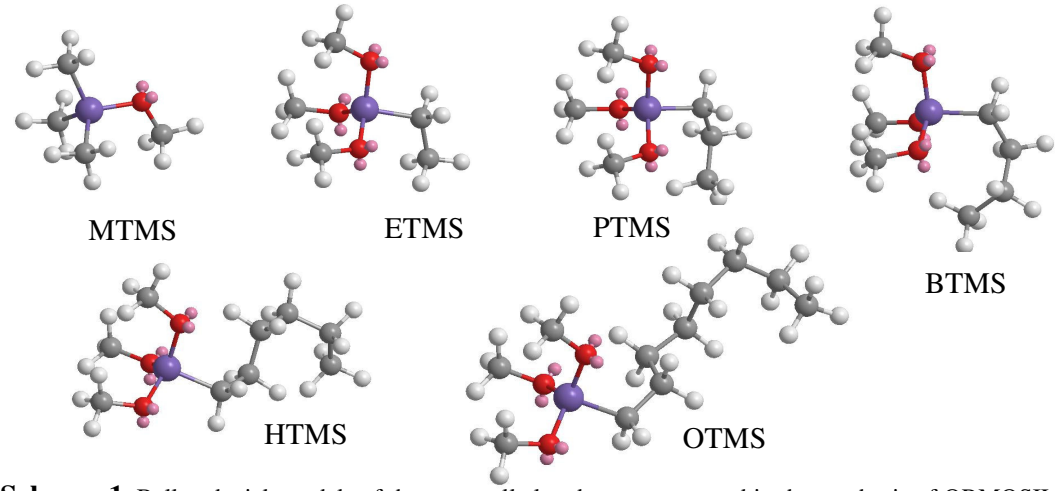
INTRODUCTION

The sol-gel encapsulation of biomaterials, from various types of enzymes to full living cells, is a field under full investigation[1-4].This process has emerged as the most successful method to encapsulate enzymes, given the mild conditions involved, which are ideal to avoid damaging the biological component[5-12]. The variety of inorganic and organic precursors available broadens the range of support properties achievable, and allows an impressive number of possible organically modified silica (ORMOSIL) matrices. The focus is mostly the design of more efficient biocatalysts, with improved activity and stability of the entrapped enzyme. In the past decade, various ORMOSILs were successfully developed as immobilization media of several enzymes and applied to a number of reactions, both in non-aqueous and in aqueous media[13-23]. Many of the enzymes that have been successfully encapsulated in sol-gel matrices are lipases[3-7,11]. The catalytic efficiency of these enzymes has been shown to depend on their local

environment, more lipophilic supports promoting higher catalytic activity. In earlier studies with *n*-alkyltrimetoxysilane precursors, Reetz *et al.* have reported a drastic enhancement of the activity of *Pseudomonas cepacia* lipase up to C_4, followed by a much smoother increase up to C_{18}.[13] In contrast, Chen and Lin observed a dramatic decrease in the activity of *Candida rugosa* lipase for alkyl chains longer than C_3.[24] Nonetheless, much remains to be explained on the reasons behind these effects.

It is the aim of the present work to correlate the structural modifications on the ORMOSIL support with the specific activity of encapsulated cutinase. This is a relatively small serine hydrolase, of approximately 45×30×30 $Å^3$ size, with 197 residues and a molecular mass of 22 kD[25]. Contrarily to many lipases, cutinase has no interfacial activation, possibly because its active site is accessible to the solvent. In lipases, substrate aggregation favors the opening of the lid that usually covers the active site, promoting an increase in catalytic activity. Previous works on cutinase encapsulated in *n*-alkylated ORMOSIL supports have revealed an activity enhancement up to *n*-butyl, a further increase in the *n*-alkyl chain length bringing about a poorer catalytic performance[23].

The different ORMOSIL structures were obtained using combinations of tetramethoxysilane (TMOS) and different monoalkylated precursors in a 1:5 molar ratio. These include methyltrimetoxysilane (MTMS), ethyltrimetoxysilane (ETMS), *n*-propyltrimetoxysilane (PTMS), *n*-butyltrimetoxysilane (BTMS), *n*-hexyltrimetoxysilane (HTMS) and *n*-octyltrimetoxysilane (OTMS), whose structures are shown in Scheme 1.



Scheme 1. Ball and stick models of the mono-alkylated precursors used in the synthesis of ORMOSILs (optimized by energy minimization - MOPAC- Chem 3D Ultra 8.0)

The local structure of the matrices has been assessed by Infrared and Nuclear Magnetic Resonance spectroscopies that provided complementary information[26].

EXPERIMENTAL

Material

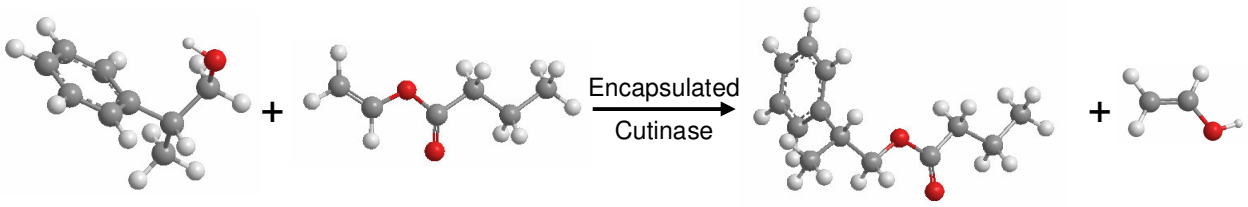
The cutinase producing strain *Saccharomyces cerevisiae* SU50-pUR7320 was constructed and provided by the Unilever Research Laboratory at Vlaardingen, The Netherlands. The production and purification of cutinase was performed at Centro de Engenharia Biológica e Química, Instituto Superior Técnico[27]. The enzyme purity was controlled by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). *(R,S)*-2-phenyl-1-propanol (97 % purity), tetramethoxysilane, methyltrimetoxysilane, ethyltrimetoxysilane, *n*-propyltrimetoxysilane and *n*-octyltrimetoxysilane were from Aldrich, *n*-butyltrimetoxysilane was from Polysciences Inc., *n*-hexyltrimetoxysilane was from Alfa Aesar, *n*-hexane, tridecane, sodium fluoride and sodium chloride were from Merck, vinyl butyrate (99 % purity) was from Fluka, polyvinyl alcohol (PVA; MW15.000) was from Sigma, and (*R*,*S*)-2-phenyl-1-propyl butyrate was prepared as previously indicated[28].

Cutinase encapsulation.

This technique was adapted from that referred by Reetz *et* al.[14] 2 mg of lyophilized cutinase was dissolved in an aqueous solution containing NaF (0.20 g L^{-1}) and PVA (4.64 g L^{-1}). The amount of water in this solution was fixed (6.86 mmol). The solution was vigorously shaken on a vortex mixer. The precursors were then added in amounts that yielded a water/silane molar ratio (R) of 8 irrespective of the type and number of precursors used (e.g. 0.142 mmol of TMOS and 0.712 mmol BTMS in 1:5 TMOS/BTMS gels). The mixture was again vigorously shaken on the vortex mixer, until it became homogeneous. It was then placed in an ice bath until gelation occurred (after a few seconds), and kept in the ice bath for an additional 10 minutes. The container with the obtained gel was kept at 4 ºC for 24 h, after which the gel was air-dried at 35 ºC for 24 h. The white gel obtained was crushed and washed (for about 10 min)/centrifuged (at 5400 rpm), first using phosphate buffer (50 mM, pH 7, 2 mL), then

acetone and finally n-pentane (also 2 mL of each). The gel was left at room temperature for 16 hours, after which it was equilibrated with a saturated salt solution at room temperature for about 3 days, to achieve the value a_W = 0.7 (sodium chloride), taken from the literature.[29] The average yield of immobilization of cutinase in the sol-gel matrix was 91 (± 8) %, as determined by the Lowry method[30].This determination was based on the amount of enzyme found in the aqueous buffer used for washing the gel. Average enzyme loading ranged from 3.2 % in TMOS/MTMS gels to 1.8 % in TMOS/OTMS gels.

Enzyme activity assays and analysis of reaction progress. Reactions were performed in glass vials (reaction volume of 750 µL) placed in a constant temperature orbital shaker set for 400 rpm. All the reaction mixture components were pre-equilibrated to a_W = 0.7 for about 3 days. The reaction studied was the transesterification of vinyl butyrate (300 mM) by (R,S)-2-phenyl-1-propanol (100 mM), as in Scheme 2:



Scheme 2. Model reaction catalyzed by ORMOSIL encapsulated cutinase

The concentration of sol-gel encapsulated enzyme was 6 g L^{-1}. The solvent was *n*-hexane. Vinyl butyrate addition marked the start of the reaction. Tridecane (15.4 mM) was used as external standard for GC analysis. The reaction conversion was measured by GC analysis performed with a Trace 2000 Series Unicam gas chromatograph equipped with a 30 m×0.32 mm i.d. fused silica capillary column coated with a 0.25 µm thickness film of 20 % 2,3-dimethyl-6-tert-butyldimethylsilyl)-ß-cyclodextrin dissolved in BGB-15, from BGB Analytik AG. Oven temperature program: 125 ºC for 2 min, 125-171 ºC ramp at 6 ºC min^{-1}, 200 ºC for 3 min. Injection temperature: 250 ºC. Flame ionization detection (FID) temperature: 250 ºC. Carrier gas: helium (2.0 cm^3 min^{-1}).

Split ratio: 1:20. No products were detected in assays carried out without enzyme. The absolute values of the specific activities of cutinase for this model reaction in different matrices were published elsewhere[23].

Matrices Characterization.

The molecular structures of all the xerogels were characterized by diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy, using a Mattson RS1 FTIR spectrometer with a Specac selector, in the range 4000 to 400 cm^{-1} (with a wide band MCT detector), at 4 cm^{-1} resolution. The spectra were the result of 500 co-added scans for each sample, ratioed against the same number of scans for the background (finely grinded KBr, FTIR grade).

Solid state NMR measurements were performed at room temperature on a Bruker MSL 300P spectrometer operating at 300.13 and 59.60 MHz for the observation of ^{1}H and ^{29}Si resonances, respectively; magic angle spinning (MAS) at 10 and 4 kHz were selected to record proton and silicon spectra, respectively. ^{1}H spectra were run using a single RF pulse with a duration of 2 μs (corresponding to 90º magnetization tip angle) and 10 s recycling delay, τ. ^{29}Si resonances were observed using the standard cross polarization – dipolar decoupling RF pulse sequence (CP-DD) with 5 ms contact time and τ equal to 60 s. The Hartmann-Hahn condition was optimized using tetrakis-trimethylsilyl-silane. Tetramethylsilane (TMS) was used as external reference to set ^{1}H and ^{29}Si chemical shift scales (δ=0).

RESULTS & DISCUSSION

Effect of the Alkyl Chain Length.

The relative activities of encapsulated cutinase for the model reaction referred above are shown in Fig. 1, as a function of the number of carbon atoms in the alkyl modifying chain. These values (in %) were determined taking as reference the maximum activity observed in the matrix TMOS/BTMS.

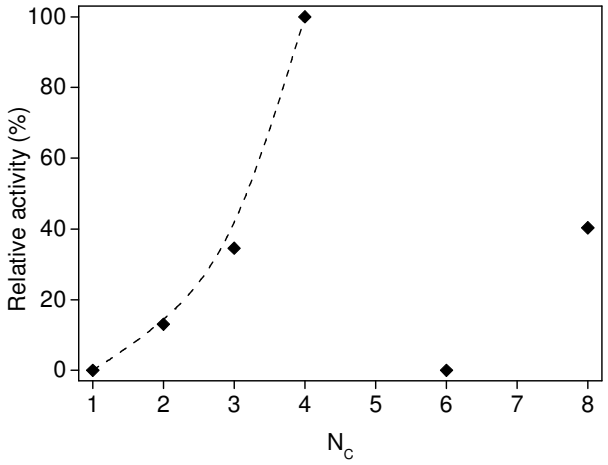


Figure 1 - Variation of the relative activity of cutinase (%) with the number of carbon atoms in the chain of the alkylated precursor.

Clearly, the activity drops for chains longer than C_4 and the ORMOSIL with HTMS is the poorest support. It is common to invoke the network lipophilicity to interpret such enzyme activity trends. However, in ORMOSILs the silica network can retain variable amounts of hydrophilic residual silanol groups that also affect the enzyme environment. Therefore, the activity changes should rather be correlated with a parameter that takes into account the two types of interactive sites, *i.e.*, the hydrophilic/lipophilic balance (HLB).[31] Spectroscopic techniques such as infrared and nuclear magnetic resonance are powerful tools that may ascertain the structural parameters to explain the dependence of the catalyst activity on the matrix.

The DRIFT spectra of the TMOS/MTMS matrix, with and without encapsulated cutinase, are shown in Fig. 2. Their almost perfect overlapping proves that the enzyme entrapment within the matrix does not alter significantly the silica backbone structure.

In the low wavenumber region, the skeletal vibrations are the asymmetric Si-O-Si stretching, ν_{as}SiOSi (1000-1200 cm^{-1}), the Si-O stretching in broken siloxane bridges, νSi-O (910 cm^{-1}), and the symmetric Si-O-Si stretching, ν_{s}SiOSi (~800 cm^{-1}). The latter is overlapped with the methyl rocking mode, ρCH_3 (780 cm^{-1}). The sharp band at 1273 cm^{-1} is assigned to the symmetric deformation mode of methyl groups bonded to Si atoms, $\delta_s(Si)CH_3$. In the high wavenumber region, only the broad hydroxyl stretching band, νOH (centered at 3326 cm^{-1}), and the methyl stretching modes, νCH_3 (asymmetric and symmetric at 2972 and 2912 cm^{-1}, respectively) are observed.

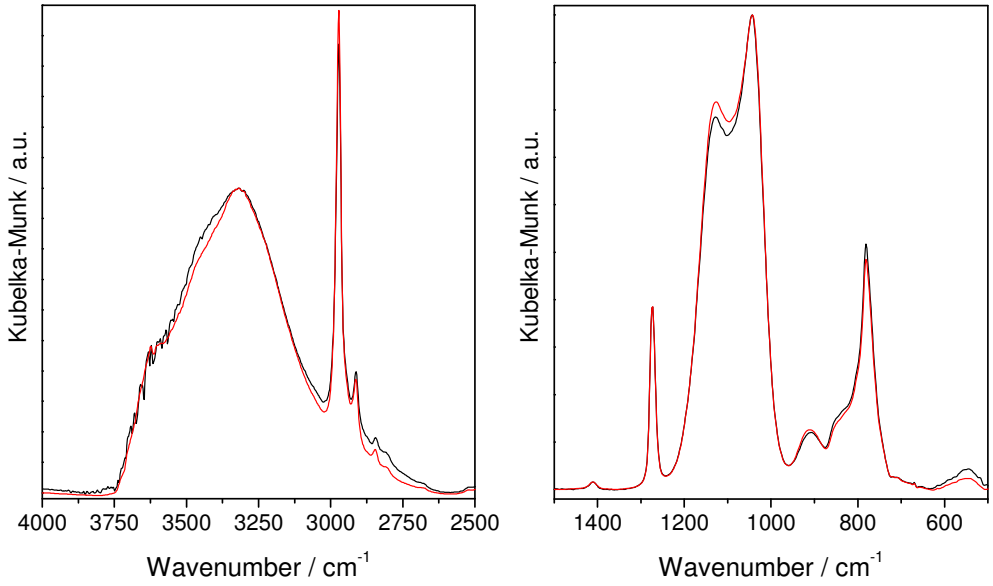


Figure 2 - DRIFT spectra of the TMOS/MTMS ORMOSIL with (black line) and without (red line) enzyme. 4000-2500 cm^{-1} region normalized to the maximum of the νOH band; 1500-500 cm^{-1} region normalized to the maximum of the ν_{as}SiOSi band.

From Figure. 2, it becomes clear that the silica network and even the type and number of hydrogen bonding between hydroxyl and silanol groups in the matrix remain unchanged upon enzyme entrapment. Additionally, the enzyme concentrations used are so low that its vibrational modes do not affect the spectra. As a result, it is reasonable to assume that the structural analysis of the matrices may be based on the DRIFT spectra of ORMOSILs with encapsulated cutinase.

The DRIFT spectra of the cutinase-containing ORMOSIL matrices with different alkyl chain lengths are shown in Fig. 3, normalized to the most intense band, the ν_{as}SiOSi, at ~1100 cm^{-1}. The primary assignments of the main spectral regions are indicated.

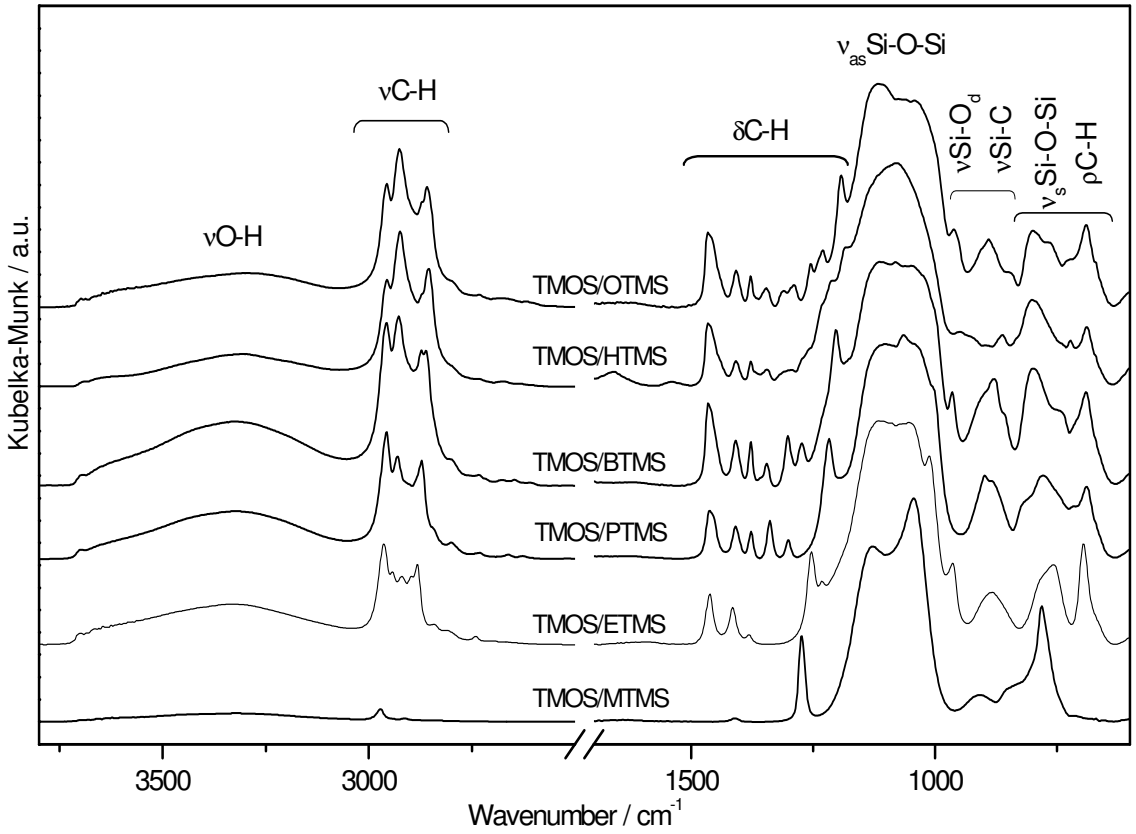


Figure 3 - DRIFT spectra of cutinase-encapsulating ORMOSILs with different mono-alkyl (from methyl- to octyl-) chain lengths. Spectra normalized to the maximum of the ν_{as}SiOSi band (~1100 cm^{-1}).

The spectrum of TMOS/MTMS is much simpler than the others, due to the absence of CH_2 groups in the matrix. Since in these spectra there were no traces of water (the δHOH that should appear at ~1640 cm^{-1} is absent), the νOH band is assigned to non-condensed silanol groups. The relative intensity of this band (with respect to the ν_{as}SiOSi) increases with the alkyl chain length up to butyl and decreases from there on. The hydrolysis and/or condensation reactions are certainly hindered by the alkyl chains, the net effect depending not only on the chain length but also on the possible rotational conformers.[32] This matter will be addressed below by a quantitative spectral analysis. The relative intensities of the νCH related bands also have a maximum for the MTMS/BTMS matrix, suggesting that the proportion of alkyl chains that effectively remain in the matrix is lower for chains longer than C_4. Possibly, phase separation occurs during the sol-gel process for excessively long chains.

The profile of the ν_{as}SiOSi band, which is the fingerprint of the silica structure, suggests that the inorganic structure is significantly modified by the participation of the alkylated precursors. In view of the partial overlapping of the Si-O stretching and the C-H deformation modes, the individual components were retrieved by spectral decomposition in the range between 1550 and 650 cm^{-1}. A non-linear least squares fitting method was used, assuming Gaussian and/or Lorentzian band profiles. The first approach to the components' maxima was previously determined by the second derivative of the spectra. The spectral decomposition of the TMOS/MTMS matrix is shown in Fig. 4-A. Figs. 4-B to D depict amplifications of the decomposition in different spectral regions for other selected matrices.

