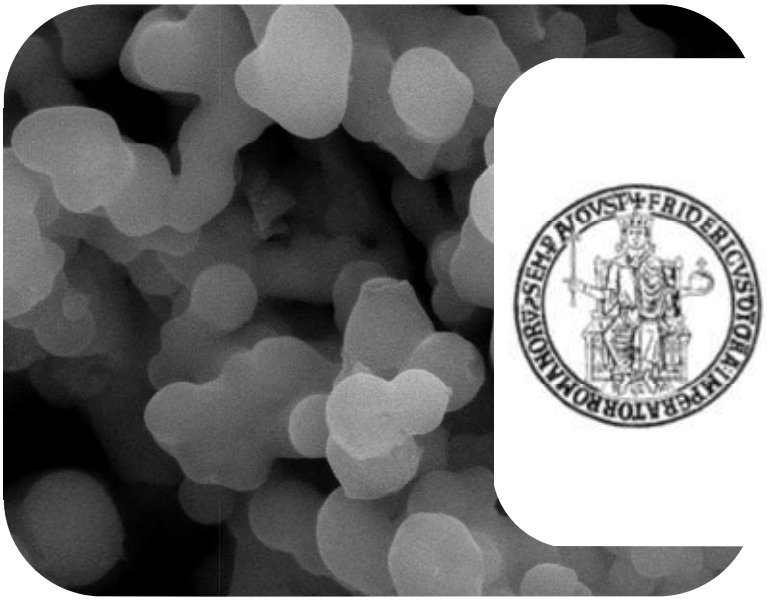

DEVELOPMENT OF MICROSTRUCTURED BIOREACTORS FOR GREEN CHEMISTRY APPLICATIONS

Thesis by

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SCHOOL OF POLYTECHNIC AND BASIC SCIENCES

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Development of Microstructured Bioreactors for Green Chemistry Applications

by

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ABSTRACT

Future generations should be able to benefit from a previous responsible economic and social growth, triggered by a good management of the sources nowadays available for the industrial production.
For decades by now, renewable sources have been a topic on the agenda overall the world, and the need to have eco-friendly processes has been a heartfelt matter, to cope with the continuous degradation, due to an improper administration of the environmental resources, which continues to affect our planet.
For this reason, in each industrial field there is a sort of challenge to gain the greenness for its own processes, in order to positively answer to some general programmes, designed to preserve the environment from further negative changes.
It is in this view that biocatalysis has made inroads in several industrial fields, but it is worthy to say that its whole employment is still far, because its widespread use in industry will require a proper conversion to achieve high space-time yield, to be competitive with the synthetic catalysis.

The aim of this thesis is to explore the capacity, efficiency and productivity of enzymatic microstructured reactors, designed adopting the sol-gel technology as the enzyme immobilisation procedure, in order to offer the industries a tool to respond to some issues regarding catalytic processes. For this purpose, many industrially interesting enzymes have been immobilised, properly tuning some parameters, in order to achieve the best microenvironment for each one of them and to take advantages from their properties, overcoming some problems related to the use of free biocatalysts, especially as regards recycling and repeatability.

The chosen enzymes for this research were a Lipase from *Candida rugosa* (CRL), a Xylanase from *Thermomyces lanuginosus* (TLX), a D-phenylglycine aminotransferase, isolated from *Pseudomonas stutzeri* ST-201 (D-PhgAT). All these enzymes are of great industrial interest, as they offer alternative methods for the synthesis of high value-added products. These methods require mild operative conditions, whereas the conventional chemical routes frequently prescribe severe values of pH and temperature. As a consequence, the enzyme processes can significantly increase the yield and the purity of refined products, often highly required.

The industrial potential of these enzymes is well known:

- a *lipase* can catalyse biotransformations of triglycerides and their derivates, in order to gain high added value products, such as natural surfactants, fats, natural flavours, but it can be also employed in the biosensors field, e.g. for triglycerides determination;

- a *xylanase* is able to catalyse the hydrolysis of glycosidic bonds within the xylan, that is a polysaccharide of the family of hemicelluloses, which complete degradation brings to the formation of smaller molecules, known as xylo-oligosaccharides (XOS), which can be considered as prebiotics;

- a *D-phenylglycine aminotransferase* can be used in transamination reactions between an amino acid and an α-ketoacid, for the synthesis of certain amino acids very difficult to obtain via synthetic route, and highly demanded especially in the pharmaceutical field.

The catalytic performance of each one of the considered enzymes has been optimized with reference to specifically selected industrial processes.
In particular, the sol-gel immobilization procedure has been modulated for every enzyme as regards the following aspects:

- choice of precursors, focusing the attention on the silane oxides;
- hydrolysis/condensation relative rate, acting on main parameters affecting the process kinetics;
- choice of the final treatment, exploring the effect of a total desiccation *versus* lyophilisation, to obtain a porous material.

Another issue addressed in this thesis is the optimization of the reactive operating conditions for the various analysed catalytic processes, both in batch and in continuous mode; in this view, a continuous reactor scheme design was accomplished on a lab scale for each enzymatic system.

Keywords: Biocatalysis, Enzyme Immobilization, Enzyme stability, Sol-Gel technology, Microstructured Bioreactors

PREFACE

This thesis describes the most important results achieved during my PhD study.

The work was mainly carried out in the Biochemical Engineering laboratory, Department of Chemical, Materials and Production Engineering (DICMaPI), University of Naples Federico II, from November 2015 to November 2018. Part of experimental activity was accomplished in the Institute of Biomolecular Chemistry, National Research Council of Italy (I.C.B.-C.N.R) and in the School of Chemistry, Industrial Biocatalysis, of the University of Edinburgh.

The thesis was submitted to the Department of Chemical, Materials and Production Engineering (DICMaPI), University of Naples Federico II on December 2018 as part of the requirements of the academic degree of Doctor of Philosophy (Ph.D.) in Industrial Product and Process Engineering.

Napoli, 11.12.2018

Maria Abagnale

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1. INTRODUCTION

1.1 WHAT IS THE *GREEN CHEMISTRY*?

The *Green Chemistry* is *the design of chemical products and processes that are more environmentally benign and reduce negative impacts to human health and the environment.*

The so-called *Green Chemistry* is neither a branch of chemistry, nor a specialisation in a particular field; it is rather an ethical approach, a sort of philosophy based on the scientific knowledge of chemistry, whose laws – according to this strategy – are applied in "a green way", in order to preserve the environment at first, and the overall cost of the process itself, also.

The *Green Chemistry* is different from the *Environmental Chemistry*, which is instead a branch of chemistry about chemical and biochemical phenomena in the natural environment, without ethical opinion or information about the political orientation, so it is not among its aims to persuade institutions to take actions against pollution.

Therefore, it is proper to say that green chemistry is just a concept developed to be used as an instrument to achieve sustainable development in the chemical industry:

Green chemistry efficiently utilises (preferably renewable) raw materials, eliminates waste and avoids the use of toxic and/or hazardous reagents and solvents in the manufacture and application of chemical products.

It is commonly known that ecological balances are nowadays compromised, though it is impossible to specifically ascribe its origin; nevertheless, the large scale production of certain required family of products implicates the realization of a great number of factories on earth, with the unavoidable generation of by-products, dangerous for the human health and for the whole environment, especially for their huge quantity (industrial scale!).

In this view, apart from the intrinsic pollution grade proper of some substances, part of the responsibility – not as an attack, but to underline that institutions and industrial units must consider this issue and try to prevent or, at least, reduce it – lies in the way the processes have been designed and carried out on large industrial scale. If the ethical reasons are not enough, more practical ones confer a higher interest: some chemical industrial processes have not an attractive yield, with a loss in raw materials and even adding costs to dispose

of the by-products (gas emissions or liquid/muddy wastes or ashes from incinerator/gasifier): in this cases, the green chemistry becomes an optimisation parameter! Anyway, these concepts are not recent, in fact they have been analyzed since the beginning of the 80s, when the United Nations founded the World Commission for Environment and Development, in 1983: its main task was to publish a report on the perspectives of long-term, sustainable and environmentally friendly development on a world scale by 2000 and after. In the first report – *Our Common Future* – come out after four years, also known as the "Brundtland Report" (after the Minister President of Norway and chairman of the commission at that time, Gro Harlem Brundtland), sustainable development is defined as *"meeting the needs of the present generation without compromising the ability of future generations to meet their own needs"*.

In 1990 in the United States a national policy to prevent or reduce pollution – wherever possible – was issued, known as *Pollution Prevention Act*, PPA: it focused industry, government, and public attention on reducing the amount of pollution through cost-effective changes in production, operation, and raw materials use; it states that:

1. Pollution should be prevented or reduced at the source whenever feasible;
2. Pollution that cannot be prevented or reduced should be recycled;
3. Pollution that cannot be prevented or reduced or recycled should be treated;
4. Disposal or other releases into the environment should be employed only as a last resort.

To preserve the *Green Chemistry* morals, John C. Warner and Paul Anastas tried to systematise them according to the following 12 pragmatic principles:

1. It is better to prevent waste than to treat or clean up waste after it is formed;
2. Synthetic methods should be designed to maximise the incorporation of all materials used into the final product;
3. Synthetic methodologies should be designed to use and generate substances that possess little or no toxicity to human health and to the environment;
4. Chemical product should be designed to preserve efficacy of function while reducing toxicity;
5. The use of auxiliary substances (e.g. solvents) should be made unnecessary wherever possible, and innocuous when used;

6. Energy requirements should be recognised for their environmental and economic impacts and should be minimised. Synthetic methods should be conducted at ambient temperature and pressure;
7. A raw material of feedstock should be renewable rather than depleting wherever technically and economically practicable;
8. Unnecessary derivisation (e.g. protecting/deprotecting groups)- should be avoided wherever possible;
9. Catalytic reagents (as selective as possible) are superior to stoichiometric reagents;
10. Chemical products should be designed so that at the end of their function they do not persist in the environment and instead break down into innocuous degradation products;
11. Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances;
12. Substances and the form of a substance used in a chemical process should be chosen so as to minimise the potential for chemical accidents.

Chemists and researchers have written these principles, aware of the role of chemistry in our society, not only as a scientific branch of knowledge, but especially for their several applications on large scale, from the industrial field to agriculture or to chemical analyses and biochemical field, and so forth.

Nevertheless, in the research field, the first step is to work onto a lab scale: when the tests give promising results, combined with a good grade of repeatability, it is possible to scale up and design the process on a larger scale. At this point, green chemistry can start playing its role, making its expertise available, through skills and ethics of people involved in the project itself. As a matter of facts, green chemistry can be very useful in the scaling up of a lab-scale route, in which so many process parameters must be taken in consideration, such as the cost of raw materials, energy and waste disposal, the public view about the settlement of a new industrial process, nearly always associated to the environment imbalance and damages to human health due to polluting emissions.

In Europe and also in Italy several organizations are raising up to professionally deal with the *green chemistry* or involved in the so called *green economy*, and year by year also some official occasions are organised in this field, as for example:

"*Chimica verde: dalla biotecnologia nuove risorse per l'industria*" – 23 July 2010, Bologna.
"*1° Festival della chimica verde*" – 27-29 April 2009, Lecce.
"*Chimicaverde*" – association founded in 2006 in order to promote non-food agricultural crops exploitation in the renewable energy area (biopolimers, biofuels, pesticides, natural dyes, vegetal fibres, cosmetics, detergents, …).
"*SusChem*", a technologic platform that – among the other skills – supports *EuChem* (European Association for Chemical and Molecular Sciences).

This kind of organizations is destined to increase in number, because nowadays the pollution prevention and a sort of sense of globe protection is very much felt.

Indeed, *Green Chemistry* is an issue of paramount concern, so that year by year more and more people are interested in its study; a noteworthy woman in this field is Prof. Frances Hamilton Arnold (born 25 July 1956), Professor of Chemical Engineering, Bioengineering and Biochemistry at the California Institute of Technology (Caltech), who has been recently awarded the Nobel Prize in Chemistry (October 2018) for pioneering the use of directed evolution to engineer enzymes. In Caltech, prof. Arnold has addressed her studies especially towards the *Green Chemistry* and the renewable energies, focusing her attention on the development of enzymes and microorganisms able to turn renewable sources into biofuel.

There are 12 Additional Principles for Green Chemistry, listed below (*Anastas and Warner, 1998*):
1. Identify by-products; quantify if possible.
2. Report conversions, selectivities, and productivities.
3. Establish a full mass balance for the process.
4. Quantify catalyst and solvent losses.
5. Investigate basic thermochemistry to identify exotherms (safety).
6. Anticipate other potential mass and energy transfer limitations.
7. Consult a chemical or process engineer.
8. Consider the effect of the overall process on choice of chemistry.
9. Help develop and apply sustainable measures.
10. Quantify and minimize use of utilities and other inputs.

11. Recognize where operator safety and waste minimization may be compatible.
12. Monitor, report and minimize wastes emitted to air, water, and solids from experiments or processes.

Strictly connected to Green Chemistry, there is the *Green Engineering*, with the following definition: *(Abraham, 2004)*

"The design, commercialization, and use of processes and products, which are feasible and economical while minimizing generation of pollution at the source and risk to human health and the environment."

As for green chemistry, also Green Engineering is based on 12 principles, reported below *(Anastas et al., 2003)*:

1. Designers need to strive to ensure that all material and energy inputs and outputs are as inherently nonhazardous as possible.
2. It is better to prevent waste than to treat or clean up waste after it is formed.
3. Separation and purification operations should be designed to minimize energy consumption and materials use.
4. Products, processes, and systems should be designed to maximize mass, energy, space, and time efficiency.
5. Products, processes, and systems should be "output pulled" rather than "input pushed" through the use of energy and materials.
6. Embedded entropy and complexity must be viewed as an investment when making design choices on recycle, reuse, or beneficial disposition.
7. Targeted durability, not immortality, should be a design goal.
8. Design for unnecessary capacity or capability (e.g., "one size fits all") solutions should be considered a design flaw.
9. Material diversity in multicomponent products should be minimized to promote disassembly and value retention.
10. Design of products, processes, and systems must include integration and interconnectivity with available energy and materials flows.
11. Products, processes, and systems should be designed for performance in a commercial "afterlife".
12. Material and energy inputs should be renewable rather than depleting.