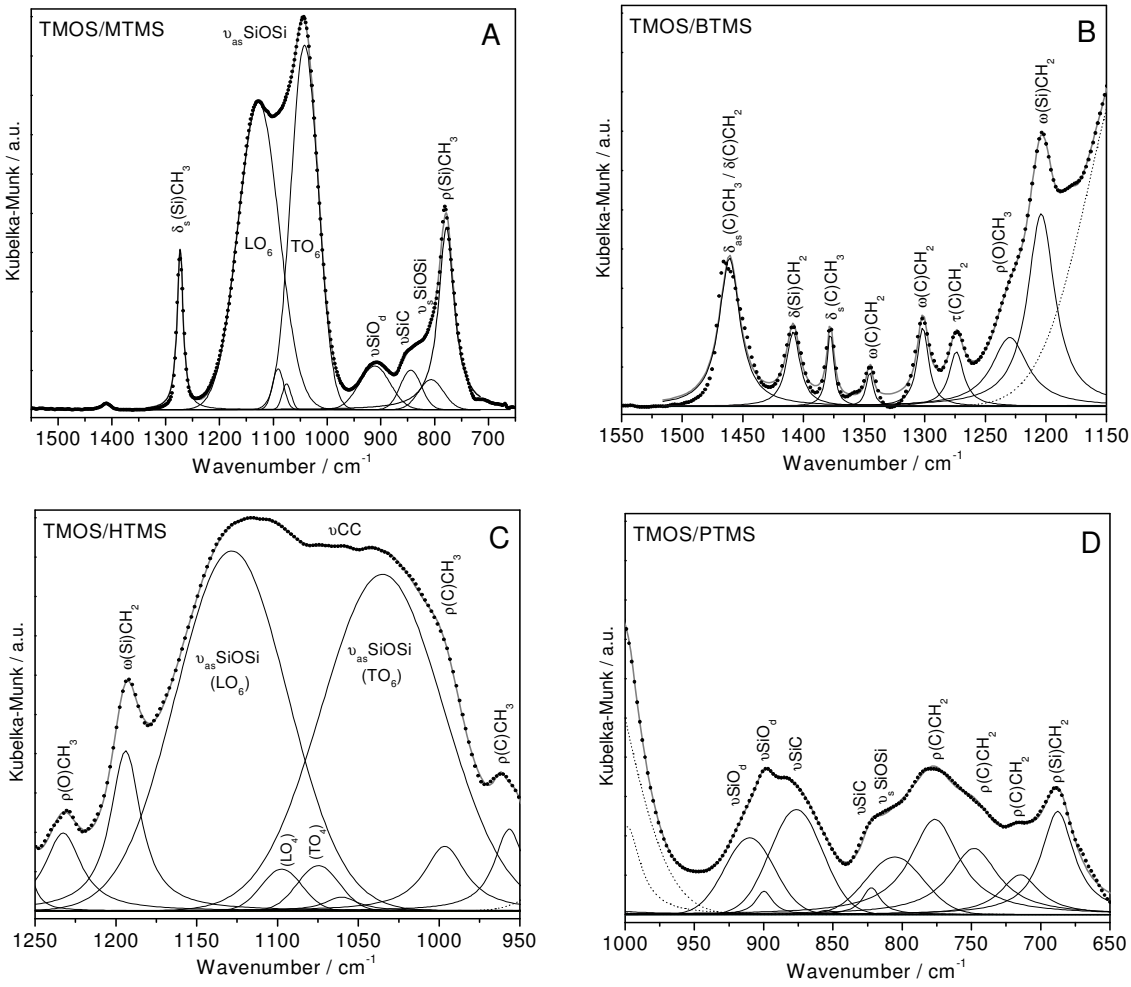


Figure 4 - Decomposition of the DRIFT spectra in the 1550-650 cm^{-1} region: A - the whole wavenumber range for TMOS/MTMS matrix; B - amplification of the region 1550-1150 cm^{-1} for the TMOS/BTMS matrix; C - amplification of the region 1250-950 cm^{-1} for the TMOS/HTMS matrix; D - amplification of the region 1000-650 cm^{-1} for the TMOS/PTMS matrix

The proposed assignment, fitted frequency and relative area of each component with respect to the fully integrated spectrum are summarized for all the samples in Table 1.

Table 1 - Assignments, positions (cm^{-1}) and relative areas (A%) of the spectral components for ORMOSIL matrices with different mono-alkyl chain lengths.

Wavenumber / cm^{-1} (A%)						Assignment[3,4]
MTMS	ETMS	PTMS	BTMS	HTMS	OTMS	
3326 (8.6)	3326 (20.3)	3324 (21.6)	3327 (21.9)	3305 (16.8)	3307 (13.7)	νOH
2972 (0.8)	-	-	-	-	-	$\nu_{as}(Si)CH_3$
-	2965/2941 (5.2)	2959 (5.6)	2960 (6.2)	2960 (5.4)	2959 (5.3)	$\nu_{as}(C)CH_3$
-	2920 (2.5)	2929 (4.1)	2927 (5.8)	2926 (7.0)	2926 (8.5)	$\nu_{as}(C)CH_2/\nu_{as}(O)CH_3$
2912 (0.3)	-	-	-	-	-	$\nu_s(Si)CH_3$
-	2880 (2.9)	2873 (3.5)	2874 (2.5)	2874 (1.7)	2873 (1.5)	$\nu_s(C)CH_3$
-	-	-	2859 (3.2)	2856 (4.1)	2854 (4.0)	$\nu_s(C)CH_2/\nu_s(O)CH_3$
-	2841 (0.5)	2839 (0.2)	2837 (0.1)	-	-	$\nu_s(C)CH_2$
-	2807 (0.6)	2799 (0.5)	2797 (0.1)	2799 (0.2)	2798 (0.2)	$\nu_{as}(Si)CH_2$
-	2740 (0.2)	2732 (0.1)	2732 (0.1)	2737 (0.1)	2730 (0.2)	$\nu_s(Si)CH_2$
-	1462 (1.5)	-	-	-	-	$\delta_{as}(C)CH_3$
-	-	1459 (1.5)	1461 (2.1)	1460 (2.7)	1460 (2.2)	$\delta_{as}(C)CH_3/\delta(C)CH_2$
1410 (0.2)	-	-	-	-	-	$\delta_{as}(Si)CH_3$
-	1415 (1.0)	1409 (0.6)	1408 (0.7)	1408 (0.7)	1408 (0.5)	$\delta(Si)CH_2$
-	1381 (0.1)	1377 (0.3)	1378 (0.4)	1378 (0.3)	1377 (0.3)	$\delta_s(C)CH_3$
-	-	1339 (0.6)	1345 (0.2)	1347 (0.3)	1348 (0.5)	$\omega(C)CH_2$
-	-	1301 (0.2)	1301 (0.6)	1310 (0.2)	1303 (0.3)	$\omega(C)CH_2$
1273 (4.1)	-	-	-	-	-	$\delta_s(Si)CH_3$
-	-	-	1274 (0.6)	1291 (0.4)	1274 (0.2)	$\tau(C)CH_2$
-	-	-	-	1255 (0.5)	1261 (0.5)	
	1254 (2.7)					$\omega(Si)CH_2$
-	1232 (0.1)	1231 (0.2)	1230 (1.7)	1233 (1.8)	1234 (2.0)	$\rho(O)CH_3$
-	-	-	-	-	1214 (2.7)	$\tau(C)CH_2$
-		1218 (4.4)	1204 (3.7)	1194 (3.2)	1187 (2.4)	$\omega(Si)CH_2$
-	1196 (0.6)	1193 (0.6)		-	-	$\tau(C)CH_2$
1127 (35.5)	1128 (22.6)	1122 (19.3)	1123 (17.0)	1128 (17.4)	1127 (20.0)	$\nu_{as}SiOSi$ (LO_6)
1091 (1.3)	1095 (0.7)	1098 (0.3)	1096 (0.3)	1097 (0.7)	1099 (0.3)	$\nu_{as}SiOSi$ (LO_4)

1075 (0.7)	1077 (0.6)	1083 (0.4)	1078 (0.5)	1075 (0.8)	1076 (0.6)	ν_{as}SiOSi (TO_4)
-	-	1065 (1.8)	1061 (0.7)	1060 (0.3)	-	νCC
1042 (28.3)	1046 (19.1)	1033 (15.3)	1032 (11.2)	1035 (17.8)	1043 (15.6)	ν_{as}SiOSi (TO_6)
-	1007 (3.0)	998 (2.1)	998 (2.3)	996 (1.7)	1001 (1.9)	ρ(C)CH_3
-	963 (2.2)	-	963 (1.2)	952 (1.4)	948 (2.5)	
911 (3.8)	901 (1.2)	910 (2.2)	910 (2.5)	910 (2.0)	910 (1.5)	νSiO_d (*)
-	-	900 (0.3)	-	-	-	
844 (2.4)	873 (2.7)	876 (2.6)	876 (2.5)	879 (2.0)	874 (1.6)	νSiC
-	-	824 (0.5)	853 (0.6)	846 (0.4)	861 (0.3)	
807 (2.1)	792 (2.7)	805 (1.7)	799 (4.3)	797 (3.2)	804 (3.7)	ν_sSiOSi
778 (11.9)	-	-	-	-	-	ρ(Si)CH_3
-	-	776 (3.5)	760 (2.2)	760 (2.0)	771 (2.9)	ρ(C)CH_2
-	752 (4.1)	748 (2.4)	739 (1.3)	-	745 (0.9)	
-	-	715 (1.1)	710 (0.7)	725 (1.4)	721 (0.8)	
-	694 (3.0)	688 (2.4)	689 (3.1)	689 (3.6)	688 (2.4)	ρ(Si)CH_2

(*) -O_d stands for dangling oxygen atoms, *i. e.*, silanol groups and broken siloxane bridges.

The maximum of the broad νOH band shifts to lower wavenumbers when the alkyl chain is longer than butyl, suggesting the presence of more interactive hydroxyl groups in these matrices. The relative intensities indicated in Table 1 (with respect to the full spectrum) confirm that the matrix with BTMS has the highest content in hydroxyl groups.

Only for the ORMOSIL with MTMS is it possible to observe the modes of methyl groups bonded directly to Si: ν_{as}(Si)CH_3, ν_s(Si)CH_3, δ_{as}(Si)CH_3, δ_s(Si)CH_3 and ρ(Si)CH_3, at 2972, 2912, 1410, 1273 and 778 cm^{-1}, respectively. No other methyl related bands were observed in this matrix. In particular, the absence of (O)CH_3 modes is a good indication that hydrolysis of both co-precursors was complete. For all the other modified matrices, the methyl modes observed may be related to (C)CH_3 and/or (O)CH_3 groups. For those groups bonded to a C atom, the assignment is straightforward: ν_{as}(C)CH_3, ν_s(C)CH_3, δ_{as}(C)CH_3, δ_s(C)CH_3 and ρ(C)CH_3 appear at ~2960, ~2874, ~1460, ~1378 and ~998 cm^{-1}, respectively. Slight shifts of these bands are observed for TMOS/ETMS, consistent with the proximity of the Si atom. The presence of non-hydrolyzed methoxy groups is definitely confirmed by the ρ(O)CH_3, at

~1232 cm^{-1}, in matrices with alkyl chains longer than ethyl, although its relative intensity becomes meaningful only for chains with C≥4. This confirms that the hydrolysis reaction is in fact hindered by the presence of alkylated co-precursors, mainly with chains longer than butyl. The assignment of the $(O)CH_3$ stretching modes is ambiguous, since they appear at the same frequencies as the corresponding methylene modes.

With the exception of MTMS, the Si atoms in the alkylated precursors are bonded to methylene groups. The frequencies and relative intensities of the corresponding stretching and rocking modes in the matrix are not much affected by the alkyl chain length: the $\nu_{as}(Si)CH_2$, $\nu_s(Si)CH_2$ and $\rho(Si)CH_2$, modes appear at ~2800, ~2735 and ~690 cm^{-1}, respectively. However, the wagging mode, $\omega(Si)CH_2$, shifts to lower wavenumbers and decreases in relative intensity as the alkyl chain increases, probably due to conformational hindering of this mode for longer chains[33]. The remaining methylene groups are bonded only to C atoms and are not all equivalent. The presence of several CH_2 rocking components, whose frequencies vary with the alkyl chain length, is a good indication that there is a distribution of stable rotational conformers within the network. However, the HTMS modified matrix is somewhat different from the others: it has only two such modes (at 725 and 760 cm^{-1}), which suggests a lower fraction of gauche conformers.

The information on the silica network is assessed mostly from the decomposition of its fingerprint band, the $\nu_{as}SiOSi$. This band is usually split into optic components, longitudinal (LO) and transverse (TO), presenting a LO/TO pair for each main type of cyclic structural unit (siloxane ring)[35]. For silica xerogels, the most common types of cyclic units have six and four Si atoms (cyclohexa- and cyclotetra-siloxane rings)[36] and the corresponding optic components are named in Table 1 as LO_6/TO_6 and LO_4/TO_4, respectively. The results summarized in Table 1 allow concluding that the silica network in all these ORMOSILs is mostly composed of cyclohexasiloxane rings (above 96%, estimated as the following ratio of % areas: $[LO_6+TO_6]/[LO_6+TO_6+LO_4+TO_4]$). If these were pure silica xerogels, this result would point to porosities above ~80%[37]. In organically modified structures, however, it must be taken into account that the pores may be partially occluded by the organic moieties, resulting in lower effective

porosities. The ORMOSIL modified with HTMS has the lowest proportion of cyclohexasiloxane rings, which is consistent with the lowest porosity.

Based on the above spectral decompositions, relevant trends of structural parameters were assessed from the following percentages: condensed silica (%SiOSi), hydrophilic Si-OH and Si-O_d groups (%OH), lipophilic -CH_x groups (%CH) using equations (1) to (3). The matrix hydrophilic/lipophilic balance (HLB) was estimated according to equation (4).

$$\%SiOSi = 100\times(A_{LO6} + A_{LO4} + A_{TO4} + A_{TO6})/A_T \quad (1)$$

$$\%OH = 100\times(A_{\nu OH} + A_{\nu SiOd})/A_T \quad (2)$$

$$\%CH = 100\times(\sum A_{\nu CH} + \sum A_{\delta CH} + \sum A_{\tau CH} + \sum A_{\omega CH} + \sum A_{\rho CH})/A_T \quad (3)$$

$$HLB = (A_{\nu OH} + A_{\nu SiOd})/(\sum A_{\nu CH} + \sum A_{\delta CH} + \sum A_{\tau CH} + \sum A_{\omega CH} + \sum A_{\rho CH}) \quad (4)$$

A_X is the fitted area of the X component and A_T refers to the total integrated area of the spectrum. The variation of these structural parameters with the alkyl chain length is shown in Fig. 5.

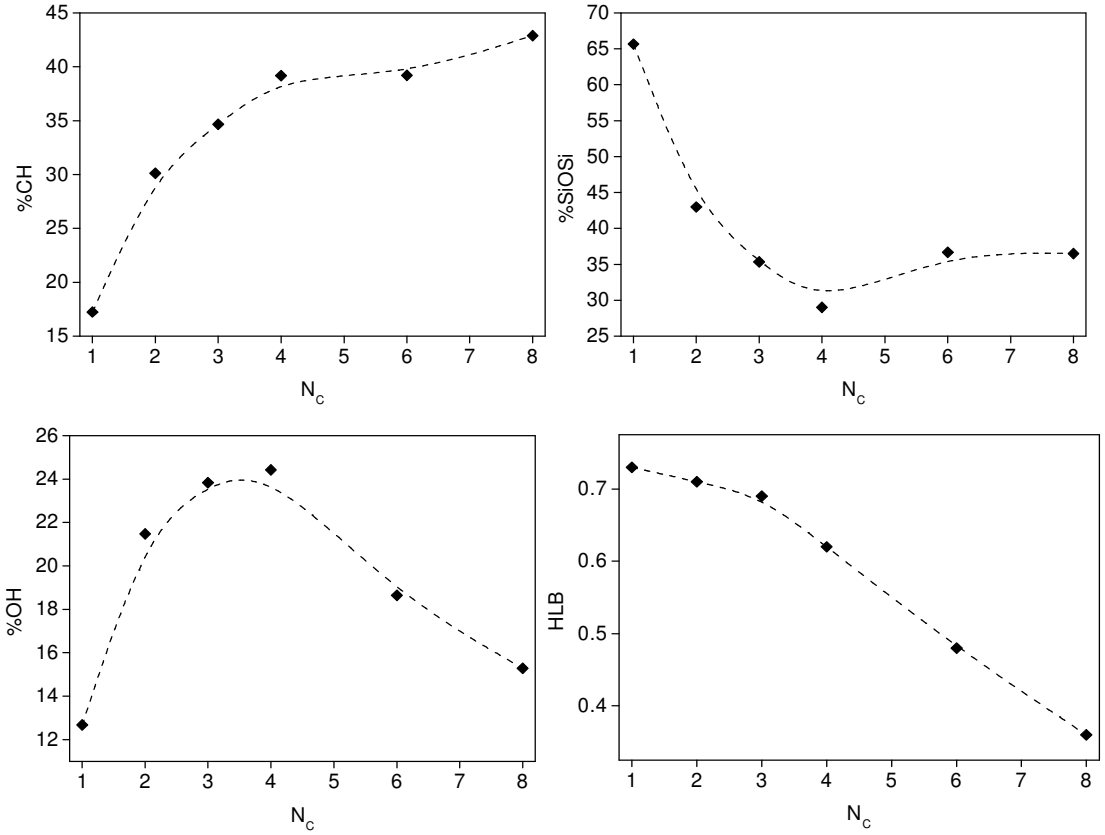


Figure 5 - Influence of the number of carbon atoms in the chain of the alkylated precursor (N_C) on structural parameters of the ORMOSIL: condensed silica (%SiOSi), alkyl (%CH) and silanol (%OH) contents, and hydrophilic-lipophilic balance (HLB).

For alkylated precursors with chains up to C_4, the expected general trend is observed: an increase in the organic content of the ORMOSIL, with the consequent decrease in the condensed silica content. This is accompanied by an increase in the residual silanol groups, which proves the fall in condensation extent with increasing chain length. Although both the hydroxyl and alkyl contents increase in the matrix, the hydrophilic-lipophilic balance (HLB) decreases, attesting the prevalence of the latter. For alkylated precursors with chains longer than C_4, the organic content of the ORMOSIL is lower than would be expected, in a clear indication that some of these precursors did not hydrolyze, and were subsequently removed upon washing. In these systems hydrolysis is hindered by the longer alkyl chains and more methoxy groups remain within the network. However, condensation is more extensive, yielding a lower content in silanol groups. Consequently HLB is determined by the hydrophilic content and keeps decreasing for matrices TMOS/HTMS and TMOS/OTMS. For alkyl chains with $N_C \leq 4$, the estimated structural parameters correlate with the relative activity of entrapped cutinase, as shown in Fig. 6.

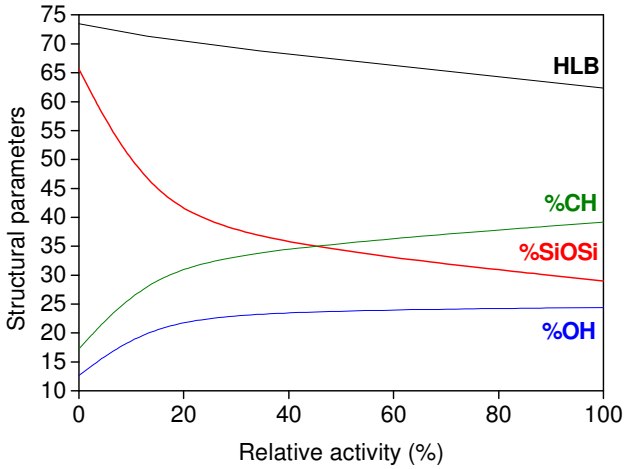


Figure 6 - Correlation between the structural parameters of ORMOSILs up to four C atoms and the relative activity of entrapped cutinase. The HLB scale has been multiplied by the factor 100.

For the model reaction studied, the activity of encapsulated cutinase increases with the decrease in the condensed HLB of the matrix. This is consistent with a diffusion-controlled kinetics, in which the solvent-matrix interactions are determinant of the enzyme activity: as the solvent is *n*-hexane, these interactions are stronger for matrices with lower HLB values [31].

For TMOS/HTMS and TMOS/OTMS matrices (N_C>4), the observed decrease in the enzyme activity, despite the continuous decrease of HLB, indicates that a pore blocking effect by the long alkyl chains plays the major role, delaying diffusion. This effect is more important for the stiffer *n*-hexyl chain, the enzyme activity becoming zero in the TMOS/HTMS matrix. The matrices TMOS/MTMS, TMOS/BTMS (where the encapsulated cutinase has the highest activity) and TMOS/OTMS (with a longer chain length but with activity different from zero) were analyzed by ^{29}Si and ^{1}H NMR. Such analysis has already proven to be a reliable tool to characterize inorganic/organic gels[7].

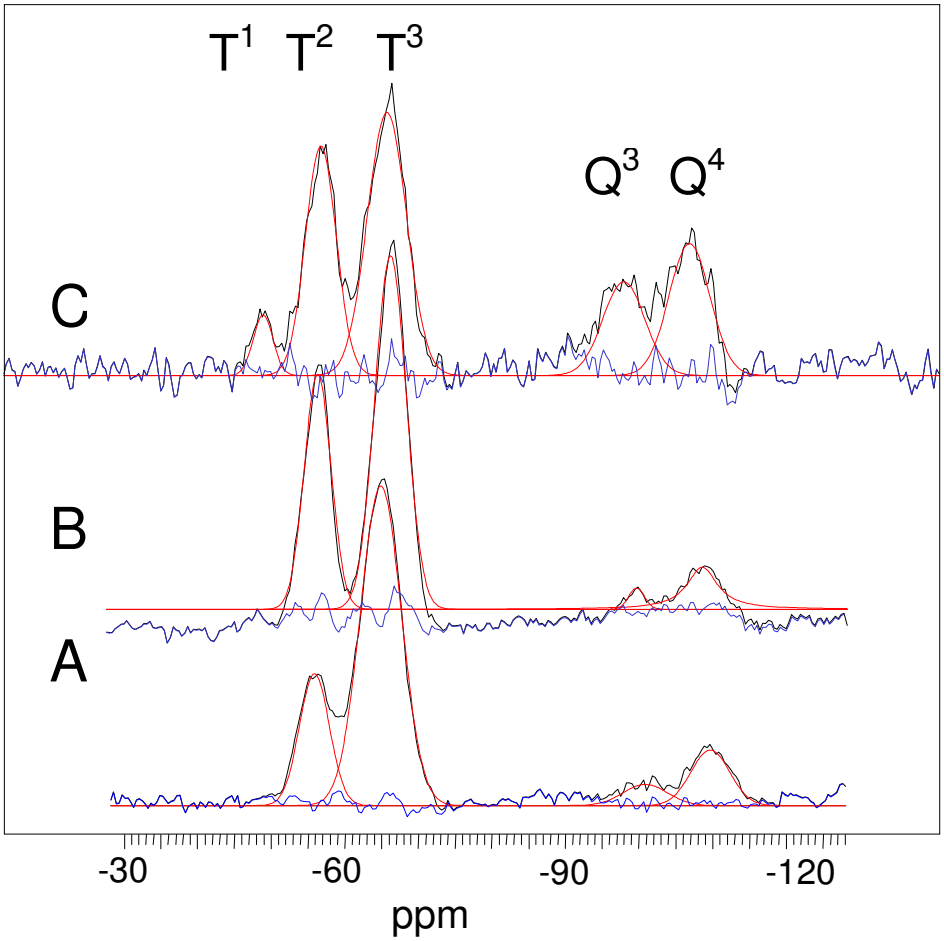


Figure 7 - ^{29}Si CP/MAS-DD spectra obtained from different matrices: A - TMOS/MTMS; B - TMOS/BTMS; C - TMOS/OTMS. The signals were decomposed using Gaussian functions; the residues are displayed in blue color.

Figure 7 shows the ^{29}Si CP/MAS-DD spectra recorded from TMOS/MTMS, TMOS/BTMS and TMOS/OTMS. At least four peaks were observed in all spectra: a)

around -100 and -110 ppm, assigned to silicon atoms in Q^3 [$(SiO)_3$-Si-OR] and Q^4 [$(SiO)_4$-Si] units, respectively; b) at about -57 and -67 ppm, from silicon atoms in monoalkylated T^2 [$(SiO)_2(OR)$Si-R'] and T^3 [$(SiO)_3$-Si-R'] units, where R stands for H or CH_3 and R' for the alkyl chain. T^1 [$(SiO)(OR)_2$Si-R'] units were only observed for the TMOS/OTMS matrix (at -50 ppm), which can be explained by hindrance of the reactive Si-OR vicinal groups by the long *n*-octyl chain. This precursor is thus less hydrolysable and co-condensable with hydrolyzed TMOS, as suggested by the DRIFT results.

The signals were decomposed using Gaussian functions, and the percent integrated areas and their ratios are summarized in Table 2. Since the ^{29}Si CP/MAS-DD technique enhances the sensitivity of silicon atoms near OR or R' groups, only the intensities of resonances recorded from similar silicon species may be compared (T^m with $T^{m'}$ and Q^n with $Q^{n'}$) on the assumption that spin dynamics is also comparable; the contact time dependencies of the ^{29}Si CP/MAS signal intensities were reported on some ORMOSILs gels.38[5] The most interesting about this analysis is that it allows attaining further insight to the reaction details for each of the two co-precursors.

Table 2 - Integrals (percent units) of the individual components obtained by decomposition of ^{29}Si spectra, distribution of silicate structures, and overall degree of reaction (η, %) estimated for TMOS and the alkylated precursor.

Sample	T^1	T^2	T^3	Q^3	Q^4	Q^3:Q^4	T^1:T^2:T^3	η^{TMOS} (%)	$\eta^{alkylTMS}$ (%)
TMOS/MTMS	0	25	61	4	10	1: 2.6	0:1: 2.4	68.1	90.3
TMOS/BTMS	0	36	54	3	7	1: 2.0	0:1: 1.5	66.7	86.7
TMOS/OTMS	8	29	34	12	17	1: 1.3	0.3:1:1.2	64.5	79.0

For TMOS derived Si atoms (Q^n units), although the Q^4 units are always predominant, its proportion decreases continuously as the chain of the co-precursor increases, in a clear indication that the hydrolytic-polycondensation of TMOS is negatively affected. Similarly, for Si atoms from the alkylated precursor (T^m units) the proportion of T^3 decreases continuously. Thus, the inhibiting role of the alkyl chain on the sol-gel reactions is felt by both precursors.

The reaction yields for the silica network from TMOS (η^{TMOS}) and alkylTMS ($\eta^{alkylTMS}$) may be estimated separately by comparing the effective Si functionality, f_{eff}, with the potential functionality (the maximum number of siloxane bridges that the Si atom can establish, assuming full hydrolysis: 4 for TMOS and 3 for alkylTMS)[26].

$$\eta^{TMOS} = 100 * f_{eff}^{TMOS}/4 \quad (5)$$

$$\eta^{alkylTMS} = 100 * f_{eff}^{alkylTMS}/3 \quad (6)$$

The effective functionality can be roughly estimated from:

$$f_{eff}^{TMOS} = \sum[n \times x(Q^n)] \quad (7)$$

$$f_{eff}^{alkylTMS} = \sum[m \times x(T^m)] \quad (8)$$

where $x(Q^n)$ is the relative proportion of Q^n species (n varying between 3 and 4) and $x(T^m)$ the proportion of T^m species (m varying between 1 and 3). The yield values obtained are listed in the last two columns of Table 2.

It is interesting to note that the condensation of TMOS is the most affected by the presence of the alkylated precursor, despite the fact that the condensation reactions of both precursors are hampered by the increase in the alkyl chain length.

Fig. 8 shows the isotropic signals of ^{1}H MAS spectra recorded from TMOS/MTMS, TMOS/BTMS and TMOS/OTMS, which exhibit different line widths. Narrow peaks are assigned to hydrogen atoms in mobile chains, and, when no spinning side bands are observed, the H-H dipolar interactions are completely averaged out under MAS at 10 kHz. Broad isotropic resonances and the corresponding envelope of spinning side bands, which spread over 30 kHz, are from rigid sites, in which case H-H dipolar interaction should be of that order of magnitude and, hence, only averaged out under MAS above 30 kHz (not available in our NMR laboratory). Consequently, from experiments performed at much lower MAS speed it is incorrect to obtain quantitative information using only isotropic signals.

The major isotropic resonances depicted in Figs. 8A and 8B are very broad (the full widths at half maximum, FWHM, are higher than 1800 and 700 Hz, respectively), as expected for rigid matrices, where many structural variations in terms of angles and distances result in inhomogeneous spectral broadening. The less intense signal observed for the TMOS/MTMS matrix (signal 1, at about 3.7 ppm, in Fig.8A) may be tentatively

assigned to unreacted (Si)OCH_3 groups of MTMS or to uncondensed (Si)OH groups involved in hydrogen bonding[39,40]. However, the latter appears to be most probable, since no residual (Si)OCH_3 groups were identified by DRIFT. The most intense signal (2, at about 0.6 ppm) results from a strong (Si)CH_3 contribution. For matrix TMOS/BTMS, resonances from (Si)OH, (Si)OCH_3, (C)CH_2, (C)CH_3 and (Si)CH_2 groups may contribute to the very broad signal (Fig.8B, signal 3, centered at about 1 ppm). The major signals from TMOS/OTMS matrix (Fig. 8C, signals 4 and 5) are significantly narrower than the resonances recorded from the other matrices (the FWHM of the convoluted signal is about 400 Hz) and are assigned to: all CH_2 groups except (Si)CH_2 (at about 1.4 ppm) and chain terminal CH_3 (at 0.9-0.6 ppm), respectively. The signal 6 (at about 0.2 ppm) is probably from (Si)CH_2 groups. This observation is consistent with the high mobility of the alkyl chain in the TMOS/OTMS matrix.

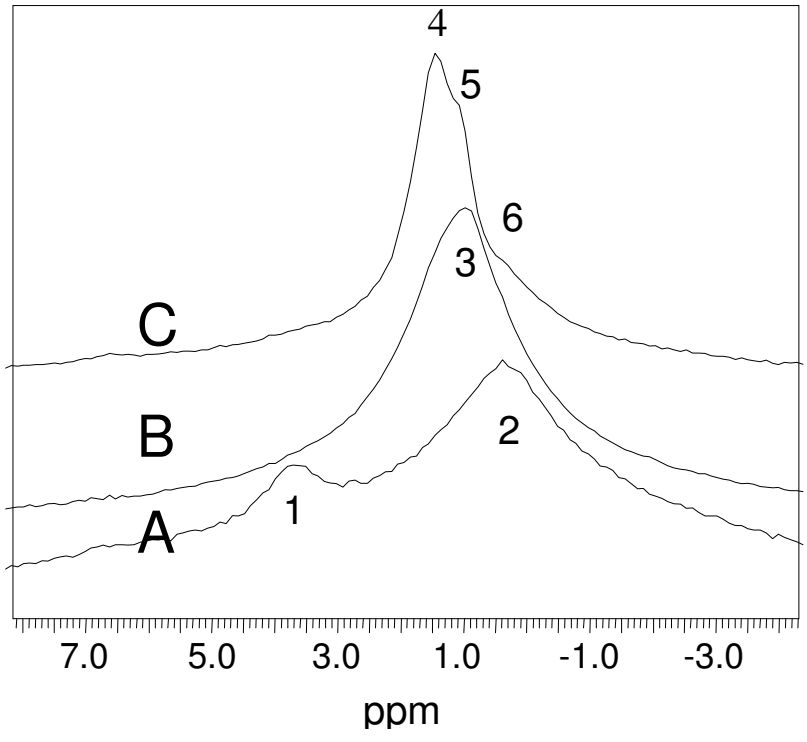


Figure 8 - ^{1}H MAS spectra obtained from different matrices: A - TMOS/MTMS, B - TMOS/BTMS; C - TMOS/OTMS.

CONCLUSIONS

The two complementary spectroscopic techniques used in the present work brought further insight into the source of the modifications in enzymatic behavior of lipases, due to encapsulation in organically modified sol-gel silicas.**Error! Bookmark not defined.**

In systems where the co-precursors are R-Si-$(OCH_3)_3$ and $Si(OCH_3)_4$ (in 5 to 1 molar ratio), it was shown that increasing R length inhibits both the hydrolysis and condensations reactions of the two precursors, with important effects on the structure of the resulting ORMOSIL matrix. Thus, dramatic modifications in the bioactivity of encapsulated cutinase result.

In non-polar media, it was observed that the activity of entrapped cutinase increases with the chain length of the alkylated precursor up to C_4, decreasing abruptly for C_6. This could only be interpreted in terms of a diffusion-controlled mechanism. In this case, the bioactivity is mostly dependent on the matrix-solvent interactions, since the accessibility to the enzyme active site plays the key role. The matrix determining parameter is its hydrophilic-lipophilic balance (HLB): the lower its value (increasing alkyl chain length of the modifying precursor), the more efficient becomes diffusion in non-polar solvents. However, partial pore-blocking by long alkyl chains hampers diffusion and the chain mobility becomes the dominant parameter on the enzyme activity: stiffer chains (as in the matrix TMOS/HTMS) are responsible for extremely low enzyme activities.

ACKNOWLEDGMENTS.

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CHAPTER IV

“Ion Jelly”

A versatile tailor-made conductor

The last chapter of this thesis started with biocatalysis but followed a completely new research line. Our initially line was to combine the best of both worlds, the capacity that ionic liquids have to modulate to enzymatic properties and the advantages of enzyme immobilization using sol-gel processing. The model enzyme was cutinase and the model reactions that we were interest in were the kinetic resolutions of secondary alcohols. This type of approach was the most logical since we could combine the results obtained in chapter II and see if we could further improve them using the immobilization techniques describe on chapter III. We have this objective in mind since ionic liquids have demonstrated to be quite good solutions to enhance enzyme properties. The relatively success of both previous chapters was not followed on this approach. The results obtained here were far from being positive since the combination of ionic liquids had a quite negative impact on cutinase activity. At the time we concluded that ionic liquid was affecting the sol-gel process, since in literature ionic liquids were described as templates of sol-gel matrices[1]. This leaded us to use different materials to accomplish the immobilization of the ionic liquids. Our first approach was to create a polymeric bead containing ionic liquid, and for that we tried to use materials like alginate or gelatin. If the first reveal unexpected difficulties and needed a further development the second was perfectly suitable for our intents. The ionic liquid was trap inside a polymeric matrix and our first goal was achieved. Nonetheless in biocatalysis we use biocatalysts and in this way only half of the way was completed. When we performed the immobilization of cutinase on these materials our expectations were high but the results did not correspond to our expectations. If fact cutinase showed a marginal activity when compared with results obtained with zeolites and sol-gel preparations. The only positive result was achieved in supercritical carbon dioxide ($scCO_2$) where we verified for the first time a higher catalytic activity in this solvent when compared with hexane, which is traditionally one of the best solvents for cutinase. Nevertheless the activity of cutinase was far from the results obtained for cutinase with sol-gel and zeolites.

Our attempts with cutinase were finished in this point. But the end of this story was actually the beginning of a completely new line our research. In this case we started with the combination of gelatin with ionic liquid which for our surprise had never been described in literature. This gave us the opportunity to patent this material was well as think in some applications for this new material. The advantage and novelty is

fundamentally related with the versatility that the combination of these two elements offers. The morphological versatility of gelatin and the chemical versatility of the ionic liquids offer this material a myriad of solutions for several different areas. Going from the separations processes up to electronic and ionic conductors. Of course that the ionic liquids play a fundamental role in these solutions and is by the liquid ionic (IL) that this chapter is going start. The composition of ionic liquids has been succinctly described on chapter II (figure 2.7).

Before going trough I would like point that all the inventions and developments that concern this new material involved the participation of Nuno Torres Lourenço which had the same contribution as my self on the conception and development of this new material.

The beginning of the XX century is considered the cradle of modern science so is no surprise that many principles of the modern high-tech application have their roots of this flourish scientific period. Ionic liquids are not an exception and the first ionic liquid to be describe (1914) was Ethylammonium nitrate, $[EtNH_3][NO_3]$. Since then many ionic liquids were discovered, for instance in late 40s it was described the first ionic liquid based chloroaluminate anion and in the 60s the tetraalkyammonium cation was firstly introduced. As we saw on chapter II this cation constitutes one of the most important families of ionic liquids and it was extensively studied during the following decades. One of the properties that attract more the attention of researchers was the solvent capacity of these salts. In the 80s this property was actually extensively studied for the chloroaluminate ionic liquids, namely to the dialkylimidazolium chloroaluminates[2]. In the 90s the discovery of a new generation of ionic liquids based on ethyl-3-methylimidazolium cation and tetrafluoroborate anion was one of the major advents of this area[2-6]. This generation showed that the use of ionic liquids was not limited to uncomfortable chloroaluminate salts. Besides that it became clear that wide range ionic liquids with different properties could be prepared using this new this cation. One of the properties that call more the attention of researchers was the fact that the polarity of the ionic liquids could be enhance by changing the type of chemical moieties presented of both cation an anion. This have the effect of changing the solvation capacity of the ionic liquid, which means that ionic liquids are power solvents that can dissolve a wide range of chemical compounds[2-6].

If we back a few centuries in history up to a period comprehend between the XV and XVIII century the eager search of alchemists was to discover a substance that have the power of dissolving every other substances, including gold, the so-called *Alkahest.* If those alchemists wave known about the ionic liquids maybe they had considered them the so desirable *Alkahest* and just for curiosity some of them are cable of dissolving some metals including gold.

This solvation capacity made IL of the pillars of green chemistry technology, and their tailor-made design made them the solvent of future[5]. One best selling properties of ionic liquids is their nonvolatility. However since the work of Earle *et* al. showing that some ILs may be evaporated and recondensed made this argument a tricky to promote the reputation of ionic liquids as green solvents[7]. But besides solubility ionic liquids have more interesting properties. One of the most interesting for this chapter is undoubtly their conductivity.

Before going trough this property in ionic liquids there some fundamental issues that regards the conductivity property that can help us to understand better this phenomenon in ionic liquids.

Electrical conductivity or **specific conductivity** is a measure of a material's ability to conduct an electric current. When an electrical potential difference is placed across a conductor, its movable charges flow, giving rise to an electric current. The conductivity (**σ)** is defined as the ratio of the current density **J** to the electric field strength **E**[8,9].

$$\mathbf{J} = \sigma \cdot \mathbf{E} \qquad \text{(Eq. 1)}$$

Conductivity has the SI units of siemens per meter ($S \cdot m^{-1}$). This property is reciprocal of **electrical resistivity** which is defined as a measure of how strongly a material opposes the flow of electric current. A low resistivity indicates a material that readily allows the movement of electrical charge The SI unit of electrical resistivity is the Ohm[8,9].

$$\mathbf{J} = \mathbf{E}/\rho \qquad \text{(Eq. 2)}$$

Ionic liquids have a broad range of conductivities 0,1-14 mS/cm, for instance higher conductivities are associated to the cation 1-ethyl-3-methyl imidazolium $[EtMeIm]^+$ where lower conductivities are associated to the ionic liquids based on tetraalkylammonium, pyrrolidinium, piperidinium and pyridinium cations (0,1 and 5 mS/cm^2). If we compare these values with conventional electrolytes we found that even the highest ionic liquid conductivity has a low conductivity value. For instance the conductivity of KOH solution (29,4 %) used in alkaline batteries is 540 mS/cm and the condutivity of the H_2SO_4 solution (30%) used in lead-acid batteries is 730 mS/cm. Nevertheless when we compared the conductivity of ILs with the conductivity of lithium solutions (10 $mScm^{-1}$) we found similar values between both[2,9].

Other interesting subject of IL conductivity is that an ionic liquid solution can if exhibit a higher conductivity when compare with IL it self. A good example is given by $[EtMeIm][BF_4]$ which have a conductivity of 14 mS/cm but when a solution of 2 M of this IL is prepared in acetonitrile the conductivity increases up 47 mS/cm[10]. This was indeed the opposite of what we might expect from a solution of salt in a solvent, since the ions are separated by solvent neutral molecules. Nonetheless at high salt concentrations all solvent molecules are involved in the ions primary solvation shell, in this case instead of having a dissolution of the salt into the solvent what we have a system called *solvent in salt solution* which resembles the properties of a classic salt solution, explaining in this way the increase in conductivity[2,10]. In this system the conductivity increases with the increase in the amount of salt up to a maximum and decreases with the further addition of salt. Of course this dilution effect could also be related with decrease in ionic liquid viscosity which is known to be reciprocal of conductivity[2,10].

These conductive properties of the ionic liquids have a major interest on electrochemical devices where ionic liquids could play an electrolyte function[2,11-18]. Of course their use is dependent on applicability of the device i.e. if we need a top conductivity like the one obtained for KOH solution or if we just need a conductivity similar to the one offered by lithium solutions. The first point that we have to consider before using an ionic liquid on an electrochemical application is its electrochemical stability, which means that the ionic liquid cannot be reduce or oxidized inside the electrochemical window of the device[2]. Before going trough this subject there are some

fundamentals that can be useful to understand and evaluate the applicability of ionic liquids on those devices.

Firstly, the concept of **electrolyte** says that an electrolyte is a substance containing free ions that behaves as an electrically conductive medium. This means that a solution containing free ions is an **electric conductor**. Usually the confusion starts at this point since an electric conductor is immediately associated to an electron current of electrons, since we intuitively associate this phenomenon of electricity[8,9,19]. On an electrolyte electric currents are flows of electrically charged atoms, i.e., ions. Nevertheless an electrolyte can also be a **pronton conductor** where electric currents results from the flow of protons[2,9,19].

This is the type of conduction that is expected for an ionic liquid and all the applications of ILs are related with this type of conductivity. The application of ionic liquids in electrochemical devices is a recent area but we can find examples of their use on the construction of batteries, capacitators, fuel cells and even dye-solar cells[2,11-18].

The physical state of ionic liquid is an important factor for some applications. For instance, on applications related with battery electrolytes and film like ion conductive materials is preferable to use liquid electrolytes. The reason for this is related with the processing and packing of those devices. Nevertheless when ionic liquids are used directly as electrolytic materials they have a serious drawback which is related with the fact that their component ions could also migrate along with the potential gradient. This means that the major fear of liquid electrolytes on batteries applications, which is the leakage from the battery, is also present in ionic liquids[11]. For that reason the major developments in this area have presented the immobilization or the solidifications of IL as potential solutions to overcome this major drawback function[15,16,18,20-20.]

This physical state can be controlled in several ways. The most obvious is by changing the chemical moieties of both cations an anion function[24] the other possibility is to solidify the ionic liquid using for instant a polymerization process function[16,20-23]. A more recent approach consisted in the introduction of polymerizable groups into the ionic liquid structure obtaining in this way a good ionic conductivity without liquid

components. These polymerized ionic liquids (PILs) have been developed for battery electrolyte and for other solid electrolyte applications[16,20-23].