Beside green chemistry and green engineering, it is worthy to mention also the *sustainability (Brundtland Commission, 1987, United Nations):*

"Providing for human needs without compromising the ability of future generations to meet their need"

with its principles *(Beloff et al., 2005):*

1. Engineer processes and products holistically, use systems analysis, and integrate environmental impact assessment tools;
2. Conserve and improve natural ecosystems while protecting human health and well-being;
3. Use lifecycle thinking in all engineering activities;
4. Ensure that all material and energy inputs and outputs are as inherently safe and benign as possible;
5. Minimize depletion of natural resources;
6. Strive to prevent waste;
7. Develop and apply engineering solutions, while being cognizant of local geography, aspirations, and cultures;
8. Create engineering solutions beyond current or dominant technologies; improve, innovate and invent (technologies) to achieve sustainability;
9. Actively engage communities and stakeholders in development of engineering solutions. There is a duty to inform society of the practice of sustainable engineering.

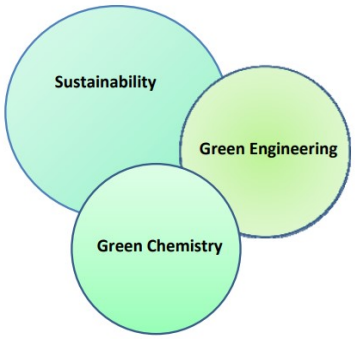


Figure 1.1: Relationship between Green Engineering, Green Chemistry and sustainability (*Abraham, 2003)*.

2. BIOCATALYSIS AND BIOCATALYSTS

2.1 GENERAL

Among the greenest technologies for chemical synthesis, biocatalysis, i.e. chemical transformations catalysed by the use of a natural catalyst, such as an enzyme, is the best-known.

Biocatalysis is part of *industrial* or *white biotechnology*. Fine chemicals, above all pharmaceuticals, are certainly essential products for our society, indeed their demand is continuously growing and for this reason the synthesis routes must constantly develop to satisfy the increasing and detailed requests. For example, one of the most frequent compounds in pharmaceuticals is the chiral amine, biofunctional group also present in natural metabolism, very difficult to obtain via synthetic route, but biocatalysis offers an easier way to obtain.

Biocatalysis is very useful in the view of the green chemistry, due to the fact that it can prevent waste generation by performing the catalytic processes with a high stereo- and regioselectivity and by preventing or at least limiting the use of hazardous reagents and solvents.

Highly energy efficient and safe processes can be designed, since operative conditions are mild (ambient temperature, pressure, physiological pH).

In recent years a combination of factors is causing a revolution in biocatalysis. Among them, there are the developments in large scale DNA sequencing, protein expression, directed evolution (rational, semi-rational, or random), high-throughput screening, structural biology and metabolic engineering.

The core of biocatalysis is with no doubt the presence of natural compounds and natural catalysts, known as enzymes, that are basically elementary or complex proteins, able to catalyse a wide range of chemical reactions.

Enzyme properties, such as substrate specificity, enantioselectivity, activity, stability, and pH optimum, can be modified to satisfy process demands, which in turn can lead to an increase in industrial enzyme applications. Notionally, they could be used in several industrial fields, as pharmaceutical, chemical and food industry, as the best catalysts for a green development, but in practice biocatalysis is still only unwillingly introduced in the chemical industry, due to the presence of diverse unsolved issues, but the point is that using biocatalysis for a synthesis step, it is possible to exploit the properties – and in

particular the streoselectivity – of a biocatalyst, in order to obtain high purity compounds (*Halim M.B., 2012, PhD thesis*).

In this way, biocatalysis have turned out to be competitive compared with classic chemical *(Drauz et al., 2012).* Among the advantages of biocatalysis, the most considerable are biodegradability, non-toxicity, highly specificity and mild reaction conditions, and from an industrial point of view, these features are of great interest.

Nevertheless, the effective application of biocatalytic processes in the chemical industry depends mainly on cost competitiveness with the existing ones, and well established chemical processes *(Sanchez and Demain, 2011)*.

Anyway, the predominant role for biocatalysis in the future lies in the pharmaceutical sector. For this reason, in the near future, biocatalysis will be fully settled as a tool for the organic (process) chemistry and will be fully implemented into chemical transformations at the retro-synthetic level.

Examining in depth biocatalysis and biocatalysts, in nature compounds are formed and broken down in the so-called controlled metabolic pathways, in which energy is rearranged and utilised for vital processes, regulated by the action of a biocatalyst.

Like all catalysts, enzymes increase the reaction rate by lowering its activation energy; chemically, enzymes are like any catalyst and are not consumed in chemical reactions, nor do they alter the equilibrium of a reaction.

Enzymes differ from most other synthetic catalysts by being much more specific; furthermore, enzyme activity can be affected by other molecules, such as inhibitors or activators.

Some enzymes are used commercially, especially in the pharmaceutical field, for example in the synthesis of antibiotics.

It is worthy to know that by the late 17^{th} and early 18^{th} centuries, the digestion of meat by stomach secretions and the conversion of starch to sugars by plant extracts and saliva were known but their mechanisms were not well identified. In 1833, a French chemist, Anselme Payen, was the first to discover an enzyme, diastase, and a few decades later, when studying the fermentation of sugar to alcohol by yeast, Louis Pasteur concluded that this fermentation was caused by a vital force contained within the yeast cells called "ferments", which were thought to function only within living organisms. He wrote that "*alcoholic fermentation is an act correlated with the life and organization of the yeast cells, not with the death or putrefaction of the cells*".

In 1877, German physiologist Wilhelm Kühne (1837–1900) first used the term *enzyme*, which comes from Greek ἔνζυμον, "leavened" or "in yeast", to describe this process.

Eduard Buchner submitted his first paper on the study of yeast extracts in 1897 *(Nobel Laureate Biography of Eduard Buchner).* In a series of experiments at the University of Berlin, he found that sugar was fermented by yeast extracts even when there were no living yeast cells in the mixture. He named the enzyme that brought about the fermentation of sucrose "zymase". In 1907, he received the Nobel Prize in Chemistry for "his discovery of cell-free fermentation". Following Buchner's example, enzymes are usually named according to the reaction they carry out, so the suffix *–ase* is combined with the name of the substrate (e.g., lactase is the enzyme that cleaves lactose) or to the type of reaction (e.g., DNA polymerase forms DNA polymers).

The biochemical identity of enzymes was still unknown in the early 1900s, because of course enzymology was in its infancy. Many scientists observed that enzymatic activity was associated with proteins, but others argued that proteins were merely carriers for the true enzymes and that proteins were incapable of catalysis.

In 1926, James B. Sumner showed that the enzyme urease was a pure protein and crystallized it; he did similarly for the enzyme catalase in 1937.

John Howard Northrop and Wendell Meredith Stanley definitively demonstrated that pure proteins can be enzymes working on digestive enzymes pepsin (1930), trypsin and chymotrypsin, and finally in 1946 they were awarded Nobel Prize in Chemistry.

Furthermore, the discovery that enzymes could be crystallized allowed their structures to be solved by x-ray crystallography. This was first done for lysozyme and the structure was solved by a group led by David Chilton Phillips and published in 1965. This high-resolution structure of lysozyme marked the beginning of the field of structural biology and the effort to understand how enzymes work at an atomic level of detail.

Going back in time, it is easy to realize that modern biotransformations have a very long development, even from 5000 B.C., as reported in the *Encyclopedia of Industrial Biotechnology (Ghisalba et al., 2010)*:

5000 B.C.: preservation of food and alcoholic drinks on Egyptian pictures, vinegar production;

800 B.C.: Casein hydrolysis with chymosin for cheese production. Homer’s Iliad mentions stomach enzymes for cheese making;

1670: “Orleans” process for the industrial bio-oxidation of ethanol to acetic acid;

1680: Antoni van Leeuwenhoek, first to see microorganisms with his microscope;
1833: Payen and Persoz investigated germinating barley and formulated basic principles of enzymes;
1874: Christian Hansen started an enzyme laboratory and company;
1878: Kühne coined the term *enzymes*;
1890: Takamine isolated bacterial amylases (what later became Miles Laboratories);
1894: Emil Fischer elaborated the essentials of enzyme catalysis;
1897: Buchner discovered yeast enzymes converting sugar into alcohol;
1897: Eduard Buchner published fermentation with cell-free extracts from yeasts;
1899: Berzelius acknowledged the catalytic reaction of starch hydrolysis by diastase;
1907: Röhm founded a company producing "Oropon" for tanning (mixture pancreatic extract and ammonium salts);
1926-1935: Summer, Northrop and Kunitz proved that enzymes were proteins;
1930: Regioselective biooxidation of sorbite to sorbose for the Reichstein Vitamine C synthesis;
1940: Sucrose inversion using an invertase;
1950: Bioconversion of steroids;
1969: First industrial use of an immobilized enzyme (aminoacylase);
1970: Hydrolysis of penicillin to 6-aminopenicillanic acid;
1985: Enzymatic process for the production of acrylamide;
1990: Hydrolysis by protease (trypsin) of porcine insulin to human insulin;
1995: 3000 tons per year for the biotransformation based synthesis of nicotinamide.

This list can continue and can be very long, due to the fact that in the last two decades much progress has been made in this field.
It is worthy to say that there are some common prejudices against enzymes, which hide a part of truth; among them, there are the following, according to which enzymes are:

- *sensitive*: this is absolutely true for most enzymes, especially if the operative temperature is very low or very high, BUT it is also true that there are some enzymes, like those from hyperthermophilic bacteria or from the bottom of the sea, that are heat stable!

- *expensive*: some of them are really expensive, because of all the operations to obtain them from the crude extract to a high purity grade, BUT if produced on a

reasonable scale, the cost of the overall process falls down; furthermore, enzymes can be recycled if immobilised on solid supports, and in addition there's the possibility to decrease more and more the cost of the overall process due to the possibility to directly use – in some cases – the crude prep, cell extract or whole cell, bypassing the purification route, that is generally the most expensive part of the enzyme preparation.

- *only active on their natural substrate*: this is true for some enzymes, BUT definitely false for the majority of them! As a matter of facts it is accepted that enzymes are much more promiscuous than previously thought (also known as *moonlighting*).

- *only work in water*: this statement is true, indeed most enzymes show their highest rates in water, BUT biocatalysts can also work in non-aqueous environment, if properly engineered/selected to function in solvents.

Enzymes catalytic action is different from the synthetic catalysts one for three main reasons:

- *efficiency*: enzymatic reactions are usually faster than reactions catalysed by synthetic catalysts;
- *specificity*: each one enzyme catalyses only one kind of reaction with a specific substrate;
- *variability*: there is the possibility to improve the enzyme action from a low to a high level.

The main and most important functions of an enzyme in a chemical process are:

- Stabilizing the transition state and lower the activation energy required to obtain the products;
- Lowering the energy of the transition state itself, without any distortions for the substrate;
- Providing an alternative pathway by forming an intermediate ES complex, that would not have been possible in the absence of the enzyme.

Moreover, in presence of an enzyme the reaction rate can be faster, even up to 10^{14} fold, compared to the one of the same process without any catalyst. This reaction rate is the

evaluation of the product – that is to say the exhausted substrate – obtained in the unit of time, and it decreases over time, due to the diminishing of the substrate concentration due to the conversion, the stacking of the product and the gradual enzyme denaturation or deactivation.

Recapitulating, there are many *advantages* when using an enzyme instead of a synthetic catalyst:

- *biodegradability*: there are no extra costs to dispose of the catalyst at the end of the process and it does not contribute to the environmental pollution;
- *efficiency* (rates can increase by 10^8 – 10^{10} fold than those of non-catalysed reactions);
- *mild operative conditions*: pH 5-8, 20-50°C are the typical ranges of pH and temperature in an enzymatic process, much less harsh than in presence of a synthetic catalyst;
- *compatibility* with each other, with the possibility to have cascade reactions in the same reaction vessel;
- *versatility* (an enzyme can catalyse many reactions);
- enhanced *chemoselectivity*, *regioselectivity*, *diasteroselectivity*, *enantioselectivity*.

Besides all these advantages, there are as well some *disadvantages*:

- *narrow operating parameters* (pH and T): in some cases, reactions are slow but it is not recommended to warm them up or change the pH, to avoid the enzyme denaturation;
- *environment-limited action*: enzymes operate best in water, so if used without further modification, they turn out to be not suitable for organic substrates;
- *substrate and/or product inhibition*: enzymes are susceptible to inhibition due to substrate or product high concentration; it is possible to overcome this inconvenience by verifying the feeding, preserving the substrate concentration roughly constant just above the quantity required, or removing the product as it is made;
- *cofactors*: in many cases enzymes require the presence of natural cofactors, such as NADH or PLP, and the ideal thing is to recycle them for an efficient and green process.

According to general knowledge, enzymes are commonly named by the reaction they catalyze or by suffixing “ase” after the name of the substrate.

The *International Union of Biochemistry and Molecular Biology* (IUBMB) developed a nomenclature for enzymes: each enzyme is described by a sequence made up of the two letters "EC", with the meaning of "Enzyme Commission", followed by four numbers, the EC numbers. The first three numbers represent the class, subclass and sub-subclass to which an enzyme belongs, and the fourth one is a serial number to identify the particular enzyme within a sub-subclass: for example, in the case of *EC 1.2.3.4*, the digits indicate that the enzyme is an oxidoreductase (class 1), that it acts on the aldehyde or oxo group of donors (subclass 2), that oxygen is an acceptor (sub-subclass 3) and that it was the fourth enzyme classified in this sub-subclass (serial number 4).

The last printed list of enzymes appeared in the year 1992. Since then it has been updated and maintained online. *(https://nptel.ac.in/courses)*.

According to this categorization, enzymes are classified into six main family classes, as reported in Table 2.1:

EC class	IUBMB Designation	Function	Typical reaction	Examples
EC1	Oxidoreductases	reduction & oxidation reactions	AH + B → A + BH (reduction) A + O → AO (oxidation)	dehydrogenase oxidase
EC2	Transferases	transfer of functional groups	AB + C → A + BC	transaminase kinase
EC3	Hydrolases	- hydrolysis & condensation reactions of various bonds - H2O is one of the substrates - no cofactors needed	AB + H_2O → AOH + BH	esterase lipase amylase protease
EC4	Lyases	- addition & elimination reactions - cleavage of C-C, C-O, C-N bonds - no cofactors needed	RCOCOOH → RCOH + CO_2	aldolase decarboxylase
EC5	Isomerases	- *cis-trans* isomerization, racemisation, epimerisation reactions - one substrate reactions (isomerization changes within a single molecule)	AB → BA	phosphorus isomerase xylose isomerase mutase
EC6	Ligases	- formation of C-C, C-O, C-S, C-N bonds (covalent bonds) - two substrate reactions - ATP required as cofactor	X + Y+ ATP → XY + ADP + Pi	DNA ligase synthetase polymerase

Table 2.1: Enzyme classification according to the International Union of Biochemistry and Molecular Biology (IUBMB).

As regards their structure, enzymes are generally globular proteins, where the sequence of the amino acids specifies the structure which in turn determines the catalytic activity of the enzyme itself. Although structure determines function, a novel enzymatic activity cannot yet be predicted from structure alone. Enzymatic structures – from the primary, which is the simplest one, to the quaternary, shown in Figure 2.1 – unfold when heated or exposed to chemical denaturants and this disruption of the structure typically causes a loss of activity, due to the fact that structure and enzymatic activity are strictly connected.

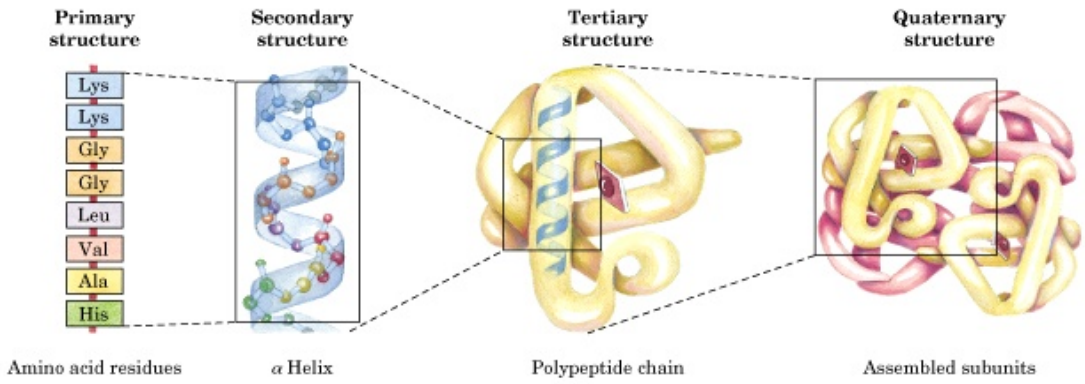


Figure 2.1: The four structures of proteins.

As a rule, enzymes are usually much larger than their substrates, and only a small portion of their structure (around 2–4 amino acids) is directly involved in catalysis, that is the catalytic site: it is located next to one or more binding sites where residues orient the substrates; they together form the so called active site, a three dimensional cavity where the substrate can bind, which in some cases corresponds to the whole polypeptide chain; the presence of the active site assures the best orientation of the substrate, letting it bind the catalyst itself in the proper way, facilitating the substrate to enter the transition state, and then to the product formation; the remaining part of the enzyme structure generally serves to maintain the precise orientation and dynamics of the active site. For some enzymes, no amino acids are directly involved in catalysis; in that case, the enzyme presents sites to bind and orient catalytic cofactors.

Enzyme structures may also contain allosteric sites where the binding of a small molecule causes a conformational change that increases or decreases activity.
As already reported above, enzymes are usually very specific as regards the reactions they catalyze and the substrates that are involved in these reactions.
Enzymes can also show impressive levels of stereospecificity, regioselectivity and chemoselectivity.
In particular, to better understand the meaning of *specificity*, the following specification should be made:

Absolute specificity: the enzyme catalyzes only one reaction.

Group specificity: the enzyme acts only on molecules that have specific functional groups.

Linkage specificity: the enzyme acts on a specific type of chemical bond regardless of the rest of the molecular structure.

Stereo-chemical specificity: the enzyme acts only on a particular steric or optical isomer.

So it is clear that enzymes are very specific to what substrates they bind and specificity is achieved by binding pockets with complementary shape, charge and hydrophilic/hydrophobic characteristics to the substrates, that is to say that biocatalysts can therefore discriminate between very similar substrate molecules and thus result to be chemoselective, regioselective and stereospecific.

Emil Fischer in 1894 suggested that enzyme specificity was primarily because both the enzyme and the substrate possessed specific complementary geometric shapes, and this is often referred to as the *lock and key* model: in this case, the active site of the protein shows a 3D jagged surface that is complementary to the substrate shape, as reported in Figure 2.2:

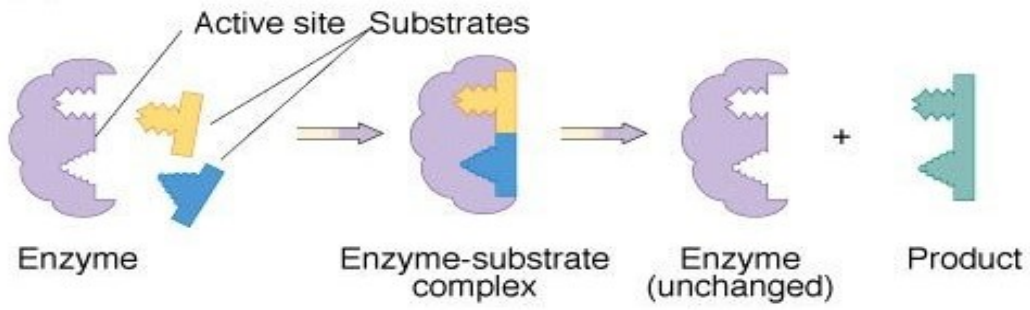


Figure 2.2: In the Lock & Key model, the active site of the enzyme and the substrate have complementary shapes: the enzyme is the "lock", the substrate is the "key" and the active site is the "key hole".

In this model, both the enzyme and the substrate surfaces are rigid; in its entirety, it seems to be a very clear way to explain the mechanism of action of the binding between the active site and the substrate, but if on one side this model clarifies enzyme specificity, on the other it fails to explicate the stabilization of the transition state that the presence of the enzyme assures to the enzyme-substrate complex.

Few decades later, in 1958, Daniel Koshland suggested a new model, according to which enzymes can adjust their shape depending on the different substrates, thanks to their flexibility: in this way, conformational changes can occur when the substrate binds during the reaction, getting the perfect match enzyme-substrate, at the transition state. Therefore, the enzyme re-shapes itself to completely fit the substrate, and so amino acid side chains

present in the active site are molded into the exact positions that assure the best catalytic performance.

To understand even better, the active site continues to modify its shape until the substrate is totally bound. This is called *induced fit* model, shown in Figure 2.3:

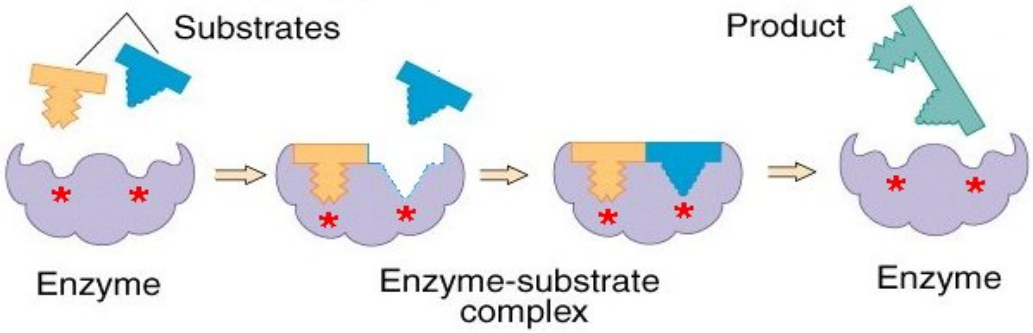


Figure 2.3: The *Induced-Fit* model: in this case, the initial interaction between enzyme and substrate is relatively weak, but it rapidly induces conformational changes in the enzyme that strengthen the binding, resulting in a strong stabilizing effect.

It is clear from Figure 2.3 that the active site modifies its shape throughout the engraftment: the binding of the first substrate induces a physical conformational shift (angular contours) in the protein that facilitates binding of the second substrate, with far lower energy than otherwise required. When catalysis is complete, the product is released, and the enzyme returns to its uninduced state, recovering its native shape.
In both cases, the binding between the active site of the enzyme and the substrate involves a range of several interactions, such as hydrogen bonds, salt bridges, Van der Waals interactions, hydrophobic effects, and all of them actively contribute to stabilize the transition state.

It is worthy to underline that as with all catalysts, enzymes do not alter the position of the chemical equilibrium of the reaction: as a matter of facts in the presence of an enzyme, the reaction runs in the same direction as it would without the enzyme, just more quickly!

Nevertheless, in order to understand the rate laws that enzyme-catalysed reactions follow, the protein-substrate binding event must be taken into account, using the scheme (2.a) for the enzymatic reaction:

(2.a) $$E + S \underset{k_1}{\overset{k_{-1}}{\leftrightarrows}} ES \xrightarrow{k_2} E + P$$

where *E* is the enzyme, *S* the substrate, *ES* the complex in the transition state, *P* the product.

This scheme, and in particular the effect of the substrate concentration on the initial rate, is well explained by Michaelis-Menten theory, postulated in 1913, proved by several experimental tests.

The model takes the form of an equation describing the correlation between the reaction rate *v* (rate of formation of the product *P*), and the concentration of the substrate *S*. Primarily, it describes the reaction between the enzyme E and the substrate S to obtain the complex ES, that is the binding step regulated by the kinetic constant k_1 and k_{-1}; then the catalytic step controlled by the constant k_2 takes place, in which the complex enzyme-substrate turns into the product, with the release of the enzyme without any modification: in this way, the overall enzyme concentration is preserved during the process.

Referring to equation (2.a), it is possible to obtain the formula (2.b) that correlates the reaction rate and the substrate concentration:

(2.b) $$v = \frac{d[P]}{dt} = \frac{v_{max} \cdot [S]}{K_M + [S]}$$

This formula, obtained in steady-state conditions, gives the possibility to define key enzyme kinetic constants:

- v_{max}, [M s^{-1}], is the maximum speed of the enzymatic reaction, at saturating substrate concentration, that is to say the maximum rate at which the reaction can run, regardless of [S], meaning that the reaction cannot go any faster even if adding more substrate, because all the active sites on the enzyme are occupied; v_{max} is observed when the enzyme-substrate complex totally disappears and turns into products:

- $$v_{max} = k_2 \cdot E_0$$

- The Michaelis-Menten constant K_M, [M], is the value of the substrate concentration, [S], required to achieve half of v_{max} and is an inverse measure of the substrate's affinity for the enzyme: a small K_M indicates high affinity, meaning that the rate will approach v_{max} with lower substrate concentration than those reactions with a larger K_M. The constant is not affected by the concentration or purity of an

enzyme. The value of K_M is dependent on both the enzyme and the substrate, as well as conditions such as temperature and pH.

$$K_M = \frac{k_{-1} + k_2}{k_1}$$

- the Turnover number, k_{cat} [s^{-1}], regulates the catalytic step and it is defined as the maximum number of chemical conversions of substrate molecules per second that a single catalytic site will execute for a given enzyme concentration, that is to say the number of substrate molecules converted into product by one molecule of enzyme active site per unit time, when the enzyme itself is fully saturated with the substrate:

$$k_{cat} = \frac{v_{\max}}{E_0}$$

- enzyme efficiency, specificity constant also called *kinetic efficiency*, [$M^{-1}s^{-1}$], is a measure of how efficiently an enzyme converts substrates into products:

$$\eta = \frac{k_{cat}}{K_M}$$

Referring to formula (2.b), displayed in Figure 2.4, it is clear to understand that the reaction order depends on the relative size of the two terms in the denominator: at low substrate concentration, [S] << K_M, there is a linear dependence between the reaction rate and the substrate concentration, while at higher substrate concentration, [S] >> K_M, the reaction assumes a zero-order kinetic and asymptotically approaches its maximum rate v_{max}, when all enzyme is bound to substrate.

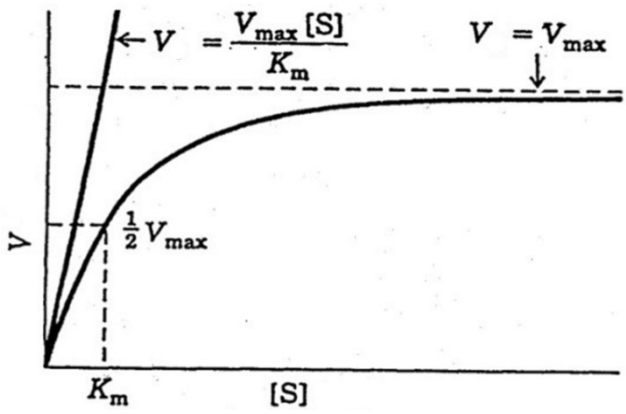


Figure 2.4: Michaelis-Menten curve.

An enzyme catalysed reaction may proceed in various ways. It is known that properly varying certain operative parameters, it is possible to get faster the desired products. Actually, the enzyme main task is to lower the activation energy (ΔG, Gibbs free energy), and it can do that in different ways, first of all by stabilizing the transition state, i.e. creating a charge distribution of the whole reaction environment complementary to that of the transition state, by providing an alternative reaction pathway, i.e. forming a provisionally covalent intermediate to provide a lower energy transition state, by destabilising the substrate ground state, i.e. disrupting substrates into their transition state condition to lower the energy required to reach the transition state and by steering the substrates toward a such arrangement to gain the reduction of the reaction entropy change.
It is also possible to act on external factors, such as substrate concentration, pH, temperature, inhibitors, to enhance enzyme function and produce the end product even faster. However, great care must be taken as regards the modulation of these parameters, otherwise the enzyme may get denatured, i.e. the amino acid bonds can be broken and so the catalytic action results damaged, so that the substrate does not fit more the active site, due to the fact that it is altered in shape, and the enzyme only slowly turns the substrates into the products, or not at all *(https://nptel.ac.in/courses)*.

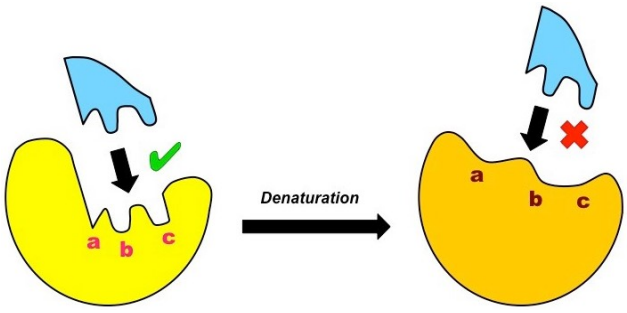


Figure 2.5: Denatured enzyme: the substrate cannot enter binding the active site, and so its catalytic activity is highly compromised.