One of the aims of the developing this type of polymers is combining the conductivity properties of a ionic liquid with the mechanical strength an flexibility of a polymer, maintaining in the end the good capacity of transporting target ions and also a controlled polar environment. One of the major concerns during the developing of these conductive materials is maintaining their ionic conductivity after the polymerization procedure[16,20-23].

A very interesting study was presented by Ohno and co-workers[16] who have introduced polymerizable groups into ionic liquid cation. They evaluated the conductivity of both monomer and PIL and found that the polymerization was responsible for a dramatic decrease on the conductivity. In some cases the decrease was from several orders of magnitude. They related this fact with the effect of the segmental motion in polymer matrix and to overcome this limitation they increase the distance between the cation and the polymerizable group (figure 4.1). The results confirmed that an increase in the length of the spacer introduced a molecular flexibility which revealed to be essential for a good conductivity on PILs.

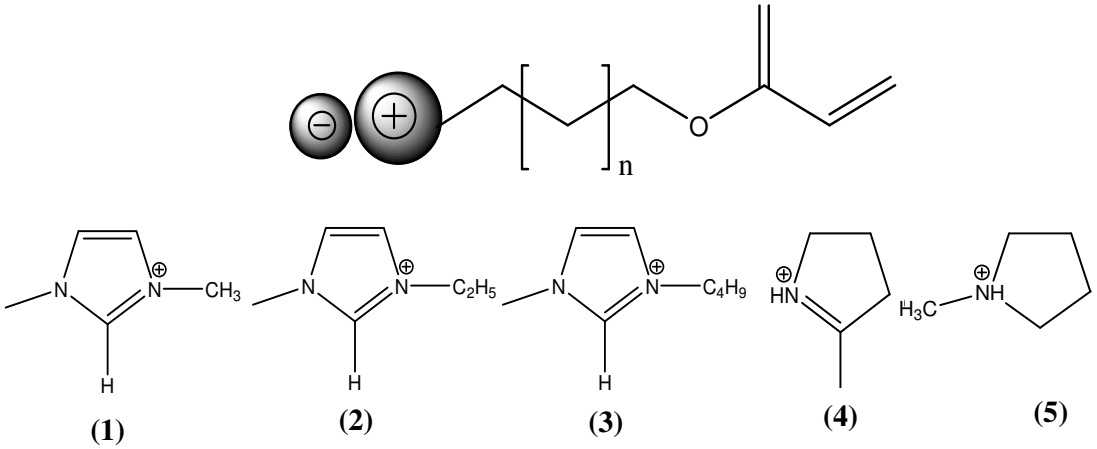

Figure 4.1 – Structure and some of the cation used in the study of Ohno and co-workers[16].

On the other hand it was also demonstrated that initial conductivity and the decrease after polymerization was correlated with the type of the cation used on PIL. They also found that the latter was also related with the mechanical properties of the PIL. For instance the PILs obtained with ionic liquids composed by the cations 2, 4, 5, were

sticky and solid where the ones composed by cations 1 and 3 were glass solid films. In this way the type cation is extremely important for the conception of electrolytes based on ionic liquids[16,2].

Polymeric ionic liquid have been investigated to be used on polymeric lithium batteries which are lithium batteries which have a lower charge density. In these batteries besides a good conductivity is necessary that specific molecules like the lithium ion could be easily transport trough the electrolyte. Combining this two factors on PILs is not a simple task since the variables, conductivity and ion transport, are related with several issues. One the major factors that can affect simultaneously the lithium transport and conductivity is the type of cation used on PIL.

Again Ohno and co-workers[23] have shown that type cation could enhance the lithium transport trough the PIL. They found that cations with the piperidinium salt structure could be and advantage for lithium ion conduction. Nevertheless these cations were less conductive then the cations presented in figure 4.1. In this way what these results suggests is that the choice of IL have to account for several factors but specially for the type of conductive specie that is gone be use with the electrolyte.

The ionic liquids can also be applied on lithium batteries with a high density charge (HDC), which differs from the previous by the type of application. In this case HDC are used as energy storage devices for uninterruptible power supplies for telecommunications of electric vehicles. The main issue with this type of batteries is their safety due to high energy load that they to store and mainly because they have in their composition organic solvents as liquids electrolytes which are flammable and in this requires safety risk procedures[27]. The ionic liquids can be a good solution to this type of application but in this case the electrochemical stability is an essential issue[25-27,29]. For instance the ionic liquids based on the imidazolium cation which were quite good options in PIL have to suffer some modifications in order to be use in this type of batteries. The problem is related with working potential of these batteries which can go up to the 5 V, for this potential imidazolium is not stable and began to decompose. The reason for this is related with the high charge density of this cation and also with its reactivity above certain potentials. The modification of this cation have the aim of reduction is positive charge and its reactivity[25-27].

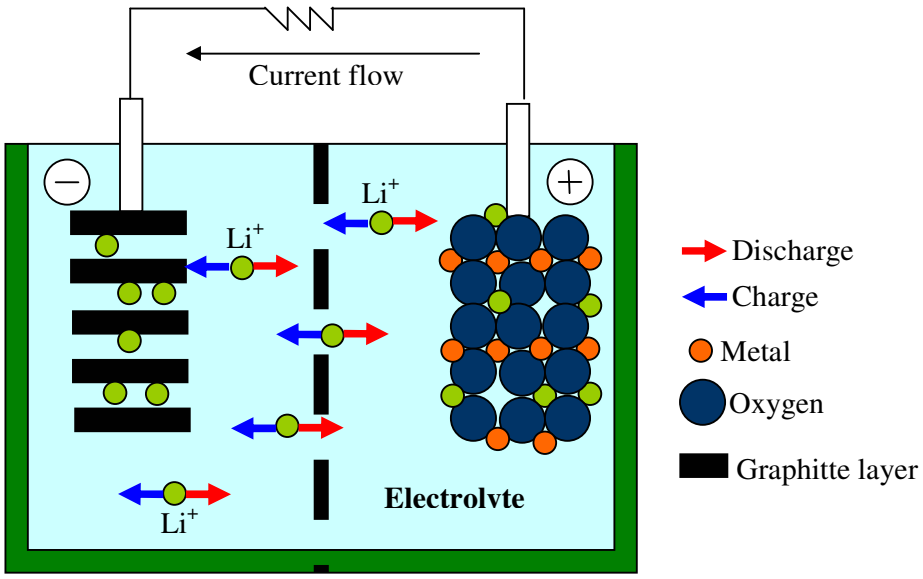

Figure 4.2 – Schematic representation charge discharge chemistry of lithium battery

A good example of the success of such approach was given by Hayashi and co-workers[26] who have performed the alkylation of all the remaining positions of ethyl imidazolium, this modification had the aim of decreasing the cation positive charge density and also create a steric hindrance which make the imidazolium less susceptible to oxidation increasing in this way its electrochemical stability.

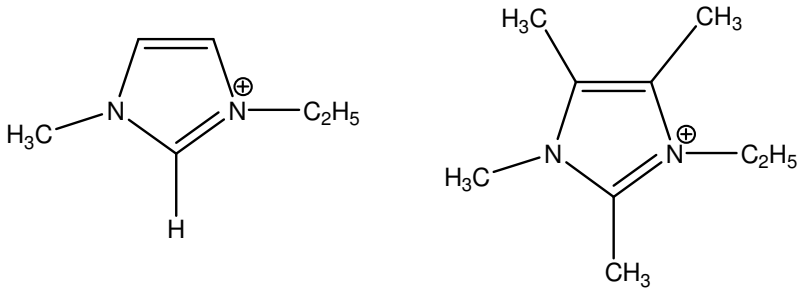

Figure 4.3 – Structure of 1-ethyl-3-methyl imidazolium and 1-ethyl-2,3,4,5-tetramethyl imidazolium.

The lithium batteries technology has motivated worldwide research efforts to develop better technological solutions for storing higher energy densities in the more reduce space possible. One of the most interesting solutions presented in this specific area came from the **lithium-metal batteries** which differ from previous by using metallic lithium instead ionic lithium[27,28]. The advantage is related with the higher theoretical energy density of metallic lithium. But the problem is that this type of batteries is

related with the growth of dendrites during the cycling which are responsible for decreasing their life cycle. For instance on the first generation of these batteries this issue was a serious drawback but the devolving of passivating layers helped to protect the electrode surface from this phenomenon. The passivating agent can be for instance a solid electrolyte which acts as a mechanical barrier or a electrolyte which controls the solid-electrolyte interface[27,28]. The latter is responsible for controlling the performance of the battery by protecting the lithium metal surface and allowing the lithium ion transport.

Recently Forsyth and their co-workers[14] have demonstrated the potential on zwitterionic ionic liquids in the formation of passivating layer of the surface of metal lithium electrodes (Figure 4.4). On the other hand these compounds were also described to largely enhance the lithium ion diffusivity in polyelectrolyte gels. If fact the results obtained by these authors show that the addition of to the zwitterion to the solid electrolyte was able to double the current density of the battery.

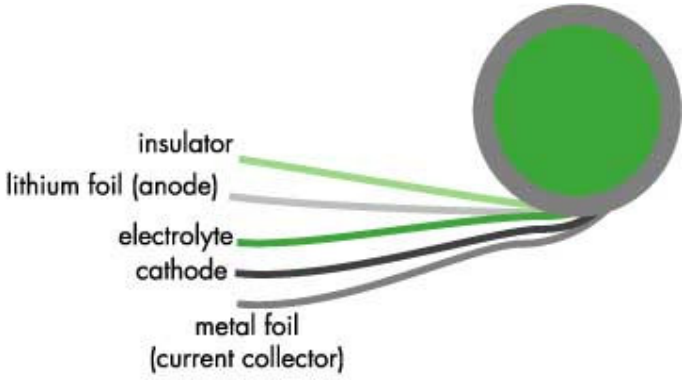

Figure 4.4 – Schematic representation of lithium-metal-battery. (solid electrolyte)

In this way there several ways to overcome the problems related with the use of ionic liquids on lithium batteries which will make them one of the most adequate choices for designing batteries specially for tailor-made applications.

Smart windows or electrochromic devices have many similarities with the lithium batteries[30]. This type of device is basically considered a transparent rechargeable battery, which the charge and the discharge conditions are associated to a color change.

In an electrochemical device the surface of each if the electrodes must be covered with n electrochromic material which color changes with its oxidation state. In this way by controlling the redox/oxidation potential of the electrode we can control the color of this electrochromic layer. The conceptual design of this type of devices is quite simple[31-34]. First we must have two transparent electrodes which the most common are made from ITO glass. This electrodes results from the deposition of a mixture of indium (III) oxide (In_2O_3) 90% and tin (IV) oxide (SnO_2), 10% over the glass. These Thin films of indium tin oxide are commonly deposited on surfaces by electron beam evaporation, physical vapor deposition, by a sputter deposition technique[33]. The surface of the electrodes must be covered with electrochromic materials which between the most popular are the Prussian blue, the tungsten oxide (WO_3) and the vanadium oxide (V_2O_5) [31-34]. A possible cell set-up is described on figure 4.5.

The use on ionic liquids on these devices in relatively recent and the aim in this case is the same as in batteries, which is to substitute the traditional or organic electrolytes. A very recent study have showed the great utility of N-butyl-N-methylpyrrolidinium bis(trifluromethansulfonyl)imide ($PYR_{14}TFSI$), on the construction of an electrochromic device[31]. This ionic liquid was interposed between two transparent films electrodes, respectively WO_3 and Lithium charged V_2O_5. The results were quite promising since they revealed a higher optical contrast during WO_3 colouration with $PYR_{14}TFSI$-LiTFSI, compared to that in a conventional non-aqueous electrolyte like PC-LiTFSI.

Another interesting study was performed by Mecerreyes and co-workers[11] who have developed a new type of tailor-made polymer electrolyte based polymeric ionic liquids. This electrolyte demonstrated a tremendous stability up to 70,000 cycles on PEDOT/electrolyte/PEDOT window which constitute an enhancement when compared with the traditional poly (ethylene oxide) electrolytes.

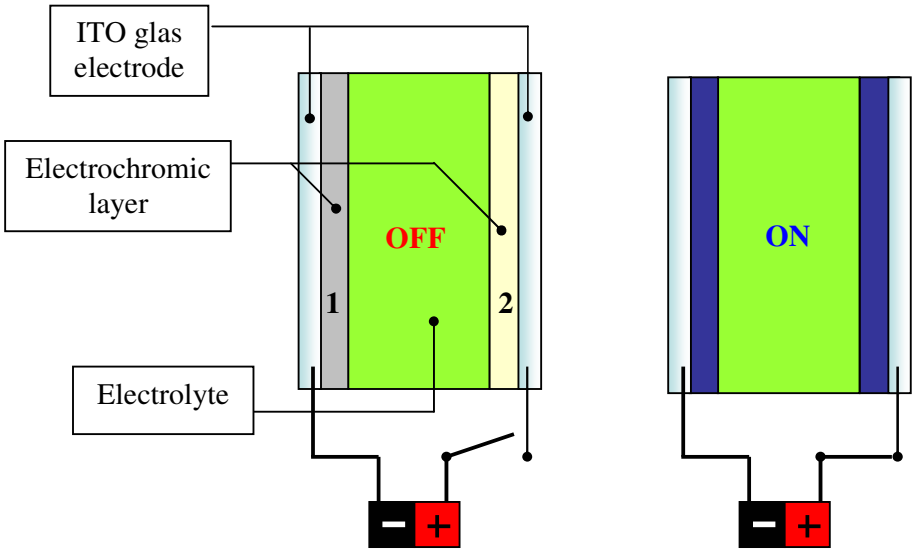

Figure 4.5 – A possible design of an electrochromic device with two different electrochromic layers. On the ON state one of the electrochromic layers is oxidized and the other is reduced. (1) is blue when is on the oxidize state and (2) is blue when is on the reduced state.

The applications of ionic liquids in electrochemistry do not extinguish in lithium batteries or electrochromic devices. Related with energy production ionic liquids have been used as electrolytes in both solar and fuel cells.

The photovoltaic energy is based on two fundamental steps, the light absorption and photon electron conversion. Today there are several ways to accomplish this. The most classical and also the most spread way to produce electricity from sun is using a **silicon wafer cell**[34-36.]This type of cell is based on the charge separation that occurs at the interface of two materials with different conduction mechanism. These materials are semi-conductors and the interface where the two semiconductor materials meet is called junction or p-n junction if it is formed by N-type and P-type semiconductors[34-36.].

These semiconductors are formed by a single atom lattice that is doped with an atoms of a different valence[38,39]. This concept is easier to understand with the example of silicon which has four valence electrons, each of which covalently bonded to other adjacent silicon atom. If this silicon lattice is doped with a trivalent atom from the group 13 such as boron, aluminum or titanium the resultant lattice will have one electron on silicon unpaired. In this way the dopant atom have to accept the electron from the neighboring silicon atom in order to complete the fourth bond. The dopant atom accepts the electron,

causing the loss of half of one the neighboring bonds which results in the formation of a "hole". The final result is a P-type conductor where the charge is majority transported by holes. In nature a blue diamonds, which contains boron impurities are good examples of this type of semi-conductor[38,39].

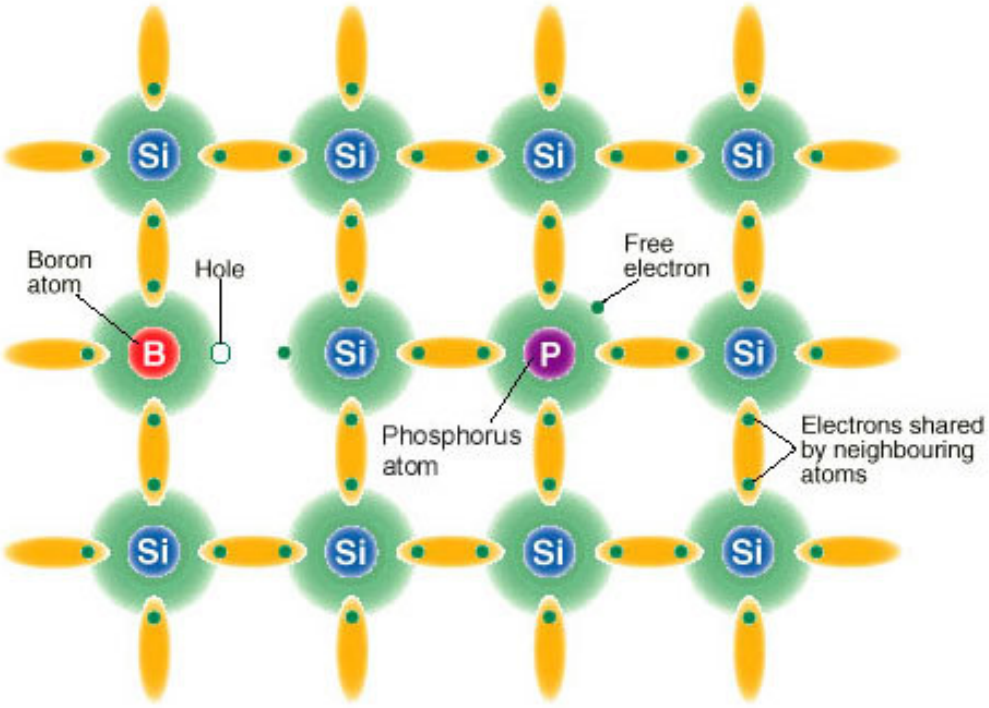

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Figure 4.6 – Schematic representation of silicon lattice doped with group 13 atoms (Boron atom) to obtain the P-type semiconductor and doped with group 15 atoms (phosphor atom) to obtain N-type semiconductor

On the other hand the N-type semiconductor is also obtained by doping but in this case the doping atom belongs to the group 15 of periodic table which includes for instance, phosphorus, nitrogen or arsenic. The atoms that belong to this group have a valence of 5. Since silicon is already full and the dopant atom have 5 electrons the result is that one electron on the dopant will be weakly bounded. In this way this electron is quite easy to excite into to the conduction band. Since excitation of these electrons does not result in the formation of a hole, the number of electrons in such a material far exceeds the number of holes. In this case the charge tranport is made by the electrons rather than holes. In both cases each hole and each electron are associated with a nearby negative-charged or positive charge dopant ion which made the semiconductor electrically neutral[38,39].

The p-n junction is in this way a nonconductor area since the electrical charge carriers in the both doped n-type and p-type attract and eliminate each other. By manipulating this nonconductive layer, p-n junctions can be used electrical switches allowing a flow of electricity in just one direction. The silicon wafer solar cell is basically a large p-n junction. When photons hit the solar cell they promote the charge separation at the p-n junction, both electrons and holes will have the tendency of being together but the p-n junction only allow the electrons to move in one direction. In this case electron will flow trough the n-type semiconductor where holes will flow trough the opposite p-type semiconductor. This externally flow of electrons and holes generates electric current (I), and the cell's electric field causes a voltage (V) [38-40].

This classical example of solar cells is on the edge of their technological improvement and has several disadvantages. The first one is related with band gap, which is the difference between valence and conduction band. This means that only photons with energy above the gap band are cable of generate electric current, the problem is that much of the energy of higher energetic photons from the blue and violet are lost in the form of heat since they have more than enough energy to cross the gap. But the main problem is that the light capture and energy production is associated to the same material. In this case in order to increase the capture of light the surface have to be fairly populated which means that a freshly-ejected electron will probably meet a hole before reaching the p-n junction. In fact this limits the efficiency of these cells up to 15% for the most common cases or up to 24% for the best lab prototypes. Another problem is that the cost of making a silicon junction is an expensive manufacturing process[40-45].