2.2 INDUSTRIAL ENZYMES

As already said above, even if it is still far their total application, enzymes are conquering step by step the interest of every industrial field, thanks to their very competitive properties and advantages, compared to synthetic catalysts. They are used in chemical industry especially when extremely specific catalysts are required, but – again – enzymes must be improved as regards their stability related to solvents and operative parameters (mainly temperature and pH); these efforts have begun to be successful and some enzymes have been designed and engineered to catalyse reactions that normally do not take place in nature (*https://en.wikipedia.org/wiki/Industrial_enzymes).*

In the following Table 2.2 some industrial applications are listed together with the corresponding enzymes used:

APPLICATION	ENZYMES USED	USES
Biofuel industry	Cellulases	Break down cellulose into sugars that can be fermented to produce cellulosic ethanol.
	Ligninases	Pretreatment of biomass for biofuel production.
Biological detergent	Proteases, amylases, lipases	Remove protein, starch, and fat or oil stains from laundry and dishware.
	Mannanases	Remove food stains from the common food additive guar gum.
Brewing industry	Amylase, glucanases, proteases	Split polysaccharides and proteins in the malt.
	Betaglucanases	Improve the wort and beer filtration characteristics.
	Amyloglucosidase and pullulanases	Make low-calorie beer and adjust fermentability.
	Acetolactate decarboxylase (ALDC)	Increase fermentation efficiency by reducing diacetylformation.
Culinary uses	Papain	Tenderize meat for cooking.
Dairy industry	Rennin	Hydrolyze protein in the manufacture of cheese.
	Lipases	Produce Camembert cheese and blue cheeses such as Roquefort.
Food processing	Amylases	Produce sugars from starch, such as in making high-fructose corn syrup.
	Proteases	Lower the protein level of flour, as in biscuit-making.
	Trypsin	Manufacture hypoallergenic baby foods.
	Cellulases, pectinases	Clarify fruit juices.
Molecular biology	Nucleases, DNA ligase and polymerases	Use restriction digestion and the polymerase chain reaction to create recombinant DNA.
Paper industry	Xylanases, hemicellulases and lignin peroxidases	Remove lignin from kraft pulp.
Personal care	Proteases	Remove proteins on contact lenses to prevent infections.
Starch industry	Amylases	Convert starch into glucose and various syrups.

Table 2.2: Industrial applications of some enzymes and their uses.

Before going onwards, a focus on the enzymes treated in the PhD period research is essential, since each one of them is very interesting from an industrial point of view:

- *lipase*: it catalyses biotransformations of triglycerides and their derivates, in order to gain high added value products, such as natural surfactants, fats, natural flavours, but also in the biosensors field, for triglycerides determination;
- *carbonic anhydrase*: it can be used for CO_2 capture from exhausted gases or from closed environments;
- *acetyl cholinesterase*: its main task is to stabilise the neurotransmitter acetylcholine level in human organism, catalysing acetylcholine conversion into thiocholine, which is fundamental to treat for example the organophosphates (OP), very dangerous compounds for the organism;
- *laccase*: this enzyme is used for the degradation of lignin, one of the most difficult compound to degrade in its components; it is also employed in the degradation of polycyclic aromatic hydrocarbons (PAH), pollutants that are very dangerous for human health and for the whole environment, which massive presence is registered above all in the industrialized countries of the world;
- *xylanase*: this enzyme catalyses the hydrolysis of glycosidic bonds within the xylan, that is a polysaccharide of the family of hemicellulose, which complete degradation brings to the formation of smaller molecules, known as xylo-oligosaccharides (XOS), which can be considered as prebiotics;
- *D-phenylglycine aminotransferase*: used in transamination reactions between an amino acid and an α-ketoacid; they are important in the synthesis of amino acids, which form proteins.

Examining in depth one at a time, it is possible to analyse their characteristics and properties.

2.2.1 LIPASES

According to the classification given by the IUBMB, lipases belong to the third group, EC 3, where the various subclasses are defined by the specific reaction they catalyse *(Svendsen A., 2000)*.

They are mainly used in the hydrolysis or in the formation of lipids, and for this reason they are often defined as lipolytic enzymes, and – because of their triacylglycerol activity – they are classified as EC 3.1.1.3, with the general reaction (2.c):

(2.c) $$Triglyceride \leftrightarrow Glycerol + FattyAcids$$

Referring to reaction (2.c), lipases are hydrolytic enzymes which hydrolyse triglycerides to free fatty acids and glycerol, but they also catalyse many other kind of reactions, among which transesterification, aminolysis and acidolysis ones *(Sangeetha R., 2011)*.

Their potential to work also in non-aqueous and micro-aqueous environments makes them a great versatile biotechnological tool, precisely because they are active at interfaces and thereby convert water-insoluble substrate.

Lipases are ubiquitous in nature – they are widely distributed in plants, animals and microbes – and especially those obtained from microbes have gained particular attention as industrial biocatalysts, besides the industrial exploitation of both fungal and bacterial lipases; all of them are associated with one another thanks to their similar characteristics: their molecular mass varies in the range 20-75 kDa, and their isoelectric point can float from 3.6 to 7.6. Moreover, lipases result to be so interesting from an industrial point of view because of their chemo, regio and enantio-selectivity properties, known since 1856, when Claude Bernard discovered the first lipase in pancreatic juice.

Lipases preferentially act on their natural substrates, i.e. triglycerides or diglycerides, and therefore have diglyceride and monoglyceride as products, rather than the glycerol and fatty acids alone. Anyway, as already said above, they are very versatile and often express other activities, such as phospholipase, lysophospholipase, cholesterol esterase, cutinase, amidase and other esterase type of activities. The fundamental striking difference is that esterases generally act on short chain triglycerides which are soluble in water, while lipases act on lipids that form aggregates in water and necessarily require a water-lipid interface to act as a catalyst.

Regardless of the kind of organism they are obtained from, lipases show a characteristic overall structural fold, but with a versatility of loop structures in contact with the substrate, and exhibits versatile substrate specificities.

The first three-dimensional structures of lipases were determined relatively late, in 1990 thanks to *Brady L. et al.* that, focusing their attention on a *M. miehei* lipase, they found out that it was an α/β type protein, with a central 8-stranded mixed β-pleated sheet, folded onto a highly amphipatic N-terminal helix, and a number of loops and helices, including a semi-external long kinked α-helix.

Furthermore, thanks to crystallographic and X-ray analyses, they revealed a Ser...His...Asp trypsin-like catalytic triad, as reported in Figure 2.6, precisely Ser 144, His 257 and Asp 203, with Ser 144 as the active residue among them, confirmed by some sequence analyses.

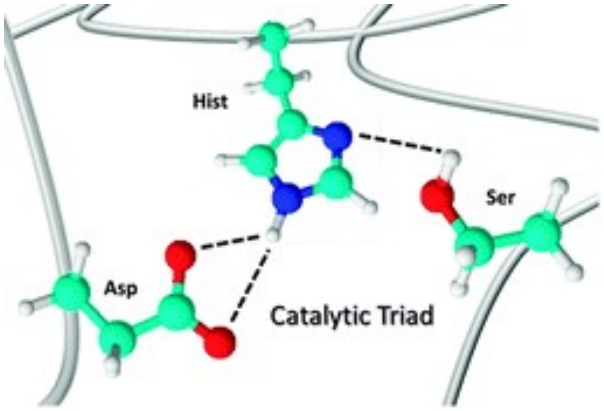


Figure 2.6: Lipase catalytic triad Serine (Ser) – Histidine (His) – Aspartate (Asp).

This catalytic triad was found to be positioned very close to the surface but not completely exposed to solvent; as a matter of facts they understood that the active serine was hidden by a short helical fragment of a long surface loop, commonly referred to as “lid”, which can be also thought about as a device to inhibit the proteolytic activity of the triad, thus protecting the enzyme itself.

Anyhow, as universally recognized, in the presence of a water-organic interphase, the lid opens and thus makes the active site accessible to substrates, according the so-called interfacial activation: the helical fragment moves away from its position, uncovering the catalytic triad, and so the catalysis can take place. Hydrophobic solid surfaces are also known to cause similar conformational changes, leading to the open-lid structure. In any case, the presence of an interface is fundamental for the catalytic activity, and in particular an essential role is played by the substrate concentration: if it is lower than the critical micelle concentration (CMC), no catalytic activity is registered, while at higher

concentrations the formation of a water-oil interface occurs, ensuring a proper enzymatic activity *(Kawakami K., 2012).*

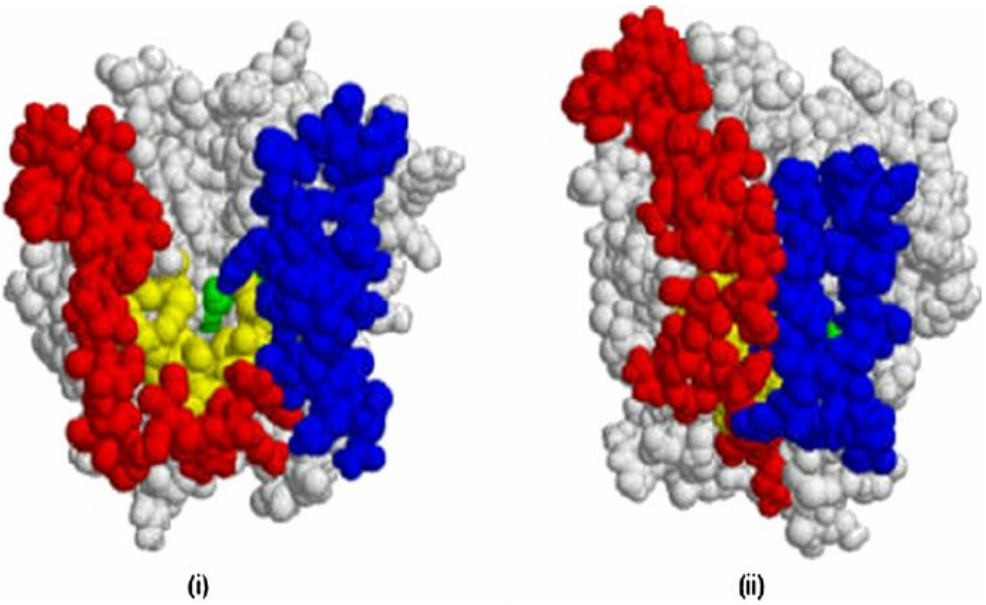
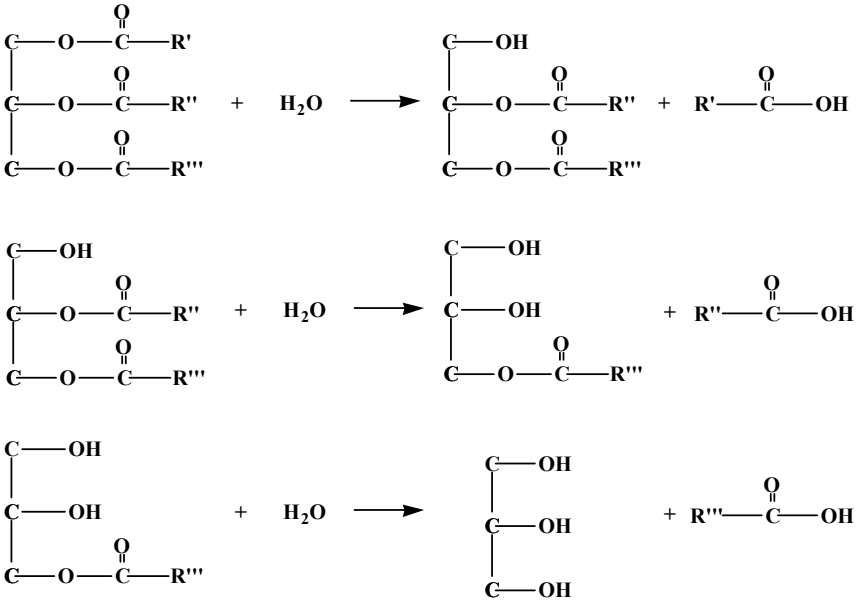


Figure 2.7: Qualitative representation of the lid movement in *Pseudomonas aeruginosa* lipase. (i) Open form of the lipase, with active site cavity. Binding pocket residues are yellow, and lid residues are red and blue. There appears to be nearly a 100% decrease in the solvent-accessible area when the lids close (structure (ii)) *(Cherukuvada et al., 2005)*.

As already said above, thanks to their enantio and regioselectivity, lipases are widely used in several industrial fields, from pharmaceutics to food industry to cosmetics, as well as into biotechnological sector (cheese ripening, detergents, polymers synthesis, pulp and paper industry, ...), but also in the biosensors area, especially for the qualitative determination of triglycerides. Definitely, lipases are so fascinating from an industrial point of view because they remain enzymatically active also in organic solvents, and this fact is absolutely not predictable for any kind of biocatalyst.