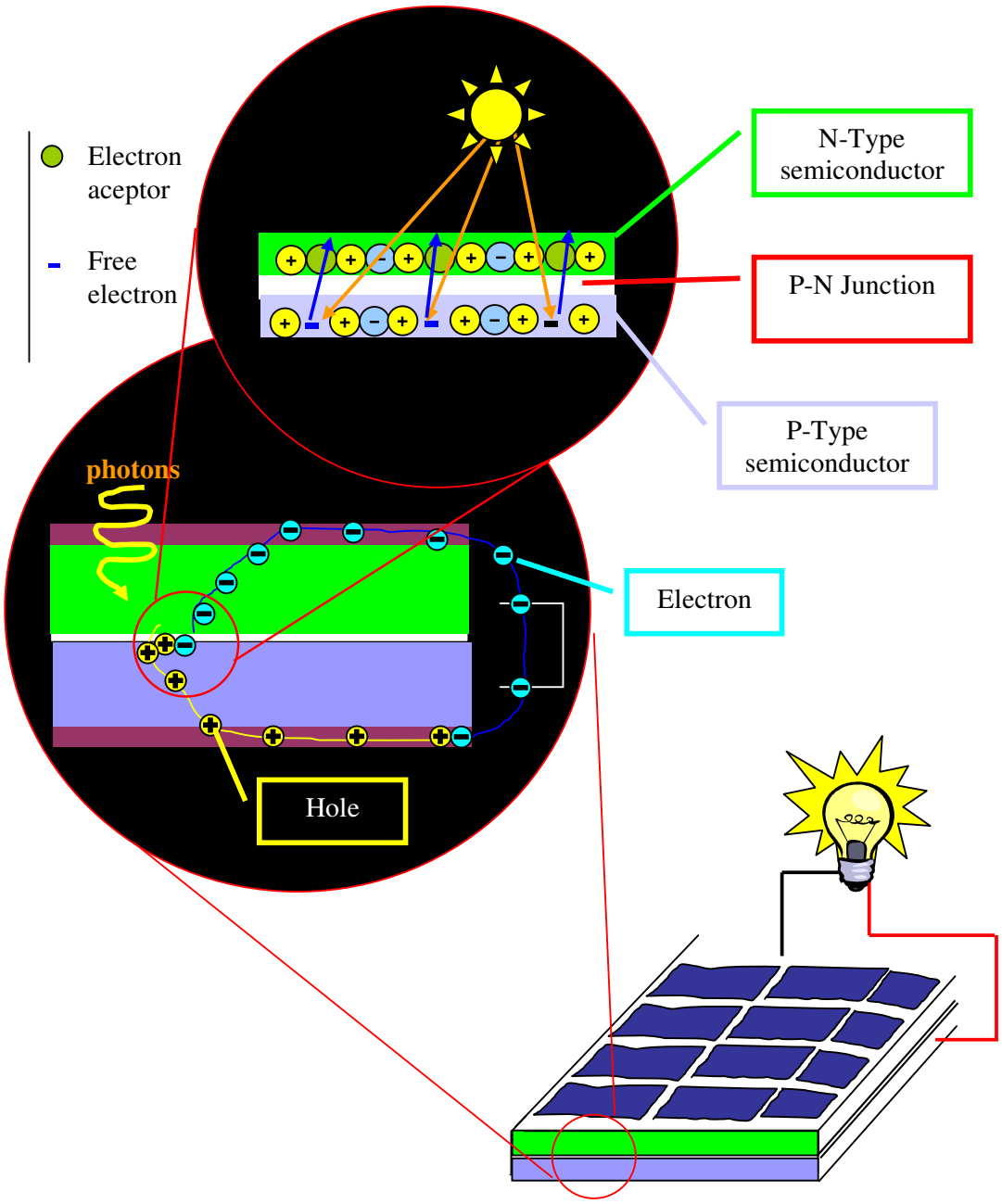

Figure 4.7 – Schematic representation of electric energy produce by a photovoltaic silicon cell.

Since energy is the engine of development many efforts have done to obtain better solutions to produce solar energy. Today we are already on the fourth generation of solar cells[38-40]. The **second generation** of photovoltaic cells is based in thin-film deposits of

semiconductors. The aim of these devices is to create highly efficient multiple junctions increasing in this way the global efficiency of the solar device. On the other hand using a thin-film cell is possible to reduce the amount of material required for cell construction. This contributed to a prediction of greatly reduced costs for thin film solar cells[38-40]. The **third generation** is a completely new area of solar cells since they are capable of producing energy without the p-n junction[40-45]. This type of cells has similarities with the photosynthetic process that occur in nature, and best examples are the dye-solar cells which also called Gratzel cells. This third generation will have a special attention on the present chapter since ionic liquids have been used to improve the efficiency of these cells. The **fourth generation** is the multi-spectrum layer which combines polymer cells from the previous generation with multi-junction from the second. The aim is to covert different light wavelengths into energy. For that propose the set-up involve different layers which covert different wavelengths into energy. This technology has been developing by NASA and other companies like Konarka Technologies.

A **dye solar cell** (**DSC)** or **Grätzel Cell** is a different concept of solar cell since they separate the two functions of a solar cell; catching the photos and converting them into energy. If in silicon cells silicon double acts as source of photoelectrons and as the potential barrier to separate charges and create an electric current, in DSC the semiconductor only have the latter function since the photoelectrons are generated by a photosensitive dye. Additionally the charge separation proceeds in a different way since in this type of cell there is a presence of a third element, the electrolyte, which complements the semi-conductor on that function[40-45].

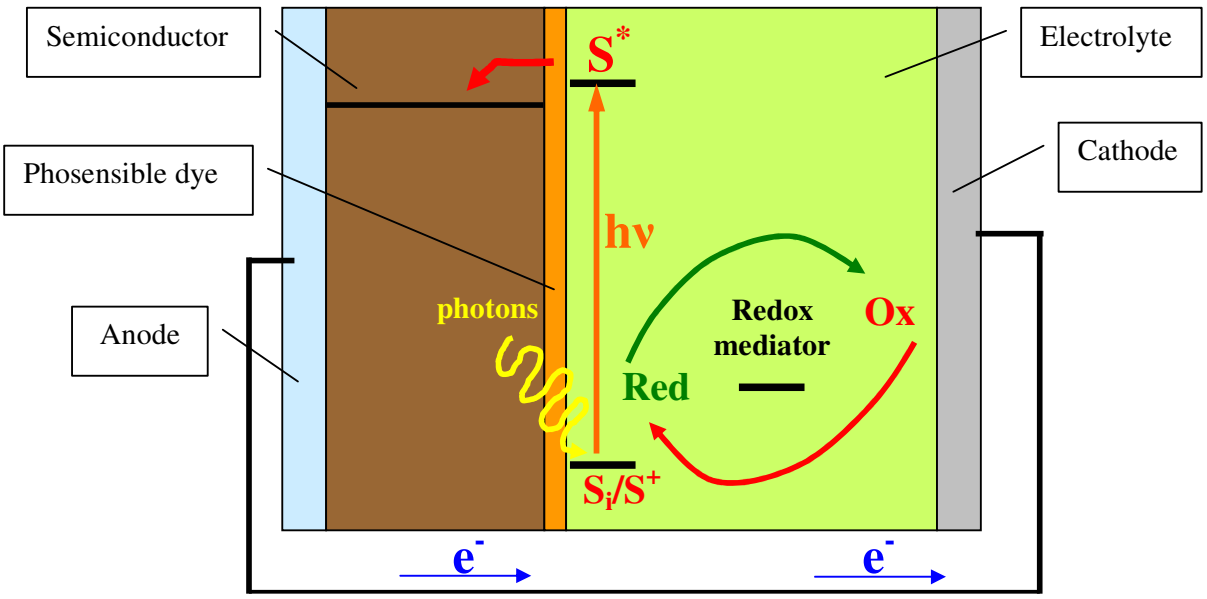

Figure 4.8– Schematic representation of a dye photovoltaic solar cell figure adapt from [42].

The heart of this cell is a mesoporous oxide layer composed by nanometer-sized particles which have been sintered together to allow a more effective electron transfer. The most common material to construct this semiconductor layer is TiO_2 commonly know as anastase. On the surface of nanocrystalline layer is deposited a layer of the photosensitive dye. The photo excitation of the dye promotes the injection of an electron into the conduction band of the oxide. The oxidation state of the dye is the then regenerated by electron donation form the electrolyte which is usually composed by an organic solvent containing a redox system. The most common redox system is iodine/triiodine. In this case iodine transfer an electron to the dye an its regeneration is made by the reduction of triiodine at the counterelectrode. The system is completed via electron migration trough the external load[40-45]

The photovoltaic efficiency to DSC is approximately 11 % which is slightly lower than the obtained by silicon solar cells, nevertheless these cells are extremely promising since they are made of low-cost materials and do not need elaborated apparatus for its manufacture making them very competitive[42,43].

Although the advantages there are still a few points that should be improved in these cells. One is related with the fact the electrolyte is base on a organic solvent which by the same reason mention for batteries should be avoid also on these devices. On the other hand the long term stability is also affected by the use of these electrolyte systems. Recently, some efforts have been done to substitute them for ionic liquids[41,46,47.]

The several approaches that used ionic liquids involve the direct substitution of the organic electrolyte acting in this way as solvent of the mediator couple which is essential for the dye regeneration. Even more interesting is the fact that the ionic liquid could work as the regenerator acting in this as an electrolyte catalytic medium. On both approaches ionic liquids had revealed a very good potential to accomplish the task. For instance Grätzel and their co-workers[41] have demonstrated that binary ionic liquids composed by 1-propyl-3-methylimidazoliumiodide and 1-ethyl-3-methylimidazolium tricyanomethanide could substitute traditional iodine/triiodine as the regeneration agent. The cell have a conversion efficiency of 8% which is a quite good result if we have in mind that the maximum obtained by DSC is 11%. Furthermore an excellent light-soaking stability was also observed during 1000 hours indicating a quite remarkable stability.

In the same field of energy production but with a completely different concept for energy production are the fuel cells[50]. This concept is much easier to understand than the previous related with solar energy. The fuel cell is basically an electrochemical reactor with two semi-cells separate by electrolyte membrane, where both reactants and products are constantly removed. What is essential is to have a reactant that can be oxidized on electrode surface in order to create a pronton gradient that migrate trough a membrane to the other semi-cell. On this second semi-cell is necessary the reduction of other reactant specie on the surface of the electrode serving in this way as the proton accepter [50]. The simplest example is the hydrogen cell fuel cell, which used H_2 as the reactant for the formation of proton gradient and uses O_2 was proton acceptor. In theory everything that can be oxidized can be used as fuel. One of the most interesting concepts was already presented on the chapter I.[49]

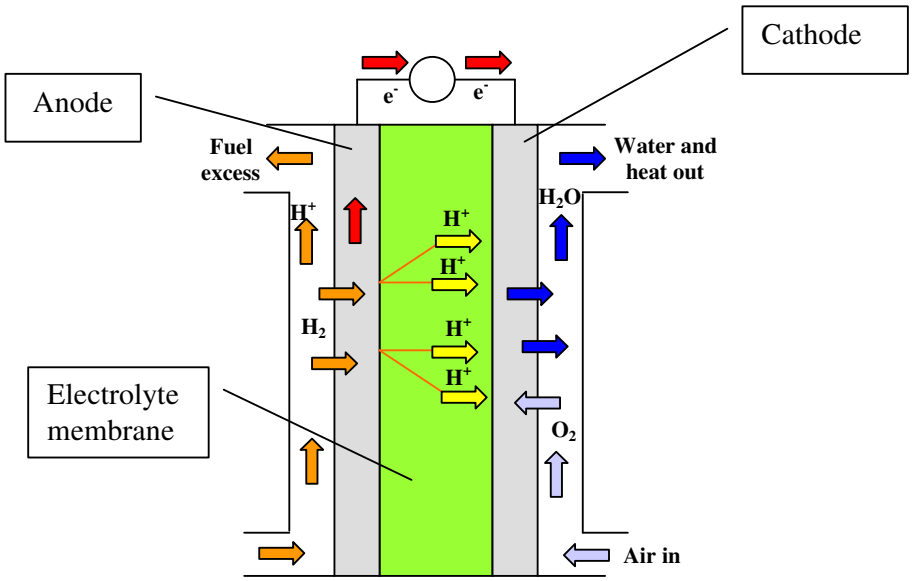

Figure 4.9 – Schematic representation of a H_2/O_2 fuel cell. This representation is a typical PEM (polymer electrolyte cell)

Of course that secret on these cells is in the membrane which has to be proton selective and very stable in working conditions. The best material or at least the most common is the Nafion® which is a sulfonated tetrafluorethylene copolymer which has an excellent thermal and mechanical stability. The problem is the high cost of of this material[48].

This fact has motivated the research on alternatives to Nafion®. The ion-solvating polymer composite membranes have been presented as possible alternatives[48,51-53]. One of those alternatives consisted on the incorporation of a KOH into a poly(ethylene oxide) (PEO) based membrane. The major drawbacks of this membrane is the high crystallinity which is responsible for conductivity reductio[51]. A more promising example is chitosan, which is a natural, low-cost and also is a weak alkaline polymer electrolyte[51,54]. Besides that it has a thermal stability up to 200 ºC with an acceptable mechanical strength. On the other hand, the presence of hydroxyl and amino groups give the chitosan membrane a high level of hydrophilicity, which is crucial for the operation. Its drawback is related with its semi-crystalline structure. For example when chitosan is on is dry state, which is its natural state is almost non-conductive. Nevertheless when fully hydrated, it can have a conductivity close to 10^{-3} S cm^{-1} which is a good conductivity but not high enough for large scale fuel cells application[55].

Fuel cells are probably one of the most well studied applications of ionic liquid on electrochemistry field with several examples that clearly show the potential of these solvents on membrane electrolytes[48,56,57]. The first experiments of ionic liquid on fuel cell membranes was with Nafion® membranes which their presence improved their already good working conditions. Nevertheless by the reason mention before Nafion® is a solution to avoid[58]. This fact motivated the incorporation of different ionic liquids in different types of materials such as polyacrylonitrile (PAN), polymethacrylate (PMA), polyethylene oxide (PEO), polymethyl methacrylate (PMMA) or polyvinylidene difluoride (PVdF)[56-58]. These studies reported very interesting results in terms of stability and conductivity for the application of ionic liquids on fuel cells membranes. For instance Doyle and co-workers[58] have shown that the incorporation 1-ethyl,3-methylimidazolium triflate and tetrafluoroborate ionic liquids on ionomer membranes enhance their conductivity up to 0,1 S cm^{-1} at 180 ºC which is an excellent result to think in an application.

Until this point we have presented several applications for ionic liquids in electrochemistry. These tailor made compounds have been successfully applied on solar cells, lithium batteries and fuel cell, most of times acting as an electrolyte. Dye solar cells are a quite good example to show that the application of ionic liquids does not extinguished on the electrolyte role. The applications of ionic liquids in electrochemistry is still given the first steps nevertheless the tailor made possibilities of these remarkable solvents made them an excellent bases for the developing creative solutions for this area.

The next part of this chapter is going to be focused on polymeric materials that can exhibit electric conduction and whose properties could be combined with the ionic liquids in order to create a more integrated solution to the previous electrochemical applications. We start this chapter by introducing basic concepts electrochemistry. At this point is also important to refer some principles of electronic transfer.

The materials can be simple divided in three different classes in respect of their conductive properties. In this way we have **isolators**, **semi-conductors** and **conductors**, and this classification determine their different target of application[8,9]. The next figure explains w why the materials exhibit these different properties. Everything is related

with the energy gap between the valence and the conductance band. When this band is too large, electrons cannot "jump" between valence and conducting band and the material is classified as an isolator. On the opposite site we have a conductor when these to bands are overlapped and the energy added to system is sufficient to propel the electrons between the two bands. The semi-conductors which were already described before, have also have a gap between the two bands, but in this case this is not so large as it is on the isolators. So if we applied an external electric field the electrons can be easily exited from the valence to conduction band and the number of free charge carriers is influence by the supplied energy. What this means is that the conductivity is controlled by the external potential.

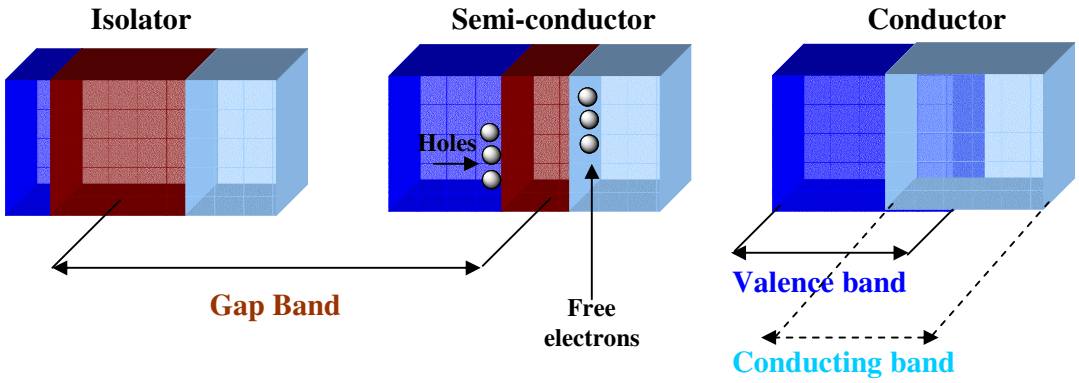

Figure 4.10 – Schematic representation of an isolator, a semi-conductor and a conductor.

Inside the conductors we have different mechanisms of charge transport (figure 4.11). For this chapter the most interesting types of conduction are the ones present on polymeric materials.In this way we have **conducting polymers** and **polymer electrolytes**[9,59,60]**.** The conducting polymers are polymeric materials that are electron conductors by virtue of the π electrons via conjugated double bounds and are designated as intrinsically conducting polymers (ICPs). On the other side we have the polymers which achieve their conductance by ion conduction. The dissociate ions are free to move within the matrix and can conduct electricity under applied voltage.

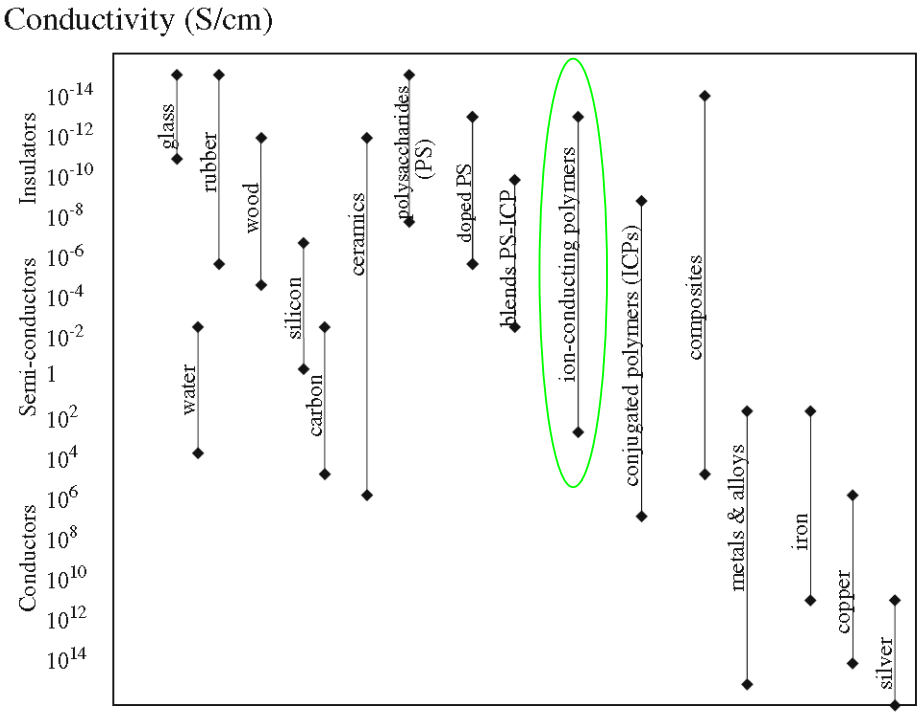

Figure 4.11– Schematic representation of the conductivity ranges for some groups of materials. Green oval are market the most relevant group for the present discussion. Figure adapt from 9.

A study that aims for the characterization of the conductivity properties of a given material results for the correlation of several physical parameters. In this way we have the **electrical resistance,** which is define the capacity of the material to resists to the flow of an electric current. The Omh's law states that, in an electrical circuit, the current passing through a conductor between two points (I) is proportional to the potential difference (V) across the two points, and inversely proportional to the resistance (R) between them. An is written by where, **V** SI units is *Volt (V)*, **R** SI units is *Ohm* (Ω) and **I** SI units is A*mpere* (A).[8,9]

$$\mathbf{I} - \frac{\mathbf{V}}{\mathbf{R}} \qquad \text{(Eq. 3)}$$

Electrical conductance (G) is the reciprocal of electrical resistance and is a measure of how easily electricity flows along a certain path through an electrical element. The SI derived unit of conductance is the siemens (S). A very important thing about conductance is that it should **not be confused** with conduction[8,9].

The **conduction** is the movement of electrically charged particles through a transmission medium (electrical conductor). This movement can form an electric current in response to an electric field and the underlying mechanism for this movement depends on the material. Nevertheless conduction in metals and resistors is also described by Ohm's Law. The easiness with which current density (current per unit area) (J) appears in a material is measured by the **conductivity** σ, which as defined by (Eq. 1)[8,9].

The correlation between resistence and conductance can be given by:

$$G = \frac{1}{R} = \frac{I}{V} \qquad \text{(Eq. 4)}$$

The conductance system for an of ion-conducting material, which is an electrolyte, depends on several factors such as, polymer matrix, the concentration of the ionic species (charge transporters), the mobility of this ions on the matrix (conductivity), the valence of the ionic species and finally from temperature. In order to simplify it can be assume that electronic contribution of these polymers to the global conductance is poorly significant when compared with the other factors. In this way we can express the conductance of this material by:

$$G_{(T)} = \sum n_i \cdot q_i \cdot \mu_i \qquad \text{(Eq. 5)}$$

Where G is the conductance of the material at a certain temperature (T), **n** is the number of charge species per unit mole, **q** is the net charge of each species, and **μ** is the ion mobility of each species [8,9].

There are several techniques that can be use to characterize the conduction and conductance of a given material distinguishing the different contributions implicated in that conductance system. In this way a system can be characterized by impedance[63], cyclic voltammetry[64] and Hall effect[61,62].

The Ohm´s Law (Eq. 3) is perfect to study ideal resistors which most of the polymer are not, for these cases **impedance** is used. Like resistance, impedance is a measure of the ability of a circuit to resist the flow of electrical current, but in this case the concept is extend to AC circuits, describing not only the relative magnitudes of the voltage and current, but also the relative phases.

Impedance spectroscopy or Dielectric spectroscopy measures the dielectric properties of a medium as a function of frequency. It is based on the interaction of an external field with the electric dipole moment of the sample, often expressed by permittivity i.e on the material ability to transmit (or "permit") an electric field. This spectroscopy uses a small amplitude signal, usually 5–50 mV, applied over a range of frequencies (0.001–100,000 Hz) [63].

Electrochemical impedance spectroscopy (EIS) uses a small amplitude signal, usually 5–50 mV, applied over a range of frequencies (0.001–100,000 Hz). With this technique is possible to measure the complex components of the impedance response of the specimen, i.e., the "real" resistance and the "imaginary" capacitance:

$$\mathbf{Z = R + jX} \qquad \text{(Eq. 6)}$$

Where the real part of impedance is the resistance **R** and the imaginary part is the reactance **X**. Dimensionally impedance is the same as resistance; the SI unit is the Ohm

Another technique commonly used is **cyclic voltammetry**[64]. In this experiment, a potential is applied to the system, and the faradic current, which is a current corresponding to the reduction or oxidation of some chemical substance, is measured. The most important fact in this technique is that the direction of the potential that is scan can be reversed, and the same potential window is scanned in the opposite direction (hence the term cyclic). This means that, for example, species formed by oxidation on the first (forward) scan can be reduced on the second (reverse) scan. This provides a simple and fast method for the characterization of a redox-active system. Moreover this technique can estimate the redox potential but also give an idea about the

electron transfer rate between the electrode and the stability of the analyte in the electrolyzed oxidation states, i.e. if they undergo any chemical reactions.

Both techniques base their measurements on alternating current (AC) nevertheless there are techniques that can evaluate materials electric properties using direct current (DC). The **Hall effect sensor** measures the voltage variation in response to the changes in the magnetic field of the analyte. This technique is based on the Hall effect principle described by Edwin Hall 1879. The **Hall effect** refers to the potential difference (Hall voltage) created by a magnetic field applied perpendicular to the current on the opposite sides of an electrical conductor through which an electric current is flowing. In other words the basic principle for the Hall Effect is the Lorentz force, which is the force that an electromagnetic field exerted on a charged particle. When an electron moves along an applied magnetic field, it experiences a force acting perpendicular to both directions and moves in response to this force and to the force applied by the internal electric field. Electrons subject to Lorentz force initially drift away from the current line, resulting in excess electrical charge on one side of the sample and giving rise to the Hall voltage, which in this way, measures the potential drop across the sample normal to the applied voltage[61,62]

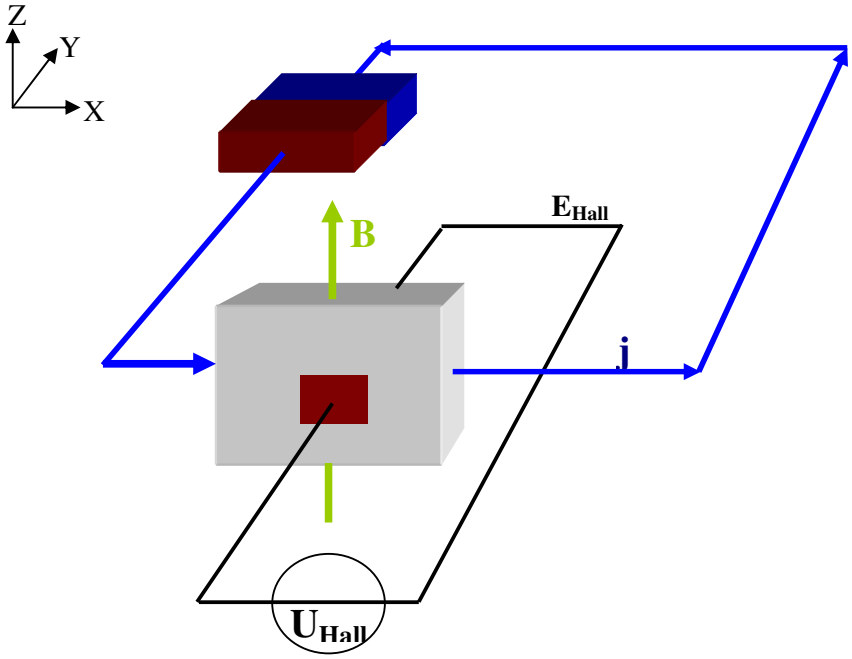

Figure 4.12 – Magnetic field **B** is employed perpendicular to the current direction j, as a consequence a potential difference (i.e. a voltage) develops at right angles to both vectors. The **Hall voltage U_{Hall}** will be measured perpendicular to **B** and **j**. An electrical field E_{Hall} develops in **y**-direction.

Hall voltage is a direct measure of the mobility μ of the carriers which are charge particles (ions or electrons). In this way can be consider a property of the material since different materials have different Hall effect coefficients resulting from the different impact that the same magnetic field have on current flow. If for a given material we had a conductivity value measured for instance by dielectric spectroscopy we can have a good characterization of the electric properties of the material.

$$R_{hall} = -\frac{\mu}{\sigma} = -\frac{\mu}{q.n.\mu} = -\frac{-1}{q.n} \qquad \text{(Eq. 7)}$$

This correlation between the Hall effect and conductivity give us directly the mobility of the carriers responsible for the conductance. The minus sign above is obtained for electrons, i.e. negative charges. If positively charged carriers are involved, the Hall constant would be positive[61,62].

For think in an application of a given conductor material either or not with ionic liquids is fundamental to know its properties, namely the ones related with the parameters that could affect the material specific conductivity. In this last part of this chapter we aimed to highlight fundamental concepts behind the conduction process as well as present some procedures to characterize this physical property. Trough the several examples that were presented related with ionic liquids applications we can say that ionic liquids have a major role as electrolyte. In fact it can be one of the best solutions to fulfill that task in any of the devices presented. Nevertheless ionic liquids have also being associated to materials where the charge transporters are electrons. A good example of this association is carbon nanotubes. The idea that that salts could enhance the conduction via electrons is not new[12,65-68], but its application and development is a quite emerging area in materials science. The materials that share both types of conduction are commonly known as the mixed conductors and the main objective of this development is related with the creation of new semi-conductors and electrodes. The next example is good application of this type o mixed conductor.

Carbon nanotubes modified with ionic liquids are commonly known as bucky gels and are obtained by simply disperse the carbon nanotubes in an imidazolium cation type

ionic liquid[12]. These suspensions become viscous affording a gel. This last psychical sate is possible related with the π-electronic interaction between the ionic liquid surface of the carbon nanotubes by means of cation–π and/or π–π interactions. Basically what the ionic liquid brings to carbon nanotubes is and an enhancement of the ionic conductivity which is usually implicated in an enhancement of electric conductivity[12]

These bucky gels have been used on the construction of electrochemical actuators. An actuator is a device for moving or controlling a mechanism or system which in the case of electrochemical devices operates by the converting the electrical energy into mechanical energy. A bucky-gel-based actuator works in air without any support of external electrolytes. Furthermore, unlike conventional polymer actuators[69] this actuator operates without deposition of a metallic electrode layer. The bucky-gel-actuator adopts a simple bimorph configuration with a polymer-supported internal ionic liquid electrolyte layer[70,71],which is sandwiched by bucky-gel electrode layers (Figure 5). The actuator film can readily be fabricated through layer-by-layer casting of electrode and electrolyte components in a gelatinous 4-methyl-2-pentanone solution of vinylidene difluoride-hexafluoropropylene copolymer (PVdF(HFP). The most interesting aspect of this device is the presence of an ionic liquid on the different components of this device.

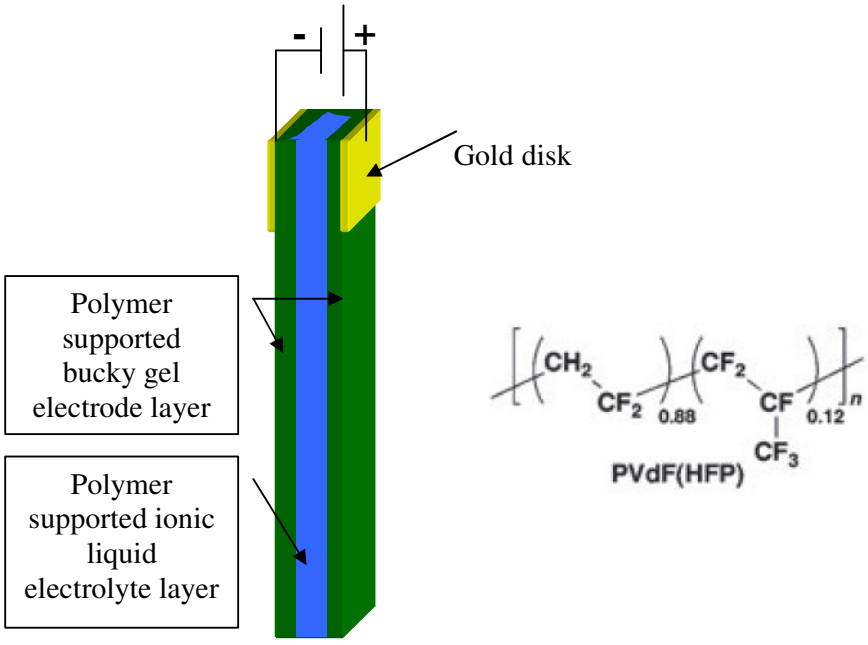

Figure 4.13 – Schematic configuration of an actuator composed by bucky gel PVdF(HFP)-supported and an ionic liquid electrolyte layer. This figure was adapted from reference 12.

The hot applications on conducting materials are related with applications that are directly involved with our interaction with the surrounding environment. The concept of "smart environments" has attracted scientists from distinct areas in order to develop the best solution to fit this concept[72-75]. The man-machine interfaces concepts are becoming every day more bidirectional, more intuitive and more imperceptible to human behaviour. In a certain way we can say that this concept is becoming bioinspired specialy if we think in our skin which is the most specialized organ and crucial for our interaction with the surrounding environment. Exploring its several layers we found different functions related with the thermal control, protection to external agents, exchange with the surrounding environment, camouflage etc. This specialized organization inspired man on the developing devices whose interaction with the user resembles a skin, in this case an "artificial skin". The new concept of electronic devices aims in this way for the development of new interfaces between these and their users creating in this way a simple and dynamic man-machine interaction.

Clothing have been considered for a long time our second skin so the artificial skin concept choose cloth was the elected raw material to create the required interactive solutions. Both modern environments and daily routines are considerably complex and full of devices that are deeply implicated in our life rhythm. Smart environments present a concept that all the devices that "participate" in our daily routine could learn more about us and take imperceptible decisions that could make our life easier. But for that three fundamental conditions have to present sensing, logical interactivity and of course energy.

A new concept always generates other new concepts and the development of conducting materials has been corresponding to aims of smart interaction. For instance the interest in cellulose and in other wovens as raw materials for new electronic concepts had an exponentially increase in the last decade.

One of most interesting examples of the advances in this area was brought by *Energy Systems Inc.* which have develop thin-film flexible batteries onto a woven substrate. This concept is commonly designated as "power fibres"[72]. These fibres cells are fabricated by coating a cellulose fibre with thin film layers, consisting in the same materials that are typically used in flat batteries, such as $LiCoO_2$ as cathode, lithium as

anode, and LiPON as solid electrolyte. The figure 4.12 (a) represents two nanotubes composite fibres that were separately coated with an electrolyte, forming in this way a supercapacitator (diameter 100 mm) providing a capacitance of (5 Fg^{1}) and an energy storage density (0.6 W h kg^{-1} at 1 V)[73]. These values are quite comparable with the larger commercial supercapacitors. Nevertheless the most interesting point of this device was the stability of its performance which remained unchanged over 1,200 charge–discharge cycles. The helically wound nanotube fibres are separated at the capacitor ends so that electrical connections can be made. These last examples together with the enormous market for these applications made this research area one of the hottest at the moment.

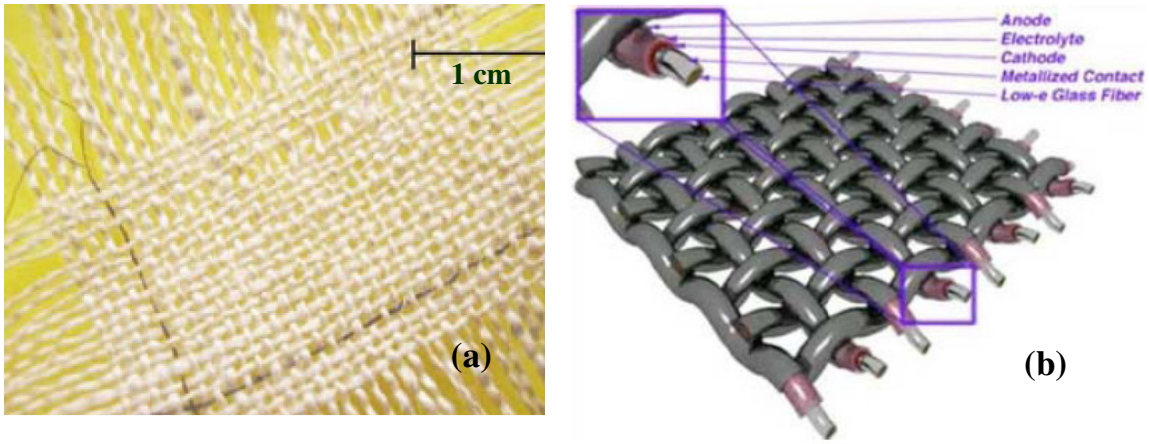

Figure 4.14 – (a) Photograph of a textile containing two nanotube-fibre supercapacitors woven in orthogonal directions; adapt from reference 73 (b) Power fibres, Current-generating materials layered a top individual fibres. Adapt from reference 72.

The materials that are presented in this thesis are been developing to meet certain requirements essential in such smart environment and interaction. Our raw material is gelatin one of the most abundant polymers in nature and our design material is based on ionic liquids. From the combination of these two results versatility. We are able to obtain material with different conducting and morphological properties just by changing the type of ionic liquid used creating in this way suitable materials for a several applications. Our study has been focused on the characterization of the material properties as well as on the development an electrochromic and electrochemical applications.

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CHAPTER IV

Experimental Section

Ion Jelly: a tailor-made conducting material for smart electrochemical devices

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CONTENTS

ABSTRACT

The highly demanding for new conducting solutions as leaded the material research toward the development of novel and creative concept materials. Here we demonstrate a novel conceptual solution to for designing polymeric conducting materials (PCMs). We have made use of the ability of ionic liquids to dissolve gelatin, which is a widely available, inexpensive and well studied gelling agent. This yields a viscous gel that can be molded into a transparent film or a block, and solidifies by cooling below 35 ºC. Our approach fuses the physical chemical versatility of the ionic liquids with the high moldability of a natural polymer such as gelatin. Creating in this way a transparent, tailor-made, conducting material ready to be used as film or as compact block. These characteristics open a wide range of opportunities for their application on electrochemical devices, such batteries, fuel cells electrochromic windows or even photovoltaic cells. Moreover ionic liquids are excellent solvents and gelatin is well-known for its biocompatibility, these combined characteristics can be explored also on bio-applications such as drug delivery.

INTRODUCTION

Polymeric conducting materials (PCMs) have been successfully applied in a wide range of electrochemical devices, such as batteries, capacitators, fuel cells, actuators, photovoltaic cells, electrochromic windows and light emitting cells[1-4]. A common feature to all these devices is the presence of an electrolyte whose properties have a pronounced effect on the device performance. A reasonable conductivity (above 10^{-4} S cm^{-1}), and a large electrochemical window (above 1 V) are some of the criteria that an electrolyte must meet to be suitable for utilization in an electrochemical device[1]. Room-temperature ionic liquids (RTILs) fill all these requirements[1,5]. Unlike traditional molecular solvents, ionic liquids are entirely made of ions. Their unique properties such as nonvolatility, nonflammability and excellent chemical and thermal stability have made them an environmentally attractive alternative to conventional organic solvents. Ionic liquids have low melting points (<100 ◦C) and remain in the liquid state in a broad temperature window (<300 ◦C)[6,7]. One of the characteristics that have attracted more

attention is the adjustability of RTIL properties through changes in the type of chemical moieties present on both cation an anion[5], and this has led to the designation "designer solvents". By changing the functionalities of cation and/or anion, properties such as polarity and solvation ability can be manipulated, and therefore RTILs are suitable for a wide range of applications that go from reaction media for chemical and enzymatic catalysis[8] to electrochemical applications[1,5,9-17]. RTIL conductivities and electrochemical windows are usually within the range 0.1-14 mS cm^{-1} and 4-5.7 V, respectively[5].

From the standpoint of device conception, solid electrolytes are preferable to liquid ones since the former allow the building of thin film devices using layer-by-layer techniques. Thus in electrochemical applications RTILs are usually coupled to supporting materials, such as organic polymers polyacrylonitrile (PAN), Polymethyl methacrylate (PMMA), polyethylene oxide (PEO), Polyvinylidene fluoride (PVdF) and Nafion®[18-26]. The synthesis of RTILs containing groups that can be further polymerized to produce PCMs is also worth attention[13,16,17]. Although combined RTIL-organic polymer materials have successfully replaced traditional electrolytes in some applications, there are still some problems to overcome. These are mainly related with the complexity of processing techniques, which in some cases such as that of Nafion®, have prohibitive costs.

Gelatin is prepared by the thermal or acidic denaturation of collagen, isolated from animal skin or bones, and also from fish skin. The triple helix of type I collagen extracted from skin and bones, as a source for gelatin, is composed of two α1(I) and one α2(I) chains, each with ca. 95 kDa molecular mass, 1.5 nm width and 0.3 μm length. Gelatin is basically a heterogeneous mixture of left-handed proline helix polypeptides and aminoacid strands, with a typical sequence of -Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro-. Dissolution of gelatine in water occurs at 30-35ºC, where these polypeptide strands undergo a coil-helix transition. Upon cooling, these helices go through an aggregation process similar to collagen, with right-handed triple-helical proline/hydroxyproline junctions[27-30]. The presence of this posttranslational modified residue favours compactness of the quaternary structure due to the formation of hydrogen bonds between the hydroxyl group and carbonyl groups of the main chain helices. Physical properties of gelatine and collagen are influenced by this interaction and higher levels of these pyrrolidine rings result in stronger gels[34].

Here we report a very simple way to obtain a polymer conducting material. We have made use of the ability of RTILs to dissolve gelatin, which is a widely available, inexpensive and well studied gelling agent. This yields a viscous gel that can be molded into a transparent film or a block, and solidifies by cooling below 35 ºC (Figure 1). The resulting material, which we call ion jelly, combines conductivity that is a characteristic of RTILs, with the mechanical flexibility of a polymer.

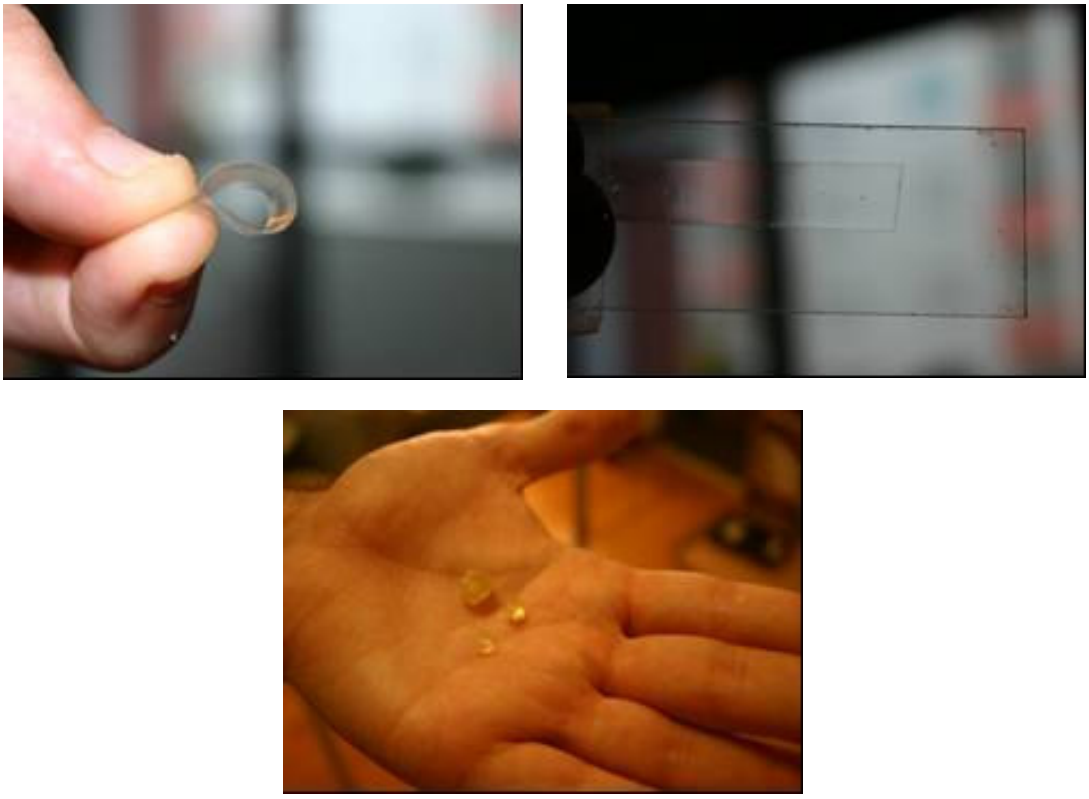

Figure 1 – Ion jelly films and blocks.