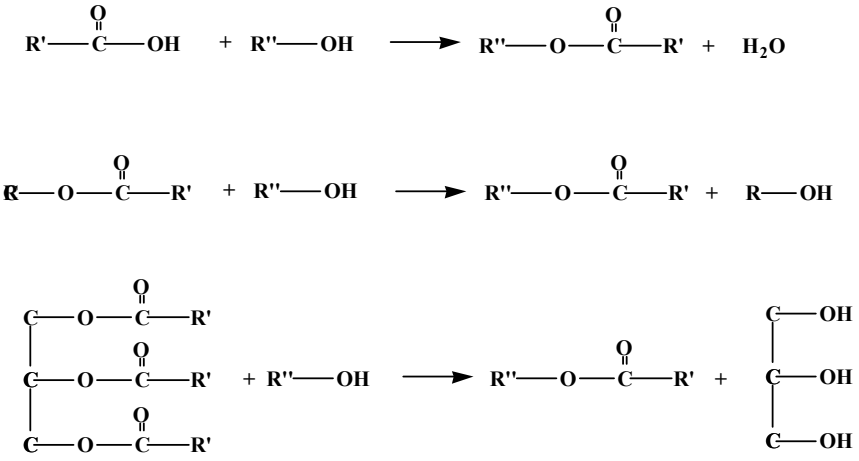
The most relevant lipase-catalysed processes are reported below:

TRIGLYCERIDES HYDROLYSIS:



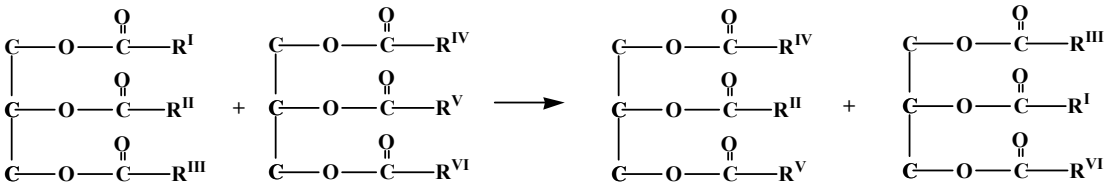
Indeed, this reaction is an equilibrium, so in lack of water the reverse process is thermodynamically favoured, and it is known as the esterification reaction:

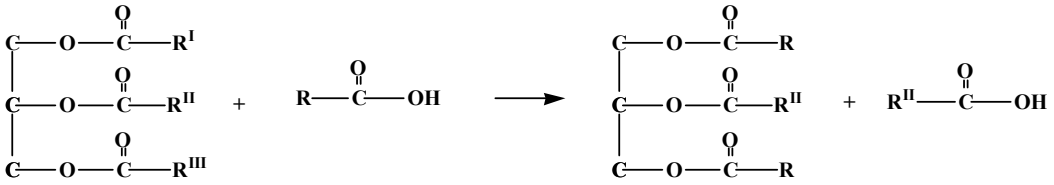
ESTERIFICATION:



In organic solvents, the transesterification reactions take place: in absence of water, an alkyl ester reacts with an acyl acceptor (acidolysis if the acceptor is an acid, alcoholysis if it is an alcohol, interesterification if it is another ester), and this is obviously valid also for triglycerides, which are alkyl esters as well:

TRIGLYCERIDES SYNTHESIS





The use of lipases in these applications allow to:

- increase raw materials sources;
- improve the processing procedures in order to obtain higher quality products;
- supply biodegradable products;
- have greener processes, with less energy used and a smaller amount of waste;
- follow reaction paths otherwise difficult (or impossible) to monitor with synthetic processes.

2.2.1.1 INDUSTRIAL APPLICATIONS OF LIPASES

As clarified in the review of *Sangeetha 2011*, many microbial lipases have been commercialized by popular enzyme producers in the world, like Novozyme (Denmark), Amano Enzyme Inc (Japan), Biocatalysts (UK), Unilever (Netherlands) and Genencor (USA). Bacterial lipases produced form the genera *Burkholderia* and *Pseudomonas* are commercially available. Lipase PS isolated from *Burkholderia cepacia* and Lipase AK isolated *P. fluorescens* are supplied by Amano and Lipase SL and Lipase TL isolated from *B. cepacia* and *P. stutzeri* are supplied by Meito Sangyo (Japan).

As commonly known, lipases are valuable biocatalysts with diverse applications, and though lipases share only 5% of the industrial enzyme market, they have gained focus as biotechnologically valuable enzymes, especially due to their versatility, indeed they play a central roles in food, detergent and pharmaceutical industries.

Lipases catalyse the hydrolysis of lipids to fatty acids and glycerol, and the reversal reaction takes place in an environment with low water content or – rather – in the presence of organic solvents. Such reactions, which substantially serve to alter the physical properties of the oil or fat and produce new esters, include esterification, transesterification and interesterification (acidolysis, alcoholysis or transesterification).

Lipases have a very wide range of application, from food industry to detergents or textile industry; below the main important industrial application of lipases are reported:

Hydrolysis of oils: Hydrolysis of oils is usually performed to concentrate the fatty acids present in the oil, successively used in other processes for which fatty acids are required, as in the production of adhesives, cosmetics and other personal care products, lubricants and coatings. Therefore, the production of fatty acids by the hydrolysis of oils and fats catalysed by lipases becomes crucial for them to be exploited by various industries. The Polyunsaturated Fatty Acids (PUFAs) are simple lipids with two or more double bonds and play diverse physiological roles which contribute to the normal healthy life of human beings. PUFAs belong to two major families namely n-3 (ω3) and n-6 (ω6). The nutritionally important ω3 fatty acids include α-Linolenic Acid (ALA), Eicosatrienoic Acid (ETA), Eicosapentaenoic Acid (EPA) and docosahexaenoic acid (DHA), while the Linoleic acid and arachidonic acid are the most common ω6 fatty acids. These are currently in high demand as they are formulated in nutraceutical and pharmaceutical products. The demand is met by concentrating these PUFAs from alga, fish oil, fish by-products and edible oil *(Chakraborty et al., 2010)*. Purification and concentration of

PUFAs can be best done using lipases and many bacterial lipases have been studied for their efficiency in enhancing PUFA content *(Chakraborty and Paulraj, 2008)*. *Kojima et al. (2006)* used two lipases (AK-lipase and HU-lipase) produce by *P*. *Fluorescens* to selectively concentrate EPA and DHA from fish oils. This method is economical for concentrating PUFAs if compared to the methods which involve more difficult and costly procedures, as distillation, chromatography and fluid extraction. *Byun et al. (2007)* have used a *Pseudomonas* lipase to hydrolyse sardine oil in the presence of emulsifiers and observed a decrease in the level of saturated fatty acids and increase in the levels of mono and poly unsaturated fatty acids after hydrolysis.

*Modification of fat and oil***:** The health potential of fats and oils depends largely on the distribution pattern of the fatty acid present in them. Tailored vegetable oils with structured triglycerides – commonly known as structured lipids – are attractive since they contribute to get health benefits. Many vegetable oils like sunflower, coconut, olive, corn and rice bran oil are rich in ω6 fatty acids and fish, linseed oil, walnuts and milk are rich in ω3 fatty acids. Though both these poly unsaturated fatty acids play a predominant role in contributing to the health of an individual, the ingested ratio of ω6/ω3 needs to be monitored. This ratio is significant and should be balanced between 1 and 4; a high ω6/ω3 ratio may pose health hazards, as reported in *Griffin, 2008*. The best strategy to improve the ω6/ω3 ratio is the enzymatic modification method which uses lipase-catalysed interesterification.

Glycerolysis: Monoacyl Glycerols (MAGs) and Diacyl Glycerols (DAGs) are surface-active molecules, widely used as emulsifiers in food, pharmaceutical and personal-care products. They possess excellent emulsifying properties and are traditionally produced by chemical glycerolysis. DAGs have also gained attention due to their positive impact on health unlike triglycerides, as described in *Cheirsilp et al., 2009* and *Kahveci et al., 2009*.

Synthesis of flavour esters: Flavour compounds that are extracted from plants are too expensive and hence are replaced by flavour esters, low molecular weight compounds, synthesized by the esterification of fatty acids, using catalysts, preferably by microbial lipases. Flavour or fragrance materials which include various aliphatic and aromatic compounds share a major market of food additives throughout the world.

Some of the esters synthesized by esterification reactions catalysed by bacterial lipases are ethyl acetate, ethyl butyrate, ethyl methyl butyrate, ethyl valerate and ethyl caprylate (*Dandavate et al., 2009*).

Tea seed oil: Cocoa butter is a primary ingredient of dark chocolates and the high price of cocoa and cocoa butter prompts to find an alternative cheaper source. Interesterified tea seed oil was identified as a best replacement for cocoa butter and chocolates made with tea seed oil resembled those made with cocoa butter. Tea seed oil is a by-product of tea processing and thus partial substitution of coca butter reduces the cost of confectionary products (*Zarringhalami et al., 2010*).

Lipolysed milk fat (LMF): The LMF is prepared from condensed milk or butter oil using lipases which release free fatty acids and give it a cheesy aroma.
LMF is used in chocolate coatings, artificial flavour additives, margarine and other similar elements. The bacterial lipases used to prepare LMF include those obtained from *Achromobacter* and *Pseudomonas* sp.

Cheese: Lipases from *Lactobacillus* are of primary importance in manufacture of bacterial ripened cheese, like Parmesan and Grana Padano cheese. *Mandrich et al. (2006)* have investigated the role of lipase/esterase from *Alicyclobacillus acidocaldarius* in milk and cheese models and they observed that the recombinant enzyme was more efficient than the native enzyme and could be used in dairy industry to impart flavour or enhance cheese ripening. Furthermore, Enzyme Modified Cheese (EMC) (a concentrated cheese flavour food ingredient produced by treating cheese curd with enzymes) has more intense flavour than naturally ripened cheese, thanks to the action of small chain fatty acids (C_2-C_6), obtained by esterification reactions *(Fenster et al., 2003)*.

Bread making: Emulsifiers are additives required as a bread improver. Bread improvers enhance bread volume and texture and dough stability. These emulsifiers are detectable in the final baked and marketed loaf, and thus find a place in the label. Enzymes on the other hand get denatured during baking, therefore providing bread improving functions without appearing on the label. Lipase from *B. Subtilis* has been proved to play a role in bread making in a study by *Sanchez et al. (2002)*.

Detergents: The most noteworthy application of hydrolytic lipases is their use in household and laundry detergents. Lipases were developed as detergent enzymes after the successful introduction of proteases in powder and liquid detergents. They are used as detergent additives due to their properties of stability at alkaline pH, solubility in water, tolerance to detergent proteases and surfactants and low substrate specificity, as already known and reported in literature *(Rahman et al., 2006)*. Genencor International introduced commercial bacterial lipases Lipomax from *P. Alcaligenes* and Lumafast from *P. Mendocina* which could be used as detergent enzymes in the year 1995 *(Rahman et al., 2006)*. Though fungal lipase was the first introduced detergent enzyme, bacterial lipases captivated the detergent market, since the acidophilic nature of fungal enzymes makes them incompatible with the alkaline wash conditions. During laundering, the lipase adsorbs on to the fabric surface to form a stable fabric-lipase complex which then acts on the oil stains and hydrolyses them. The complex is resistant to the harsh wash conditions and is retained on the fabric during laundering *(Hasan et al., 2006)*.

A detergent stable lipase was isolated from *B. cepacia by Rathi et al. (2001)*. The enzyme was found to meet all the criteria necessary for a detergent additive and exhibited better stability than Lipolase, a detergent stable lipase marketed by Novo Nordisk, Denmark. *Suzuki (2001)* has patented an alkaline *Pseudomonas* lipase which remains active at low temperatures and has improved wash performance. The patent also pertains to a detergent composition containing such lipase.

Many other cases can be found in literature, a clear evidence of how much lipases are suited to industrial applications in the detergents field.

Tannery: Leather processing operations are classified in to three groups, as also reported in *Thanikaivelan et al., 2004*:

- pre-tanning, which cleans the hides or skins,
- tanning, which stabilizes them,
- post-tanning, which adds aesthetic value to it.

All these stages use many chemicals and enzymes. Enzymatic leather processing was at the research level in the early 19th century and only in the year 1980 did industrial scale operations were done. The different stages of leather processing are:

- curing,

- soaking,
- liming,
- dehairing,
- bating,
- pickling,
- degreasing,
- tanning.

Lipases are employed in soaking, bating and degreasing stages, as can be found in *Choudary et al., 2004*. In particular, often a synergic action of various enzymes is required to accomplish the various stages.

Textile industry: Denim culture has spread all over the world and India has witnessed alluring changes in denim fashion. Garment processing promises a refined and polished look to the finished fabric. Desizing is such a process and is required to remove the size material which has been impregnated from the fabric prior to weaving. Traditional desizing uses acid or oxidizing agents which damages the cellulose material in the fabric. Enzymatic desizing has multiple advantages over the traditional process and uses enzymes like cellulase, amylase, protease and lipase depending on the sizing agent. Bacterial lipases can be used if the size material is a synthetic sizing agent like polyesters. *Lund (2001)* has patented a process for combined desizing and stone-washing of denim. Lipolytic enzymes from *P. cepacia*, *P. fluoresecens*, *P. fragi* and *P. stutzeri* were evaluated and the lipase of *P. cepacia* was selected as the best for this purpose.
Polyethylene Terephthalate (PET) fabrics are known for their strength and wrinkle resistance. The lack of dyeability is its undesirable feature which is because of its hydrophobic character. Any attempt to increase its moisture regaining nature will make the PET fabrics more fashionable. *Kim and Song (2006)* have used lipases from *P. cepacia* and *P. fluorescens* in an eco-friendly method to improve the moisture regain of PET fabrics and found it to be more effective than the alkaline method.

Synthesis of polymers: Polylactate (PLA) fibres are made of lactic acid obtained by fermentation of natural sugars. It is an eco-friendly material which has low energy consumption and high bio-degradability. It is used in textile fabrics, packaging material, bio-medicine and as bio-plastics, as described in *Drumright et al., 2000*. PLA fibre is

hydrophobic and thus the affinity of dyes and chemicals to it is less. This necessitates the modification of the surface of the fibres which makes them more wettable. Since ester bonds are present in poly-lactate, the surface can be modified enzymatically using lipase.
The mechanical properties of PLA are similar to those of polystyrene and polyethylene tetraphthalate and can be obtained from different isomers of lactic acid. For this reason, the enzymatic synthesis of Poly-L-Lactic Acid (PLLA) – which is more potential than amorphous PLA – has spread out in the industrial field, using lipase from *B. cepacia,* as reported in *Matsumura et al., 1997*.

Pharmaceutical applications: many uses of lipases can be found in the pharmaceutical field, but only few are listed below.
Miyazaki and Fujikawa (2009) have patented methods to treat skin, scalp disease and hair-loss using compositions that contain a protease, lipase and a metallic salt. The lipase could be obtained from a fungal or bacterial source, preferably *Pseudomonas*. In another paper, *Sani (2006)* has proposed that bacterial lipases can replace pancreatic lipases generally employed to treat cystic fibrosis and pancreatitis. Pancreatic lipase is used as a digestive aid in lipid malabsorption disorders, but its application is limited as it loses its efficacy at low pH and in the presence of protease. Many bacterial lipases are hence promising alternatives to pancreatic lipase.
Phenolics are natural antioxidants which lose their activity in stabilizing oils. This disadvantage is due to their hydrophilic nature. Solubility of phenolics in a fatty region becomes necessary for it to exhibit its action and thus production of lipophilic phenols will make the complete usage of the antioxidant properties of phenols. Biocatalytic synthesis of cinnamoyl esters using *Pseudomonas* lipase has been reported in *Buisman et al., 1998* and cinnamoyl esters have higher free radical scavenging activity than cinnamic acid.
A *Serratia marescens* lipase was used for the bioresolution of racemic ketoprofen, a non-steroidal anti-inflammatory drug, as demonstrated in *Long et al., 2007*.
Hydrocinnamate esters are precursors for the synthesis of 1,3,4,9-tetrahydropyrano [3,4-b]indole-1-acetic acid which is used as analgesic, antipyretic and anti-inflammatory agent; these esters also act as inhibitors of HIV-protease, and they have been synthesized using *P. cepacia* lipase by *Priya and Chadha (2003)*.