MATERIALS & METHODS

Materials

All the ionic liquids used in this work were kindly offered by SOLCHEMAR (www.solchemar.com). [bmim][Cl] -1-butyl-3-methyl-Imidazolium chloride; [bmim][$N(CN)_2$] - 1-butyl-3-methyl-imidazolium dicyanoamide; [C_{10}mim][Cl] - 1-decyl-3-methyl-Imidazolium chloride; [mmim][Cl] - 1-methyl-3-methyl-Imidazolium chloride; [im][Cl] - imidazolium chloride; [Emim][$EtSO_3$] - 1-Ethyl-3-methylimidazolium n-ethylsulphate. Metallic Copper (Cu, Fluka), metallic zinc (Zn, Fluka), copper sulphate ($CuSO_4$, Fluka), zinc sulphate ($ZnSO_4$, Fluka), potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$, Fluka), Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$, Merck), potassium chloride (KCl, Pronalab) were certified p.a. grade and used without further purification, gelatin.

Ion jelly

100-300 µL of ionic liquid was heated at 40 ºC and under magnetic stirring 1 equivalent mass of gelatin was added. The mixture was left under magnetic stirring in order to obtain a homogenous solution of gelatin. Depending on the ionic liquid the stirring time varied form 1 to 24 hours. The solution was then spread over a glass surface or used to fill a mold. The jellification occurred at room temperature. The R indicates the ratio (w/w) between ionic liquid and gelatin.

Prussian Blue film preparation

PB films were electrogenerated on glass indium tin oxide transparent electrode (ITO, acquired from Institute for Chemical Education – Chemistry Department). The electrochemical polymerization was performed in a conventional three-electrode cell. An aqueous solution of 5mM $K_3[Fe(CN)_6$, 5mM $FeCl_3.6H_2O$, 5mM HCl and 0.2M KCl was used as PB polymerization solution. Electropolymerization conditions was a constant applied voltage, 0.55V (vs Ag/AgCl), for 300s. After deposition the electrode was gently rinsed with distilled water.

WO_3 film preparation

WO3 films were prepared from a tungsten (W) target with 99,95% purity using the technique of direct current magnetron sputtering[44].

Electrochromic Window Assembly

The electrochromic window set-up was constructed using two ITO which one covered with a different electrochromic layer respectively WO_3 and Prussian Blue. The ion jelly was prepared and spread over the WO_3 ITO using a pipette forming a homogenous film. The film was left to jellify for approximately 10 minutes before being covered with the Prussian Blue ITO. After the window was compress with two cramps and was left to jellify for 24 hours. Before sealing with an epoxy glue the window was left under vacuum for more 24 hours.

Electrochemical measurements

Electrochemical polymerization and choronocoulometry/chronoabsorptometry were performed with a conventional three-electrode cell using a computer controlled computerized potentiostat-galvanostat Model 12 Autolab, from Eco-Chemie Inc. The collection of data was controlled by the GPES Version 4.9 Eco Chemie B.V. Software (Utrecht, The Netherlands). The working electrodes was the WO3-ITO electrode, auxiliary electrode and the reference electrode were the PB-ITO modified electrode.

Spectroscopic measurements

UV/Vis absorbance chronoabsorptometry were recorded in a Shimadzu UV2501-PC spectrophotometer. All spectroscopic measurements of the electrochromic cell were done in situ.

Conductivity measurements

The conductivity of several ionic liquid gel films was measured using and impedance analyzer (Alpha-N Analyzer from Novocontrol GmbH), covering a frequency range from 0.1 Hz to 1 MHz; films were placed between two gold-plated electrodes (diameter 20 mm) of a parallel plate capacitor. The temperature control, with a precision of ± 0.1 K, was assured by the QUATRO Cryosystem also supplied by Novocontrol GmbH. Frequency dependent ionic conductivity of the different gel films was measured from -110 up to 40ºC.

“Jelly Wire” experiments

To evaluate the possibility of the ion jelly as wire a simple apparatus was constructed (figure 7). Two ion jelly films were placed over a glass surface without being in contact with each other. This two were connected to a current generator and their junction was made by a LED. The current intensity as well as the potential was measured. AC a DC.

“Jelly Battery” experiments

A [emin][SO_3]$_{(R=3)}$ film was left to jellify over a glass slide. Before the jellification occur both ends on this film were doped different metallic solutions. These were prepared by mixing either the pair zinc/zinc sulphate or the pair copper/copper sulphate in the same ionic liquid used to prepare the film. Gelatin was added To these mixtures on the same ratio that film. The preparation was left to jellify at room temperature.

Hall effect measurement

The hall effect was measured using a Biorad HLJ500PC. A sample (square 1x1 cm) as placed on the analyzer and Ag glue was used as an electrode. The messurement were conducted at atmospheric pressure and room temperature.

X-ray diffraction

The X-ray diffraction measurements were carried out on a Enraf-Nonious X-ray generator equipped with a Cu-Rotating anode. The diffraction patterns were collected, by rotating the sample 360° with a step size of 1° and an exposure time of 1h, with a detector to sample distance of 90 mm.

RESULTS & DISCUSSION

Being a natural polymer, gelatin can be easily modified *via* functionalization by chemical or biological agents. Using transglutaminase, it is possible to cross-link lysine to glutamine residues[36], while glutaraldehyde promotes cross-linking between lysine residues. On the other hand, thermal cross-linking between gelatin amino acid residues can also occur[31] when temperature increases above a certain range. In fact, this is a very common technique to link gelatin to other materials, such as chitosan or sugars[38,39]. Cross-linking can considerably alter the gel properties.

The formation of an ion jelly should occur in much the same way as the formation of a water-based gelatine gel. Certain RTILs are known to dissolve proteins in considerable amounts[37]. Both gelatin and RTILs have ionic character and this leads to strong interactions between the two species, and a high solubility of gelatin. During the renaturing or annealing process, the polypeptide strands will have a tendency to rearrange into the most thermodynamically favourable structure, which, in the case of gelatine, is the left-handed proline helix conformation. However in the presence of RTIL these conformational rearrangements seem be modified.

X-ray diffraction experiments have been preformed to water-gelatin and RTIL-gelatin films (Figure 2) and significant differences have been observed. Similar to collagen, water-gelatin films (Figure 2a) exhibit three main diffracting rings at approximately 12 Å, 4 Å and 2.8 Å resolution when exposed to X-ray radiation. It has been suggested that the high resolution ring arises from the residues periodicity of each helices turn. The rise per residue along the helical axis is 2.9 Å [34]. Regarding the low resolution ring, it corresponds to the diameter of the super helix aggregate of the film. However, in the diffraction images of RTIL-gelatin films (Figure 2b-c) the referred rings can no longer be found. This feature indicates serious modifications in the conformation of the gelatin left-hand helix, as well as with the right-handed superhelix.

Several authors have shown that the structure and properties of water-based gelatin gels could be changed by adding different types of polyelectrolyte compounds, such as surfactants or sugars, during the jellification process[31-33]. The reason for this effect is generally attributed to the type of interaction that is mediated by the added species. The

two major contributions are ionic and hydrophobic interactions[32,35]. It was found that gelatin and surfactants could form either soluble complexes or precipitates as a result of the interactions established. E.g. Miller and co-workers[40] reported that gelatin interacts with sodium dodecyl sulphate (SDS) not only through electrostatic attraction between the cationic residues of gelatin and the anionic surfactant head-groups, but also via hydrophobic interactions between the aliphatic residues and the exposed surfactant hydrocarbon tails. In the case of the ion jelly, ionic interactions are the most likely, since with the exception of [omim][Cl], the RTILs used have a low content of hydrophobic chains. As shown in Figure 2, an increase in the RTIL-gelatin ratio from 1 to 3 had little effect on the structure of the material, as observed also upon changing the polarity of the RTIL. Such interactions would occur with side chain charged atoms, namely hydroxyproline, hence interfering with the hydrogen bonding network of the gelatine triple helix. Furthermore, the presence of these large ions bound to the gelatine strands would also prevent the formation of the triple superhelix and the macro assemblies typically seen in these molecules, can no longer arise.

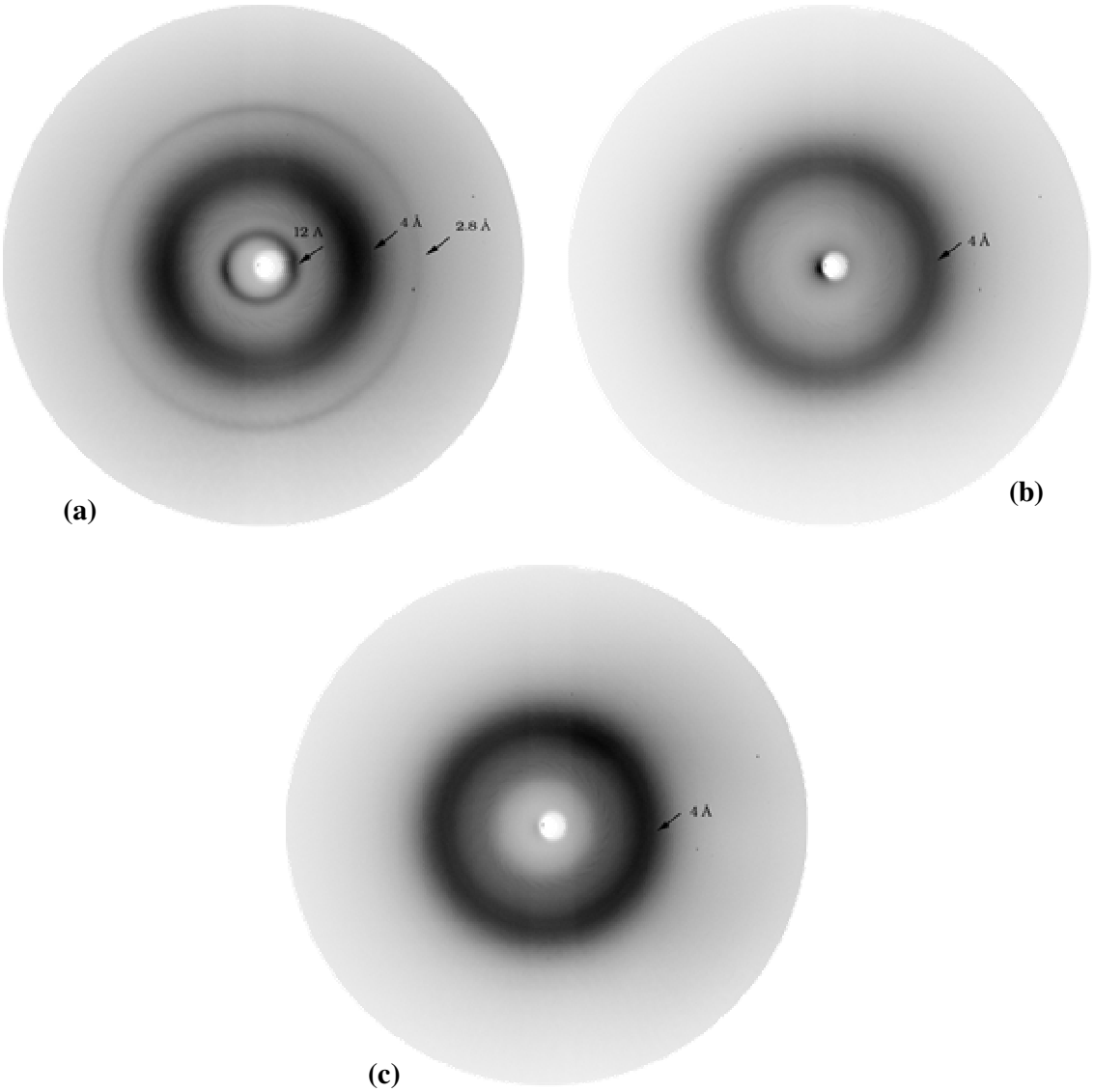

Figure 2 – X-ray diffraction patterns. (a) – water-based gelatin gel ; (b) – ion jelly with [bmim][$N(CN)_2$] ($R_{RTIL/gelatin}$ = 3) ; (c) – ion jelly with [emim][$EtSO_3$] ($R_{RTIL/gelatin}$ = 3);

Differential scanning calorimetry (DSC) presents additional evidence for an impact of the RTIL on the structure of the gelatin gel (Figure 3). The observed increase in the decomposition temperature of the material suggests a stronger interaction between the polypeptide chains of the gelatin tripe-helix structure. Temperature plays a critical role in the properties of water-based gelatine gels, in the switching between the denaturation and renaturation processes.. An important result of the present study is related with the fact that once the ion jelly is formed a further raise in temperature does not reverse the material to its initial viscous form. Since reversibility is associated with the rupture of

proline/hydroxyproline junctions, our results suggests a stabilizing effect of the RTIL on the ion jelly structure[31].

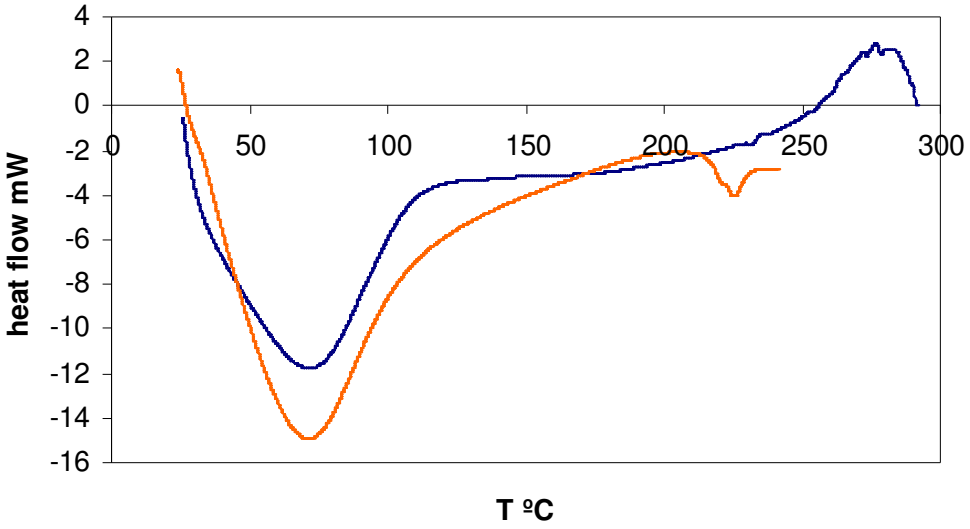

Figure 3 - Differential scanning calorimetry analysis. Blue – ion jelly with [bmim][$N(CN)_2$] ($R_{RTIL/gelatin}$= 1); orange – water-based gelatine gel.

The conductivity of the ion jelly materials was strongly affected by the RTIL used. To quantify the conductivity response of ion jelly films, frequency dependent ionic conductivity was measured at 25ºC, as shown in Figure 4. Several systems present, over a wide frequency range, very reasonable conductivity values that are of the order of 10^{-5}-10^{-4} $S.cm^{-1}$. By analyzing the frequency dependence of the conductivity in Figure 4, it is obvious that the ion jelly materials conform only partially to the usual conductivity pattern observed in disordered conductive systems[41,42,43]. For such systems, the spectra at the lowest temperatures is independent of frequency and shows a plateau, due to a diffusive ion transport; at higher frequencies, a bend off occurs into a dispersive regime, with a strong increase of the conductivity. The absence of an extended plateau in the conductivity plot in the low frequency region, where a decrease in conductivity is observed, means that ionic conduction becomes blocked due to electrode polarization, i.e, ions accumulate in the sample/electrode interface.

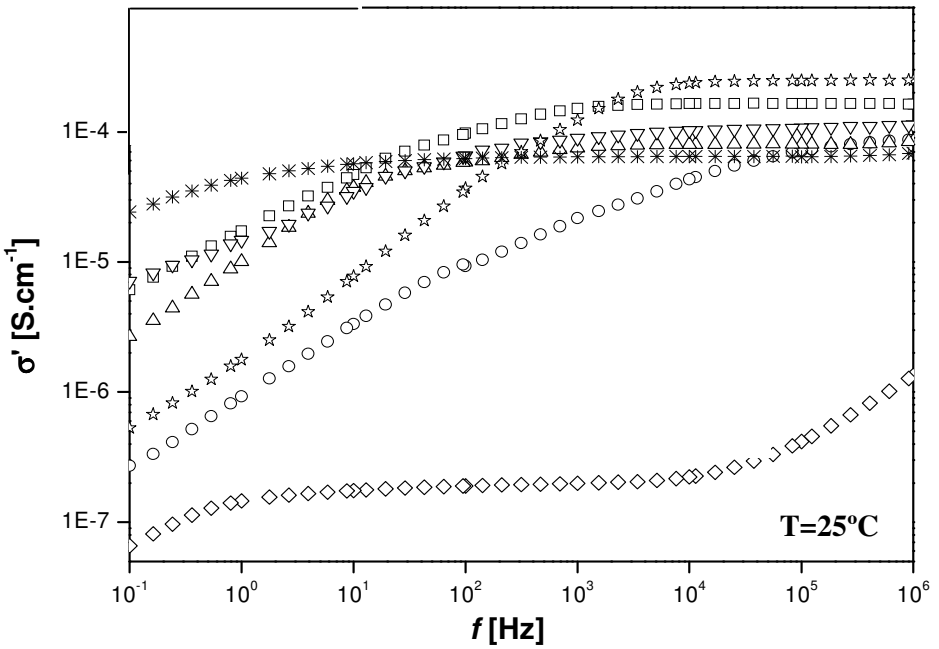

Figure 4 - Conductivity spectra of ion jelly films at 25ºC. □ [bmim][$N(CN)_2$]$_{(R=3)}$; ○ [bmim][$N(CN)_2$]$_{(R=1)}$; △ [bmim][Cl]$_{(R=1)}$; ▽ [mmim][Cl]$_{(R=1)}$; ◇[C_{10}mim][Cl]$_{(R=1)}$; ☆ [bmim][$EtSO_3$]$_{(R=1)}$; ✳ [im][Cl]$_{(R=1)}$.

The very reasonable value of conductivity in a large frequency range led us to study the possibility of using ion jelly as a conductor. For that purpose, we built an electrochromic window based on Prussian blue (PB) and WO_3 as electrochromic layer. There are many applications described in the literature of RTILs as electrolytes[45-48], usually with ionic conducting species solubilized, such as lithium salts. Here we used an ion jelly instead. Although this type of electrochromic window has been designed to work with a highly concentrated electrolyte solution, electrochromic windows built with an ion jelly performed reasonably well (Figure 5) in what concerns contrast, stability and switching velocity. This suggests that the ion jelly network has a charge transport mechanism with sufficient degree of freedom.

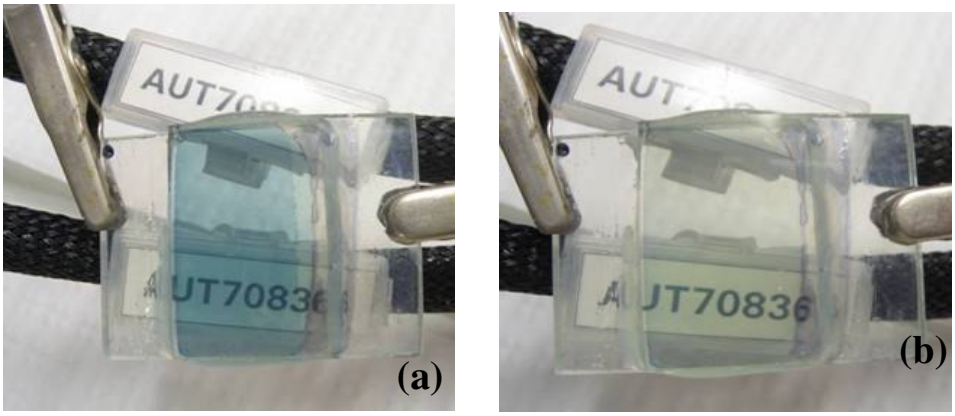

Figure 5 – Glass-ITO/WO3/ion jelly/PB/ITO-Glass.(a)- Colored state; (b) – Bleached state.

The stability of the electrochromic device was evaluated (Figure 6) by repeatedly switching the WO_3/ion jelly/PB system between -1 V (180 s) and 1.5 V (100 s), for 350 cycles (ca. 27 hours). After that time, a decrease in the overall absorbance of the cell was observed. The loss of coloration in PB electrochromic cells has been related with the decrease in the thickness of the PB film during the cycling. The "etching" of the PB layer can be due to some wash effect from the gel electrolyte, this problem was already reported and a possible solution is the protection of the electrochromic layer with the deposition of an inorganic layer like LiAlF[10]. The lower switching velocity can be explained by the absence of an electrolyte salt, but can be partly circumvented by incorporating ionic species in the ion jelly via their solubilization in the RTIL.

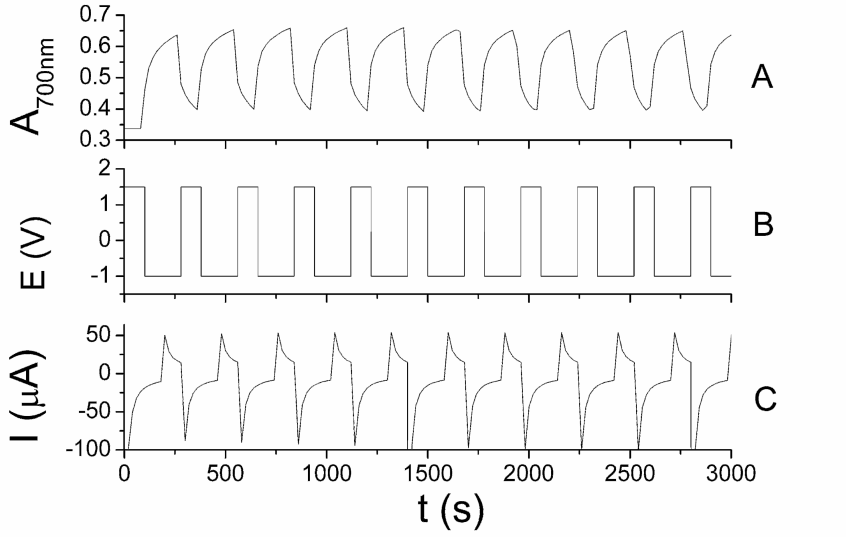

Figure 6 – *In situ* spectroelectrochemical cycling data for Glass-ITO/WO3/ion jelly/PB/ITO-Glass. A - chronoabsorptometry recorded at 700nm; B - Square-wave switching between -1V (step duration 180s) and +1,5V (step duration 100s) (vs PB); C – Current.