Biosensors: Qualitative and quantitative determination of lipids and lipid-binding proteins is possible with the help of biosensors, which may be of chemical or biochemical in nature.

Bacterial lipases have been used as biosensors and this exploits immobilized lipases. Such biosensors are used to detect triglycerides in food and clinical samples, pollution analysis like pesticide contamination and pharmaceutical industry. *Huang et al. (2001)* immobilized a lipase on a micro-emulsion based gel to fabricate a glass-electrode-based lipase biosensor; *Setzu et al. (2007)* fabricated a potentiometric biosensor using a lipase immobilized on a mesoporous silica matrix.

Agrochemical industry: Lipases are used to synthesise intermediates involved in the production of pesticides, insecticides and other agrochemically useful compounds. Transesterification and resolution by *Pseudomonas* lipases was studied to produce insecticides, fungicides and secondary alcohols like (R, S)-HMPC [(R, S)-4-hydroxy-3-methyl-2-(2'-propenyl)-2-cyclopenten-1-one] (*Xu et al., 2005*).

Cosmetics and personal care products: Production of flavours by transesterification and resolution of racemic intermediates by lipases boosts the cosmetic and perfume industry. Lipases produced by *P. cepacia* have been used to resolve the racemic rose oxides produced by the bromomethoxylation of citronellol, as explained in *Taneja et al., 2005*.
Esters of aliphatic and aromatic acids, alcohols including terpene alcohols, aldehydes, phenols are commonly present in the flavour materials used in perfumes and other personal care products. *Priya and Chadha (2003)* studied the synthesis of hydrocinnamic acid esters by *P. cepacia*, which are used in perfumes and sunscreens. Mouth washes and shaving creams contain menthol to provide a peppermint flavour and a cooling sensation: menthol can be artificially produced by the esterification process when there is a dearth for the natural menthol. *Chaplin et al. (2006)* have patented a process to produce menthol esters and similar compounds and this process utilizes lipases of *P. fluorescens* and *P. cepacia*.

Biodiesel production: Biodiesel is an alternative fuel for petroleum-based diesel and is biodegradable, renewable, non-inflammable and non-toxic. Biodiesel is defined as monoalkyl esters of long chain fatty acids, usually a methyl ester of fatty acid and commonly known as FAME (fatty acid methyl ester). Biodiesel is used for diesel engines and heating systems and its demand has increased due to the soaring petroleum prices. Biodiesel is produced from vegetable oils such as soybean oil, rice bran oil, sunflower oil, palm oil, cotton seed oil and jatropha oil, animal fat, algae, waste edible oil and industrial acid oil. It could be synthesized by chemocatalytic, thermocatalytic and biocatalytic

approaches where, the latter employs lipases as biocatalysts. The lipase catalysed transesterification reaction takes place between a lipid and a short chain alcohol to produce an ester and glycerol, as reported in literature (*Chen et al., 2009*; *Dizge et al., 2009*; *Raita et al., 2010*).

The production of biodiesel requires a micro-aqueous environment because the presence of water does not promote transesterification, rather favours the hydrolysis of oil. Hence immobilized lipases are employed to function in a micro-aqueous of solvent-free system. *Wang and Zhang (2009)* studied the presence of compatible solutes to improve methanolysis for biodiesel production. Methanol competitively inhibits lipase and also denatures it and thus decreases biodiesel production. A solute like ectoine decreases the affinity of the lipase for methanol but increases its affinity for the triglyceride and this strategy improved the yield of biodiesel.

Glycerol, the by-product of biodiesel production is converted to 1,3 propanediol which is a monomer for the synthesis of novel polymers like polymethylene terephthalate. Bacterial species like *Klebsiella*, *Citrobacter*, *Clostridium*, *Enterobacter* and *Lactobacillus* can convert glycerol to 1, 3 propanediol, as reported in *Xu et al., 2009*.

Environment management: Waste-water treatment processes remove lipidic residues by air-floatation and discard it in sanitary landfill dumping yard. These pose a threat to the waterbeds and ground water by decreasing the oxygen transfer rate and hence bio-remediation is adopted to control pollution. Oil spills in the soil and water during rigging and refining can be handled using lipases, as explained in *Pandey et al., 1999*.

Environmental studies: Biogeochemical studies include the analysis of the relationship between Dissolved Organic Matter (DOM) and bacterial dynamics, the response of bacteria to environmental conditions, and other similar issues. These studies utilize various parameters for analysis and bacterial activities are one among them. Bacteria use the readily available pool of DOM for their growth and breakdown major molecules enzymatically when the small molecules deplete. Thus the analysis of bacterial enzyme activities like amino peptide hydrolysis and polysaccharide degradation *in situ* was studied as indicators of DOM dynamics. Recently *Bourguet et al. (2009)* studied the relationship between the levels of lipids and *in situ* lipase activities during different environmental conditions like Spring and Summer.

Honestly, the industrial use of lipases is much more productive with continuous operations. As a matter of facts, a continuous system ensures an easier monitoring of some deactivating mechanisms otherwise not detectable with batch reactors. In particular, a catalyst deactivation due to contaminants present in the substrate solution stream could not be observed in a discontinuous process, especially if the impurities concentration is considerably lower than the biocatalyst one; on the other hand, the continuous feed of the same substrate solution provokes a gradual enzymatic activity reduction, so the kinetics can be correlated with the feed flow rate.

As well, the substrates stabilizing effects lose its strength in batch systems, due to the huge variability of substrates concentration during discontinuous tests.

In wider terms, only in the continuous mode operative conditions it is possible to characterize the effects due to chemical species potentially present in the feeding stream or generated as reaction products, which could compromise the enzyme catalytic action.

Furthermore, since organic solvents represent in most cases the reactive operative environment, and because of the fact that they are highly flammable substances, the use of flow reactors – microstructured reactors, in particular – allow to use reduced amounts of potentially hazardous substances, to lower down the production costs and to work in safer conditions.

2.2.2 XYLANASE

Xylanases, classified as EC 3.2.1.8, are glycosyl hydrolases that break down the backbone of xylan in plant cell walls and are generally secreted by micro-organisms that live by degrading plant biomass, including several phytopathogens *(Lo Leggio et al., 1999)*.

Xylan is the second most abundant polysaccharide in nature, consisting of a backbone mainly formed by β-1,4-linked xylopyranosyl units, though β-1,3 xylans are also found in some algae, and depending on the xylan source, the xylopyranosyl units can be substituted by glucuronopyranosyl, acetyl or arabinofuranosyl groups.

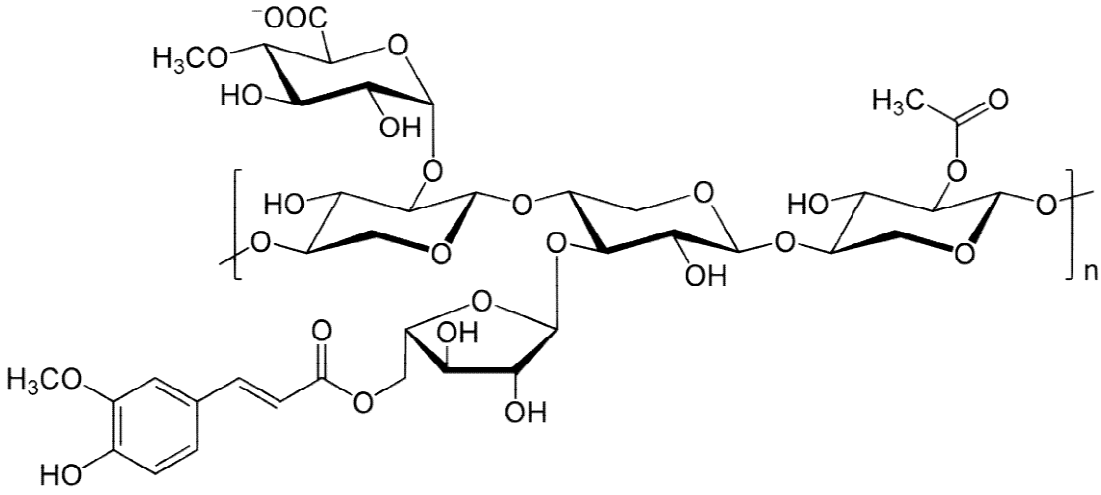


Figure 2.8: Xylan backbone.

Xilan is the most abundant hemicellulose present on the earth surface *(Dhiman et al., 2008)* and Schulze, in 1891, was the first to introduce the term 'hemicellulose' meaning the fractions isolated or extracted from plant materials with dilute alkali *(Beg et al., 2001)*. Hemicelluloses include xylan, mannan, galactan, and arabinan as the main heteropolymers, and the classification of these hemicellulose fractions depends on the types of sugar moieties present.

Focusing the attention on xylans, their monomeric unit is mainly D-xylose, with traces of L-arabinose. In plants, xylans or the hemicelluloses are situated between the lignin and the collection of cellulose fibers underneath. Consistent with their structural chemistry and side-group substitutions, the xylans seem to be somehow interconnected with lignin by covalent linkages and by non-covalent interaction with cellulose, and this fact may be important in maintaining the integrity of the cellulose in situ and in helping protect the fibers against degradation to cellulases *(Uffen 1997)*.

As already stated above, most xylans occur as heteropolysaccharides, containing different substituent groups in the backbone chain and in the side chain. The common substituents are acetyl, arabinosyl, and glucuronysyl residues, and the presence of acetyl groups in the backbone of xylan is responsible for its partial solubility in water.

D-xylose , the main component of xylan, is a five-carbon sugar that can be converted to single cell protein and chemical fuels by microbial cells, which can be considered the cheapest 'chemical factories'. Because of its complexity, xylan complete breakdown requires the action of a complex of several hydrolytic enzymes, with diverse specificity and modes of action, that can be specifically chosen time by time, according to the particular considered process. The xylanolytic enzyme system carrying out the xylan hydrolysis is usually composed of a repertoire of hydrolytic enzymes: β-1,4-endoxylanase, β-xylosidase, β-L-arabinofuranosidase, β-glucuronidase, acetyl xylan esterase and phenolic acid (ferulic and *p*-coumaric acid) esterase, as showed in Figure 2.9.

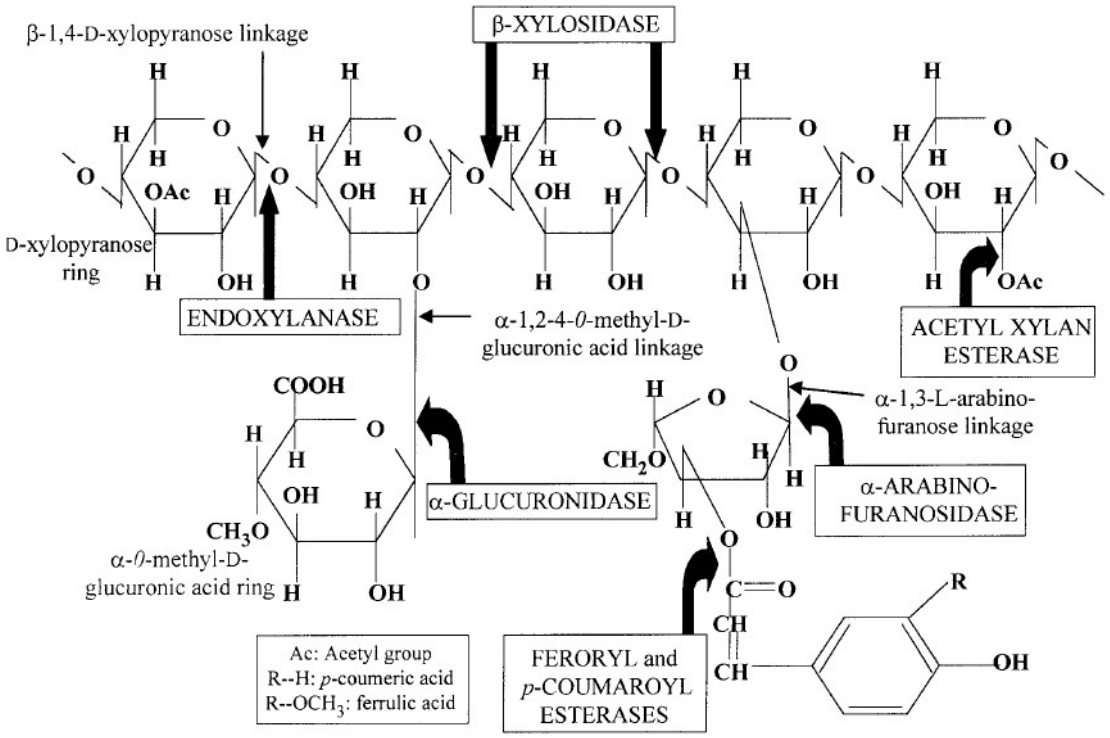


Figure 2.9: Hypothetical plant xylan structure, with sites of attack by microbial xylanases on different substituent groups *(Beg et al., 2001)*.

All these enzymes act cooperatively to convert xylan into its constituent sugars, and it is immediate to understand that it is such a multifunctional xylanolytic enzyme system.

Generally speaking, xylanases can hydrolyze the β-1,4-linkages between the xylose units which form the backbone into two ways, using an endo approach, by attacking internal linkages, or in an exo mode, by acting on the external bonds on the polysaccharide chain.

Studying the interaction of xylanases (and polysaccharidases in general) with their natural substrates at the molecular level is not easy, because first of all the substrates are extremely complex, due to their polymeric and heterogeneous nature. Moreover, often it is very difficult to understand and define the exact composition of the xylans used for enzyme assays, as well as the structure; for this reason, the main analytical methods that give the possibility to obtain useful information about the treated polysaccharide and its interactions with polymeric substrates are high-performance liquid chromatography, HPLC *(Bray et al, 1992)* and thin-layer chromatography, TLC (*Biely et al., 1981)* (see Materials and Methods section).

These methods can provide detailed data related to the bond-cleavage frequencies and the preferred cleaved bond in oligosaccharides, besides the fact that they can be used for precise studies of the enzyme kinetics of xylanases. On the other hand, they require either specialized equipment for high-performance liquid chromatography or radiolabeled oligosaccharides for quantitative thin-layer chromatography studies, that are not commercially available.

2.2.2.1 INDUSTRIAL APPLICATIONS OF XYLANASES

When using a xylanase, what happens is the degradation of the long xylan chain into smaller fractions, until obtaining the so-called xylo-oligosaccharides, industrially interesting due to the fact that they can be used as prebiotics, but not only. Various biotechnological applications have been suggested or are already in use for xylanases, from biobleaching of paper pulp *(Viikari et al., 1994)* and improvement of the digestibility of animal feeds *(Bedford et al., 1995)* to applications in the food industry, e.g. as a flour additive.

As regards pulp and paper industry, only in the past two decades microbial enzymes have been commercially exploited, as reported in *Beg et al., 2001*. The use of xylanases in this industry has increased significantly with the discovery of *Viikari et al. (1986)*, and since then researchers worldwide have focused their attention toward newer microbial isolates to use in this field.

The scientific interest in this area is continuously growing up, and indeed several research papers have been published during recent years dealing with xylanases from newer sources, as well as bleaching experiments reported using various hemicellulases, pulps, and bleaching sequence.

In particular xylanases have been reported mainly from bacteria (*Gilbert and Hazlewood 1993)*, fungi *(Sunna and Antranikian 1997)*, actinomycetes *(Ball and McCarthy 1989)* and yeast *(Hrmovà et al., 1984).*

Year by year the number of possible applications of xylanases in pulp and paper industry is in continuous growth and some of them are in commercial use. Currently, the most effective application of xylanase is in prebleaching of kraft pulp, to minimize use of harsh chemicals in the subsequent treatment stages of kraft pulp.

Besides their utilization in pulp and paper field, xylanases are also used in many other industrial sectors:

- as food additives to poultry (*Bedford and Classen 1992)*,
- in wheat flour for improving dough treatment and quality of baked *products (Maat et al. 1992)*,
- for the extraction of coffee, plant oils, and starch *(Wong and Saddler 1992),*
- in the improvement of nutritional properties of agricultural silage and grain feed *(Kuhad and Singh 1993)*.

It is easy to understand that these enzymes have an undoubted commercial potential. Here some important industrial applications in various fields for xylanolytic enzymes are reported *(Beg et al., 2011; Juturu et al., 2012; Viikari et al, 1994)*:

Pulp and paper industry - prebleaching of kraft pulps: currently, it represents the most promising application of xylanases; enzyme application improves pulp fibrillation and water retention, reduction of beating times in virgin pulps, restoration of bonding and increased freeness in recycled fibers, and selective removal of xylans from dissolving pulps. Xylanases are also useful in yielding cellulose from dissolving pulps for rayon production and biobleaching of wood pulps.

Animal feed industry: incorporation of xylanase into a rye-based diet of broiler chickens results in reduced intestinal viscosity, which is strictly connected to the depression in weight gain and feed conversion efficiency.

Bakery: xylanases improve the quality of bread, further enhanced when amylase is used in combination with xylanase.

Waste water management: xylanases are used for conversion of xylan into xylose in waste water, thanks to the fact that xylan is present in large amounts in wastes from agricultural and food industries.

Phytosterols: xylanase treatment of plant cells can induce glycosylation and fatty acylation of phytosterols.

Clarification of fruit juices: xylanase are used concurrently with cellulase and pectinase for clarifying must and juices, and for liquefying fruits and vegetables, and in the pretreatment of forage crops to improve the digestibility of ruminant feeds and to facilitate composting.

New surfactants industry: Alkyl glycosides are one of the most promising candidates for new surfactants. Commercially, they are produced from monomeric sugars such as D-glucose and a fatty alcohol, but using a xylanase in the direct glycosylation using polysaccharide provides a challenging opportunity for many reason, first of all because hydrolysis of polysaccharide and subsequent steps can be omitted, saving time and money!

It is reported that xylanase from *Aureobasidium pullulans* has been used for direct transglycosylation of xylan, 1-octanal and 2-ethyl hexanol into octyl-β-D-xylobioside, xyloside, and 2-ethylhexyl-β-D-xylobioside, respectively.

Food processing: α-L-Arabinofuranosidase and β-D-glucopyranosidase have been employed in food processing for aromatizing musts, wines, and fruit juices.
A recent application of a truncated bacterial xylanase gene from *Clostridium thermocellum* has been demonstrated in rhizosecretion in transgenic tobacco plants (*Borisjuk et al. 1999*).

Biological fuel production: xylanase in synergism with several other enzymes, such as mannanase, ligninase, xylosidase, glucanase, glucosidase, etc., can be used for the generation of biological fuels, such as ethanol and xylitol, from lignocellulosic biomass starting from delignification of lignocellulose to liberate cellulose and hemicellulose from their complex with lignin, followed by depolymerization of the carbohydrate polymers (cellulose and hemicellulose) to produce free sugars, and finally fermentation of mixed pentose and hexose sugars to produce ethanol.

Degumming of plant fiber sources (flax, hemp, jute, and ramie): A potential application of the xylanolytic enzyme system in conjunction with the pectinolytic enzyme system is in the degumming of bast fibers such as flax, hemp jute and ramie.

2.2.3 D-PHENYLGLYCINE AMINOTRANSFERASE

Aminotransferases, also named transaminases (TAs), are classified as EC 2.6.1.X and are pyridoxal 5'-phosphate (PLP) dependent enzymes, in the sense that they utilize the PLP cofactor to catalyze the transfer of an amino group between an amino donor and an amino acceptor, i.e. they are responsible for the catalytic action in a transamination reaction *(Koszelewski et al., 2010)*.

In the last decades, aminotransferases have gained increased attention mostly in the pharmaceutical industry, due to their potential for the preparation of chiral amines and unnatural amino acids *(Yi et al.)*, which are challenging reactions to achieve by conventional synthesis; in particular they are gaining attention due to their application in the manufacture of pharmaceutical intermediates on a large scale *(Planchestainer et al., 2017).*

The coenzyme pyridoxal-5'-phosphate (PLP), shown in Figure 2.10, is a derivative of vitamin B6 and it is used by several enzymes for some catalytic processes, such as decarboxylation, racemisation, retro-aldol cleavage and transamination *(K. Engelmark Cassimjee, 2012, PhD thesis)*:

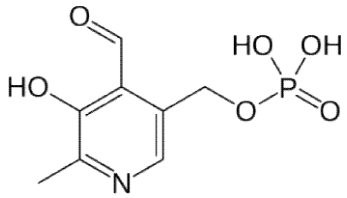


Figure 2.10: Pyridoxal-5'-phosphate (PLP) coenzyme structure.

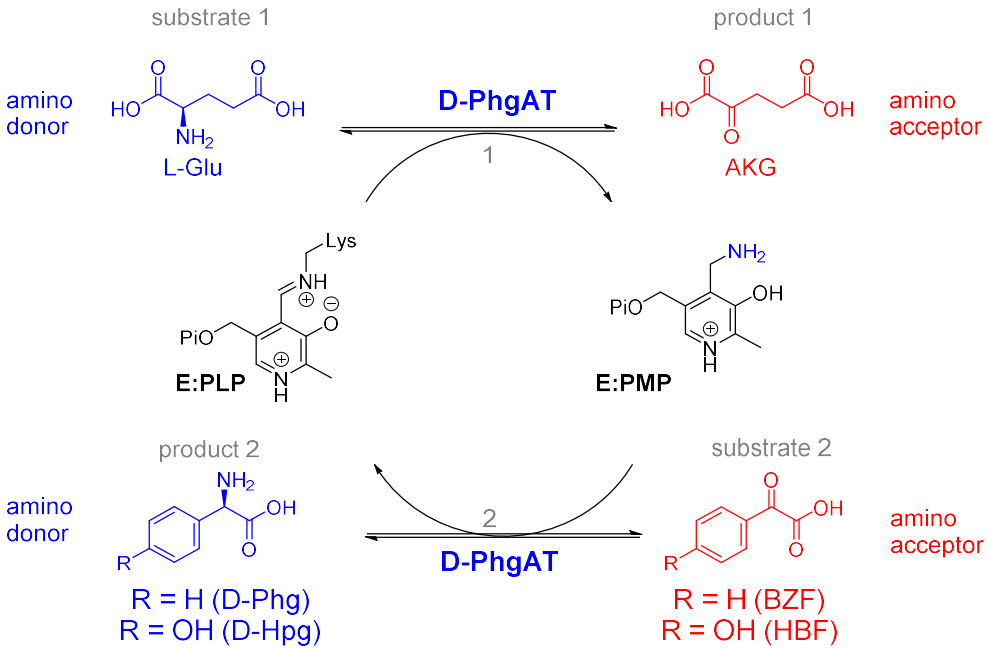
PLP readily forms Schiff bases with a lysine residue in the active site; the presence of a Schiff base structure confers stability to the involved chemical species.

Certainly, effective catalysis cannot be performed only thanks to the presence of the enzyme with its cofactor, but also enough space for the substrate is essential, and even more the substrate ability to be positioned in a catalytically productive manner.

Analyzing in detail, as already mentioned above, in a living cell an amino acid can be formed from the corresponding keto-acid, by PLP dependent transaminase enzymes. In this case the transaminase is labelled by the Greek letter α, having an α-amino acids and α-keto acids as substrates and products; on the other hand, transaminases which are able to accept aliphatic ketones and amines as their substrates, i.e. not only keto acids and amino acids, are labelled with the Greek letter omega (ω-transaminase, EC 2.6.1.18).

Amines are found in living cells. Dopamine, adrenaline and serotonin are examples of amines present as signal substances in mammals. Amines are also very common in pharmaceuticals. Synthesis of a wide range of enantiopure amines are of great interest in a pharmaceutical industry point of view, and for this purpose ω-transaminases has been shown to be an attractive option, but anyway also α-ones are gaining growing interest in the industrial field, although its investigation is still at its first steps.

In 1997 a stereoinverting D-phenylglycine aminotransferase (D-PhgAT), that is the one chosen for this work, was isolated from the soil bacterium *Pesudomonas stutzeri*; D-PhgAT catalyses the reversible transamination of L-glutamic acid (L-glu, amino donor) with benzoylformate (BZF) as amino acceptor, yielding D-phenylglycine and α-ketoglutarate (AKG), as showed in the Scheme 2.1.



Scheme 2.1: Simplified mechanism of D-PhgAT transaminase action. In the first half of the reaction, D-PhgAT L-Glu donates the amino group to the PLP cofactor generating PMP and AKG; in the second half, BZF (or HBF) accepts the amino group from PMP yielding D-Phg or D-Hpg. Amino donors are highlighted in blue while the amino acceptor in red.

Hence, it is worthy to say that D-PhgAT represents a strong target enzyme for the synthesis of enantiomerically pure D-Phg in a single step, using a cheap amino donor such as L-glutamic acid, providing a new faster and more sustainable route to their biosynthesis than the previous ones.

2.2.3.1 INDUSTRIAL APPLICATIONS OF TRANSAMINASES

Transaminases are perfect biocatalysts to be employed in the pharmaceutical industrial field. As a matter of facts, during the years, the development of efficient cascade systems involving ω-transaminases has provided an efficient biocatalytic entry to α-chiral primary amines, many of which are important for the pharmaceutical industry and that were difficult to obtain via synthetic route. Consequently, ω-transaminases have quickly entered industrial applications *(Savile et al., 2010)*.

More traditionally, α-transaminases and amino acid dehydrogenases (AADHs) are employed for the production of optically active amino acids. Besides, monoamine oxidases (MAOs) and amino acid oxidases (AAOs) are available for the enantioselective irreversible oxidation of amines and amino acids, respectively, and these enzymes have become widely used biocatalysts in chemo-enzymatic deracemization and stereoinversion systems based on the cyclic oxidation/reduction *(Koszelewski et al., 2008).*

Further investigations must be done as regards transaminases properties, to widen their range of industrial applications, although in any case they have a prominent role in the biological/pharmaceutical field.

3.IMMOBILISATION TECHNIQUES

3.1 TREND AND STRATEGY IN ENZYME ENGINEERING FOR INDUSTRIAL APPLICATIONS

It is well known, as already pointed out above, that the use of enzymes successfully accomplishes the challenge to have *bio*-route, ensuring *green* processes, but it involves massive costs; for this reason, research has turned its attention to the request of novel biocatalysts, that can continue to offer the same impressive results, but with lower costs, thus avoiding to burden the finance of the whole considered process, making the employment of the enzyme itself useful and convenient, especially from an economic point of view.

For this reason, *(Choi et al., 2015)* over the past decades, enzyme-based processes have continuously substituted traditional chemical procedures in many areas, especially in fine chemical and pharmaceutical industries. More and more solutions have been studied and proposed to gain an increased development of new technologies, to further expand the industrial use of enzymes, especially as regards their catalytic and biophysical properties, such as catalytic efficiency, substrate specificity, and stability.

Enzyme engineering is a good way towards the aim to obtain new biocatalysts with improved properties. Current trend in enzyme engineering is based on the focused-directed evolution in conjunction with computational methods, and they are destined to continue and even accelerate. Computational algorithms for systematic approach, such as ProSAR (a strategic computational method that statistically analyzes the sequence-activity relationships of proteins *(Fox et al., 2007)*), will be more optimized for easy applications and new algorithms analyzing the sequence-function relationship will be explored for generating more systematic and diverse libraries. In any case, this optimization requires a great attention, indeed a wrong choice in certain engineering step could jeopardize a whole project, and – on the other hand – accumulation of successful stories in enzyme engineering will provide proper rules in choice.

Focusing the attention on the enzymes, even though it would be arguable, stability of enzymes against heat and organic solvents are usually considered most critical, owing to harsh industrial process. Thermo-stability is often related with tolerance against organic solvents and destabilizing mutations *(Huisman et al., 2010; Reetz et al., 2010; Vazquez-Figueroa et al., 2008)*, and enhancing thermo-stability is a prerequisite for industrial

applications *(Bloom et al., 2006)*. Current trend in engineering thermo-stability is to combine the structure-based rational design and computational methods in conjunction with directed evolution. Extremophiles have been considered useful sources of industrial enzymes with high stability against heat, salts, and pH *(Elleuche et al., 2014; Illanes et al., 2012; Kazlauskas and Bornscheuer, 2009)*. For example, directed evolution in conjunction with ProSAR, was successfully used in the development of ketoreductase (KRED) and R-selective transaminase (ATA-117) to enhance the catalytic activity and enantioselectivity toward industrially relevant substrates *(Savile et al., 2010; Liang et al., 2009; Huisman et al., 2010).*

Also other computational modeling approaches, such as calculation of the free energy perturbation, substrate docking simulation, molecular dynamics (MD) and hydrogen bond energy calculation, have been employed in a rational design *(Krieger et al., 2002; Bommarius et al., 2006; Schwab et al., 2008; Kazlauskas and Lutz, 2009; Illanes et al., 2012)*. In particular, MD simulations can predict unstable residues useful for altering either activity or thermo-stability. Considering the importance of dynamics in enzyme reactions, MD simulation will provide valuable information about the flexibility–function relationship of enzymes, which has not been possible by crystal enzyme structures.