To try and elucidate the type of electrical conduction in ion jelly materials, we set up the experiment shown in (Figure 7), where two ion jelly films were placed on a glass surface and connected to a current generator. The two films were connected via a LED. To avoid decomposition of the material, we limited the applied potential to the electrochemical window of the RTIL used (e.g. -2,5 – 3,4 V for [bmim][$N(CN)_2$], -2,5 – 3,4 V for [bmim][$EtSO_3$]).

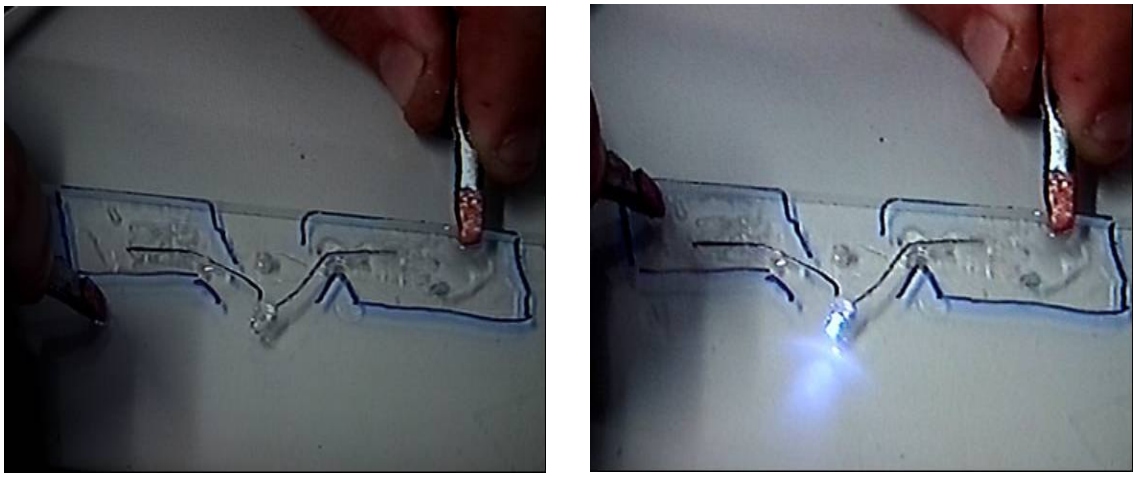

Figure 7 – Ion jelly working as "jelly wire". The electron transfer in mediated by the ion jelly conducting material.

The fact that the led lit up suggests an electronic transfer mechanism similar to that found in other conducting polymers such as polypyrrole or polythiophene, i.e. a delocalized π-system resulting from the overlapping of the π-orbitals. In a very simple way this system is based on the removal ("p-doping") or the addition ("n-doping") of electrons from the π-system. Basically when an electron is removed from the system, a free radical and a spinless positive charge are produced. The coupling of both species originates a polaron and new localized electronic states in the gap between the valence and conduction which are ultimately responsible for the conduction[45]. In the case of the ion jelly, we have an imidazolium center with a delocalized π-system that could form the basis of the charge transfer mechanism. The diffraction patterns for these materials are indicative of an ordered structure. Such type of organization is essential to create an extended π-system with a filled valence band through which the charge is transported. The Hall effect can give information about the type of charge transporters involved in the process, as well as their mobility in the material. In the case of the [emin][SO_3]$_{(R=1)}$

ion jelly, the number density of charge carriers, N_v, was $2x10^{13}$ cm^{-3}, their mobility, μ, was 9.83 cm^2 V^{-1} s^{-1}, and the Hall voltage was $1x10^{-4}$ V. The latter indicates that the charge is carried by p-transporters, and suggests the ion jelly behaves as a semi-conductor. Figure 8 shows a battery made of ion jelly modules. Each end of the ion jelly film was either doped with mixture of $Cu/CuSO_4$ or with a mixture of $Zn/ZnSO_4$ in order to create an electrode system. As shown in the figure, the battery yielded a voltage of 0,5 V with a current of 23,4 μA

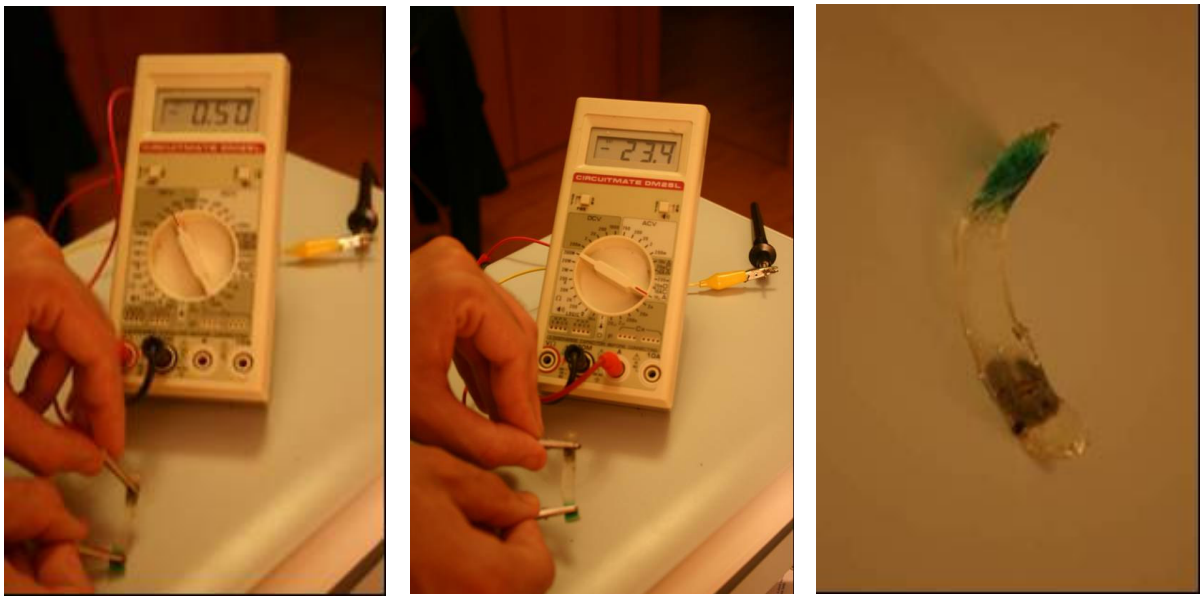

Figure 8 – Jelly battery. Green electrode ($Cu/CuSO_4$) ; Grey electrode ($Zn/ZnZO_4$); 0.5 V with 23,4 μA.

CONCLUSIONS

Our findings are significant in the area of conducting polymers because we present a very simple way to obtain such materials through the combination of gelatin with RTILs. Gelatin is a natural polymer that can be easily modified via functionalization by chemical or biological agents[36,38,39]. E.g. the ability that gelatin has to interact with cellulose opens the possibility to incorporate the ion jelly into the concept of smart clothing. RTILs themselves can be custom made. This factor lend even greater versatility to ion jelly materials, and makes possible the tailoring of the ion jelly properties to meet desired applications. Through the choice of RTIL, transparent,

flexible ion jelly films can be synthesized that behave as conducting polymers, or else that behave as an electrical insulator. Additionally RTILs have the ability to solubilize both organic and inorganic compounds. This characteristic can be exploited in applications that demand enhanced electrical conductivity, as illustrated above, but bio-applications such as drug delivery or the building of living cell scaffolds for tissue engineering applications can also be envisaged.

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CONCLUSIONS

Biocatalysis today. This thesis and the future.

Over the last twenty years the application of biocatalysis to industrial processes has been looked upon with many reservations, especially due to two major concerns: the perceived lack of availability of biocatalysts to fulfill the necessities of reaction processes, and the lack of stability of enzymes during those processes. For example, conformational changes of less than one Ångstrom can cause a dramatic decrease of enzyme activity, and for that reason the retention of enzyme activity is a stringent criterion for the use of enzymes in a chemical process. On the other hand, the price of enzymes was always referred as a major drawback. Nevertheless the improvement of the methodologies for enzyme production and purification has considerably decreased the costs of implementation of this technology. For example, today we do our laundry with enzymes.

The catalytic requirements of some enzymes, such as the need for co-factors, were frequently indicated as a drawback that this technology had to overcome in order to become easily available and user-friendly. These two conditions are very important for the implantation of any technology, and biocatalysis is not an exception. With this in mind, researchers directed their research towards the understanding of enzymes as catalysts. They tried to answer very simple questions, such as "why do enzymes loose their activity during the process?", "what are the major factors that affect their catalytic performance?", or "can we generalize our findings for one enzyme to a large number of biocatalysts?". A lot of work has been published in an attempt to answer those questions, and more should still be under way.

We can single out two important milestones for biocatalysis as we know it today. The first one happened in the 1980s, when new frontiers for enzyme applications in synthetic reactions were established, by extending to nonaqueous media the types of solvents for conducting biocatalysis. This was a great breakthrough, since everyone's conviction was that enzymes could not work in such media, but in fact they do. The number of applications of enzymes suffered an exponential increase in that decade, especially motivated by the high enantioselectivity that some enzymes exhibit in nonaqueous media. Since then, many conceptual ideas of enzyme applications have been based on that enzymatic property.

The second milestone happened in the 1990s with the introduction of DNA technology, which not only allowed the discovery of novel enzymes, but also made it possible to change the aminoacid sequence of many of them, thus improving their efficiency through increases in their activity, selectivity or stability. DNA technology redefined in this way the rules of process design, since it became possible to obtain the desired biocatalytic solution directly from nature.

Today almost all the types of reactions that we know in organic chemistry can be accomplished with an enzyme, the exception being the hetereo-cope rearrangement. This should not be cause for surprise, especially if we keep in mind the complexity of the living organisms in our planet and the enormous diversity of the environments where we can find them. If we look upon this diversity on a molecular level, we can see it translated into a vast number of proteins that confer to these organisms the characteristics to live at such conditions. This goes totally against the idea that there are a limited number of enzymes for the development of biocatalytic processes. On the other hand, the idea right now is that there are enzymes sought for many more reactions than there are enzymes available. To illustrate this idea, we can use the example of serine proteases that in nature are hydrolases, but *in vitro* can catalyze transesterifications, esterifications, epoxidations, etc.

Biotechnology and biocatalysis differ from conventional chemistry and catalysis not only in the type of catalyst used, but especially in their conceptual basis. For example, chemistry emphasizes a *molecularly-oriented perspective* dominated by the compounds and their transformations, whereas chemical engineering favors a *process oriented perspective* and biology favors a *system-oriented perspective* focused on the organism as well on its evolution. The conceptual basis of biocatalysis is to put all these perspectives together, acting as a technological melting pot. This fusion of knowledge allows the development of more creative approaches that can result in a new or alternative transformation process. For example, the raw materials for a biotechnology-based process are significantly different, since such processes can be built on sugars, lignin, or even on animal and plant wastes. The resulting products from the first transformation can be readily used or further transformed into fine chemicals with enzymes. This versatility of solutions can in fact change the way the chemical industry works, since from the same substrate we can obtain a wide range of products just by

changing the process pathway. The integration of multiple pathways based on biocatalysts can also be considered a bioinspired idea, since it mimics the metabolic pathways of the living organisms, which are considered the most efficient, selective and regulated processes in nature. With biocatalysis, we can achieve new goals in chemistry, such as novel, high-yield, shorter process routes with increased selectivity, and at the same time "go greener" by using natural wastes as raw materials.

The conceptual idea of biocatalysis as a technological melting pot is expanding to several other areas, such as materials science or even astrobiology. Materials science suffered a boost in the last decade with the development of a wide range of new materials for a large number of applications. Biotechnology has also played a role in these recent developments, especially in what concerns the synthesis or biosynthesis of polymers from natural substrates. The way that living organisms produce or polymerize certain substrates has also inspired scientists in the development of novel synthetic routes to new materials. The precipitation of silica in microalgae is quite a good example of this. Scientists identified the proteins involved in the deposition of silica in the microalgae membrane, and used them *in vitro* to synthesize different forms of silica. The result was the achievement of better structured materials, using a more controlled process.

Today biocatalysis has a set of tools to overcome the major drawbacks for its implementation. For example, we have molecular evolution tools which can be used to increase enzyme activity, stability or selectivity without the need for information on enzyme structure. Nevertheless through the rationalization of the practical results obtained, we can develop faster and better tools to obtain the same improvement in enzymatic properties. There is still a lot of fundamental research to be done before biocatalysis becomes a user-friendly technology that can be applied systematically in chemical transformations.

The approach of the present thesis was focused on fundamental issues related with the performance of cutinase in nonaqueous media. We looked at all three major enzymatic properties of activity, selectivity and stability. In what concerns enantioselectivity, we used different approaches to generate data that could contribute to a better understanding of that property. Molecular modeling studies offered insight on how

enzyme enantioselectivity depended on the relative stabilization of the transition states for the two enantiomers. For example, 2-phenyl-1-propanol and 1-phenylethanol differ only in a methyl group. This difference in substrate chain length was enough to change dramatically the enantioselectivity of cutinase towards these two alcohols. The position of the chiral center of 2-phenyl-1-propanol in the active site of cutinase allowed for a higher conformational freedom of the two enantiomers, which explains the lower discrimination of cutinase toward this substrate.

With this in mind, we used DNA technology to introduce point mutations at specific locations near the enzyme active site. Our approach was to decrease the preference of cutinase towards the (*R*)-enantiomer of 1-phenylethanol, by increasing the accessibility of the active site, and thus decreasing the hindrance to the stabilization of the *(S)*-enantiomer of this alcohol. Differences in the free energies of activation for the two enantiomers ($\Delta\Delta G_{R\rightarrow S}$) were calculated for potentially interesting mutants. We have seen in Chapter II that two mutants, respectively L189A and Y119A, were selected to continue this study. Despite the similar $\Delta\Delta G_{R\rightarrow S}$ values, replacement of a tyrosine for an alanine (Y119A) not only creates space in the active site, but also eliminates favorable van der Waals interactions between the (*R*)-enantiomer and Tyr 119, which were found to strongly stabilize the transition state for the wild-type enzyme. This could account for the observed loss of discrimination ability of cutinase towards that enantiomer.

In this work we also directed our attention to reaction temperature, which is a very efficient parameter to fine tune enzyme enantioselectivity. Through a decrease in temperature, we were able to partially recover the loss of enantioselectivity of the Y119A mutant relative to the wild-type enzyme. We were also able to increase cutinase enantioselectivity towards 2-pentanol and 2-octanol. In the case of 2-butanol, which cutinase resolved with poor enantioselectivity at room temperature, lowering the latter parameter hardly affected the discrimination ability of the enzyme. In this study we used vinyl esters of varying chain-length. Our results should help develop the existing model for cutinase.

Chapter III was focused on the improvement of cutinase activity and stability. For that purpose, we immobilized the enzyme and tried to elucidate the differences in enzyme activity afforded by the different supports. We used the sol-gel process that yields a

mechanically robust preparation and affords control of the enzyme microenvironment. Enzyme activity matched that obtained via immobilization at the surface of zeolites, which is a good immobilization technique for cutinase. However, enzyme stability in the sol-gel was greatly improved. Also cutinase activity could be enhanced relative to adsorption on zeolites, by using several types of additives. This was particularly useful in the case of supercritical CO_2 where cutinase activity is poor when compared to other solvents. One of the additives used in this study was zeolite NaY. To try and understand the effect of the presence of added zeolite NaY on cutinase activity, we turned to spectroscopic techniques.

The first idea that came to us was to try and correlate the performance of the enzyme with the structure of the material. There are several reports in the literature that indicate a modification of sol-gel structure in the presence of different materials such as surfactants and ionic liquids. The bio-mineralization process also shows that some species can lead to the formation of structurally different silica materials. We thus considered the possibility that zeolite NaY could act as a template for the sol-gel matrices. In fact, even enzymes are sometimes reported to have that effect. However, both Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectroscopy and Solid State ^{29}Si and ^{1}H NMR showed that the molecular structure of the sol-gel materials was insensitive to the presence of either additive or enzyme. DRIFT provided information on the hydrophilic-lipophilic balance (HLB) of the matrices, the degree of condensation and percentage of free silanol groups. The enhancement of cutinase activity was found to correlate with a decrease in HLB, which suggests that the enzymatic reactions performed were controlled by diffusion through the matrices. Lower cutinase activity when sol-gel precursors with alkyl chains longer than C = 6 were used suggested that the alkyl chains acted as pore blockers in that case.

Since DRIFT spectroscopy and solid state NMR could not throw light on the enzyme activity enhancements when added zeolite NaY was used, we decided to use a spectroscopic technique that looked specifically at the enzyme. We thus applied fluorescence spectroscopy based on the emission of the single tryptophan of the cutinase molecule, to probe the microenvironment of the enzyme. Our results indicated that the enzyme sensed the polarity of the surrounding matrix, but did not feel the presence of the zeolite. On the other hand, the presence of the zeolite was important

because it increased enzyme activity. This raised the question of the location of both enzyme and additive in the sol-gel matrix. The fact that zeolite NaY is a good support for the immobilization of cutinase by physical adsorption, and the fact that enzyme and zeolite are allowed to contact during the genesis of the sol-gel matrix suggested that they might be in close proximity in the material. Scanning electron microscopy and energy dispersive X-ray spectroscopy revealed that the zeolite particles were segregated to the pores of the matrices. Optical microscopy following the staining of the protein with a fluorescent dye showed that the enzyme was distributed throughout the material, and tended to accumulate around zeolite particles. By promoting the accumulation of the enzyme at the pores of the material, the zeolite should improve the accessibility of the enzyme to the substrates and lead to a higher activity of the entrapped enzyme. On the other hand, it is possible that the zeolite protects the enzyme from unfavorable interactions with the forming matrix. These could be covalent interactions, often referred as responsible for enzyme deactivation.

Chapter IV of this thesis was dedicated to the development and characterization of a novel material made of gelatin and an ionic liquid. The work that led to the "ion jelly" started with an attempt to immobilize cutinase in the presence of an ionic liquid, following previous findings with combined ionic liquid/supercritical CO_2 systems that suggested a protective effect of ionic liquids on cutinase. Our first strategy was to immobilize the enzyme together with an ionic liquid using the sol-gel process. However, cutinase activity was always lower than in the matrices without ionic liquid, and in some cases we did not even detect enzyme activity. At the time we concluded that the ionic liquid was affecting the sol-gel process, since in the literature ionic liquids are described as templates for sol-gel matrices. Next we mimicked a well known strategy to improve enzyme activity in nonaqueous media that consists in lyophilizing the enzyme in the presence of a highly concentrated salt solution. To that end, we freeze-dried the enzyme in the presence of varying concentrations of ionic liquid. This did not yield satisfactory results either. Finally we combined an ionic liquid with a natural polymer, gelatin. This was motivated by reports in the literature on ionic liquids that were capable of dissolving proteins and polymers in high concentrations. The idea was to use the ionic liquid to create a favorable ionic environment for the enzyme. It turned out that the ion jelly with cutinase did not afford any improvement in cutinase activity relative to

immobilization on zeolites or in the sol-gel. Nevertheless the end of this story was actually the beginning of a completely new line of research.

The combination of gelatin with an ionic liquid had never been described in the literature. This gave us the opportunity to patent the ion jelly as well as to think about some applications for this new material. Its novelty and advantages are fundamentally related with the versatility offered by the combination of its two main constituents. The morphological versatility of gelatin and the chemical versatility of the ionic liquids open up a myriad of potential solutions. The study presented here consists in the characterization of the ion jelly in order to look for suitable applications for this new material. Our results show that it is possible to modulate the ion jelly conductivity by changing the type of ionic liquid used to produce it. We also show that the ion jelly can work as an electrolyte in electrochromic applications, and that it can be used as an electron conducting material – the jelly wire. Our findings are significant to the area of conducting polymers since we present a very simple technique for producing tailor-made conducting polymers for different electrochemical applications. The fact that the concept of the ion jelly is based on a natural polymer such as gelatin can make its commercial application feasible when compared to conducting polymers. Moreover the ability that gelatin has to interact with other materials, such as cellulose, makes the ion jelly a very attractive material for building devices that fit into the concept of smart clothing or smart environments.

In the present thesis we start with an enzyme and end with the characterization of a novel material. This shows how versatile and multidisciplinary biocatalysis can be. The numbers of scientific areas that interface with biocatalysis are a good indication of the even greater impact that biocatalysis will have in the near future. Its importance greatly exceeds the many added-value compounds that can be produced with it. The social impact of biocatalysis can be far more significant, since the sustainable processes that are designed with that technology constitute essential guidelines for the way we want to live our lives in the future. Biocatalysis is one of the routes to green chemistry, which is what we want chemistry to be in the future.

Our need to understand certain aspects of our research can drive us to completely different areas of knowledge. By trying to go as deeply as possible in the analysis of the problems at hand, we can find solutions or partial solutions to those problems, and very often we also find solutions to problems that were not even ours, and that become doorways to the future.