A recent NMR experiment joined with mutagenesis has been applied to the study of enzyme catalysis *(Doucet, 2011)*. Like MD, this approach will provide some insight into the flexibility. Ultimate goal in rational design of industrial enzymes is to generate enzymes with new and robust catalytic functions for industrial process.

Many other techniques are in continuous development, all of them in order to offer a final product with no squandering both in terms of time and money.

Anyhow, these methodologies used to obtain engineered enzymes are not enough to sustain the industrial requests, especially as regards – for example – the preparation of expensive enzymes, for which recycling is compulsory to run economic viable processes (*Koszelewski et al., 2010)*; therefore, new procedures have been still developing, with the common aim of achieving the goal, trying to offer a tool which suites both the strictly chemical and economical sides of an industrial process.

3.2 IMMOBILIZATION: THE ANSWER TO INDUSTRIAL DEMANDS

The concept that seems to be perfect for this purpose is immobilization: it is possible to immobilize proteins according to various procedures, without altering the internal structure, and so preserving their catalytic nature. Furthermore, immobilized enzymes are considered to be more stable with wide potential applications ranging from chemical synthesis to biotechnology and medicines.

As reported in *Tischer et al., 1999*, the first intentionally prepared immobilized enzymes were obtained in the 1950s, obtaining biocatalysts with restricted mobility; from that moment on, studies on immobilization procedures started in a systematic way, and various techniques were studied, from the inclusion into polymeric matrices to binding onto carrier materials, or cross-linking, either by cross-linking of protein alone or with the addition of inert materials.

In the last decades, several methods of immobilization on a variety of different materials have been developed, and binding to pre-fabricated carrier materials appears as the preferred method so far. Immobilized enzymes are currently the object of considerable interest, and also the number of applications of immobilized enzymes is steadily increasing.

Immobilized enzymes are used in organic syntheses to fully exploit the technical and economical advantages of biocatalysts based on isolated enzymes.

For practical applications, immobilization of microorganism or enzymes on solid materials offer several advantages, and either the whole cell immobilization of organism to a solid support is performed, or sometimes the enzyme itself is immobilized on some reversible soluble-insoluble polymer.

There are many methods available for immobilization, which span from binding on prefabricated carrier materials to incorporation into carriers prepared *in situ*. Operative binding forces vary between weak multiple adsorptive interactions and single attachments through strong covalent binding. Which of the methods is the most appropriate is usually a matter of the desired application. Anyway for all of them, two main targeted benefits are easy separation of the enzyme from the product and reuse of the enzyme: without immobilization, using the wild enzyme in the reaction mixture, it is impossible to obtain that. To be more precise, regardless of the chosen immobilization method, various advantages can be identified:

- easier manipulation and separation of the enzyme from the effluent stream;
- easier recycling and re-use of expensive catalysts;
- cost saving;
- better control on undesired transformations of products;
- limits of products contamination;
- higher stability (mechanical and related to operative parameters);
- higher efficiency of the overall process (lower T and/or p, less amount of expensive reagents, less waste products);
- minimal protein contamination;
- improved control over the whole reaction process;
- possibility to skip from batch to continuous processes.

These advantages are true for immobilized enzyme preparations that provide a well balanced
overall performance, based on reasonable immobilization yields, low mass transfer limitations, and high operational stability.
The choice of the proper enzyme immobilization route must be done with the utmost care, because enzyme catalytic properties can be strongly affected by the physico-chemical and morphological properties of the solid supports used for their immobilization; as a matter of facts it is obvious that the properties of immobilized enzyme preparations are governed by the properties of both the enzyme and the carrier material, and their specific interaction provides an immobilized enzyme with distinct chemical, biochemical, mechanical and kinetic properties, thus obtaining a new biocatalyst with its own properties.
For this reason, each enzyme must be immobilized according to an accurate procedure, studied *ad hoc* to avoid its denaturation and to promote instead its catalytic nature, thus facilitating its proper application in the dedicated industrial process.

Once immobilized, some parameters must be evaluated, first of all the yield of enzyme activity: after immobilization, it depends not only on losses caused by the binding procedure, but may be further reduced as a result of lowered availability of enzyme molecules within pores or from slowly diffusing substrate molecules. Such limitations, summarized as mass transfer effects, lead to lowered efficiency. On the other hand, improved stability under working conditions may compensate for such drawbacks, resulting in an overall benefit. So, altogether, these interactions

are a measure of productivity or of enzyme consumption, for example, expressed as enzyme units per kg of product. If “enzyme units” is replaced by “enzyme costs”, the essential product related costs is obtained.

As regards the overall costs, they are constituted by:

- costs for biomass plant and animal sources/microbial fermentations;
- costs for downstreaming (larger fermentation scale);
- costs for the immobilization procedure;
- costs for enzyme manufacture and maintenance.

Therefore, apart from the potential advantage of easier removal of the enzyme from the product formed, immobilized enzymes so far provide no cost benefit. Anyway, cost savings will be achieved by multiple reuse of an immobilized enzyme, and this can be monitored by consideration of the amount of enzyme required per kg of formed product.

Before focusing the attention on the immobilization techniques, it is worthy to underline that over time enzymes have been optimized for specific metabolic reactions in living cells, but operative conditions therein are different from those in industrial bioreactors, so it is not easy to skip from the laboratory scale to an industrial scale just considering the wild enzyme.

Immobilization gives the enzymes such characteristics that make them tolerant to even harsh reaction environments.

Obviously, the first aim of immobilization is to use a proper procedure to avoid any kind of distortion of the enzyme conformation, moreover ensuring an appropriate catalytic flexibility.

In order to achieve a good immobilization and obtain a high performance biocatalyst, it is essential to study the structural characteristics of both the enzyme and the carrier; in particular the latter can have inorganic or organic origins.

The second immobilization issue – after the success of the enzyme binding – is the mass transfer: it is clear that enzyme immobilization implicates a restriction of the mobility of the enzyme, which can also involve mobility of the solutes. The various phenomena, referred to as mass transfer effects, can lead to a reduced efficiency as compared to the free enzyme, and it can also depend on the choice of the type of reactor for that specific process. For example, in stirred tanks external diffusion plays a minor role as long as the

reaction liquid is stirred enough. The most crucial effects are observed in porous particles, due to internal or porous diffusion.

For Michaelis–Menten based enzyme kinetics, the extent of mass transfer control is usually expressed by the efficiency coefficient or effectiveness factor η, expressed as reported in (3.a):

$$\text{(3.a)} \quad \eta = \frac{v'(substrate\ conversion\ rate\ of\ immobilized\ enzyme)}{v(substrate\ conversion\ rate\ of\ free\ enzyme)}$$

Numerically, calculated values of η are available when only substrate diffusion in Michaelis–Menten type kinetics is considered.
η can be also expressed as a function of the Thiele modulus Φ_R and of a dimensionless substrate concentration (3.b), or also as a dimensionless Michaelis-Menten constant β (3.c), according to *Lee and Tsao, 1974*:

$$\text{(3.b)} \quad \Phi_R = R \cdot \sqrt{\frac{V_m}{D_{eff} \cdot K_M}}$$

$$\text{(3.c)} \quad \beta = \frac{S}{K_M}$$

where:
R is the particle radius of swelled carrier, [μm]
V_m is the accessible catalyst volume, [cm^3]
D_{eff} is the effective diffusion coefficient [cm^2s^{-1}]
K_M is the intrinsic Michaelis-Menten constant, [mM]
S is the substrate concentration, [mM]

Another parameter to keep under control within the study of diffusion limitations in the substrate concentration: low substrate concentrations occur in hydrolytic reactions when substrate conversion is close to completion. Under these conditions, other factors – such as various kinds of product inhibition – will govern the reaction rate, and evidently this will further affect the mass transfer.
For this reason, to control this potential additional limitation, it is very common to assay the enzyme activity at varying substrate concentrations, and generally it has been found

that at low substrate concentrations, diffusion restrictions are expected to be predominant. Anyway, to avoid diffusion limitations it is worthwhile to assay the enzyme activity under more drastic conditions, that also mean increasing stirrer speed to exclude external diffusion, crushing the particles to reduce porous diffusion, increasing the substrate concentration to about at least 100-fold of K_M-value to avoid lack of substrate at the centre of the particles or adding buffer to avoid pH-shifts.

Hence, some general advices can be considered:

- decreasing the particle size of the carriers;
- lowering of enzyme loading is recommended for enzymes with high specific activity;
- preferential binding on the outer shell of carrier materials will enable increased efficiencies.

The third issue to face downstream of an immobilization procedure is the stability, but it is useful to distinguish storage from operational stability: storage stability is provided by an appropriate formulation, and in most cases it is achieved by the use of proper additives which protect the enzyme from denaturation; on the other hand, the operational stability is related to the working conditions, i.e. pH, temperature, solvents, impurities, and other factors that can induce protein denaturation or remarkable modification of functional groups, thus enhancing inactivation.

It is evident that to achieve the cost benefits of the overall process, as already mentioned above, it is essential to benefit from the operational stability of the immobilized enzyme.

With regard to purity, quality, and formulation, and therefore to cost considerations, it can be useful to define “productivity” as the fermentation volume required to prepare the immobilized enzyme activity needed to synthesize a certain amount of product. This is functional when the overall performance of an immobilized enzyme catalyzed process has to be competitive with other technologies *(Tischer et al., 1999)*.

3.3 IMMOBILIZATION TECHNIQUES: THE MAIN PROCEDURES

As stated above, upon time several immobilization techniques have been developed and are being used extensively today. A wide range of support materials has also been employed for enzyme immobilization.

The use of immobilized enzymes normally offers several advantages if compared to free enzymes, such as increased stability, localization, and retention of the molecules at the material surface, which enables easier handling, repeated use, and low costs. Other important advantages of using therapeutic immobilized enzymes are the prolonged blood circulation lifetime without the loss of specific activity and the lower immunogenicity. This advantage is particularly important for delivering enzymes or other biomolecules and may constitute an alternative and suitable method for the enzyme replacement therapy.

Anyway, some limitations have been attributed to the use of immobilized enzymes, such as mass transfer resistances, adverse biological responses of enzyme support surfaces (*in vivo* or *ex vivo*), fouling by other biomolecules, greater potential for product inhibition, and sterilization difficulties.

However, immobilized enzymes are continuously gaining attention from several industrial fields, and so these limitations are challenges for the research, which is working on them to find proper solutions.

Immobilization techniques can be divided into different categories: physical, chemical, enzymatic, and genetic engineering methods and, among all of them, the following will be analyzed *(Costa et al., 2004)*:

- Adsorption;
- Ionic binding;
- Covalent Binding;
- Crosslinking:
- Entrapment.

3.3.1 ADSORPTION

The adsorption of an enzyme onto a support or film material is the simplest method to obtain an immobilized enzyme. The enzyme is attached to the support material by non-covalent linkages and does not require any pre-activation step of the support, indeed the interactions between the enzyme and the support material will be dependent on the existing surface chemistry of the support and on the type of amino acids exposed at the surface of the enzyme molecule. Enzyme immobilization by adsorption generally involves weak interactions between the support and the enzyme, such as ionic or hydrophobic interactions, hydrogen bonding and Van der Waals forces.

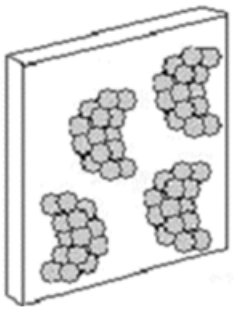


Figure 3.1: Enzyme adsorption on a suitable carrier.

Evidently, most of the support materials have appropriate surface-charge properties suitable for immobilization by adsorption, and they include inorganic carriers (ceramic, alumina, activated carbon, kaolinite, bentonite, porous glass), organic synthetic carriers (nylon, polystyrene), and natural organic carriers (chitosan, dextran, gelatin, cellulose, starch). The method consists of simply mixing an aqueous solution of enzyme at a certain concentration with the support material for a chosen period of time, after which the excess enzyme is washed away by a proper washing route. The procedure requires strict control of pH and ionic strength, because these can alter the charges of the enzyme and the support and, therefore, affect the level of adsorption. A simple shift in pH can cancel ionic interactions and promote the release of the enzyme from the support. The main advantages of adsorption are:

- method simplicity;
- little effect on the conformation/activity of the biocatalyst;
- possibility of regenerating inactive enzyme by addition of fresh enzyme.

The main disadvantage is the desorption of the biocatalyst from the support due to the weak interactions; unfortunately, it can easily occur by changes in the environment medium such as pH, temperature, solvent, and ionic strength.

3.3.2 IONIC BINDING

Ionic binding immobilization is mainly based on ionic bonds between enzyme molecules and the solid supports containing ionic charges, even if in some cases physical adsorption may simultaneously take place. The main difference between ionic binding and physical adsorption is the strength of the interaction, which is much stronger for ionic binding, although less strong than covalent binding.

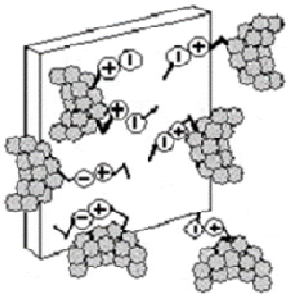


Figure 3.2: Enzyme immobilization via ionic bonds on a suitable carrier.

The preparation of immobilized enzymes using ionic binding is based on the same procedure as described for physical adsorption, so it is very easy, since it consider a mixing of an enzymatic solution with the support material for a chosen period of time, after which the excess enzyme is appropriately removed. Also in this case, the binding forces between enzyme and support depends on pH variations, support charge, enzyme concentrations, and temperature. The supports used for ionic binding may be based on polysaccharide derivatives (e.g., diethylaminoethylcellulose, dextran, chitosan, carboxymethylcellulose), synthetic polymers (e.g., polystyrene derivatives, polyethylene vinyl alcohol), and inorganic materials (e.g., ambertite, alumina, silicates, bentonite, sepiolite, silica gel, etc.).
The immobilization by ionic binding assures the minimal distortion of the enzyme conformation, resulting in an immobilized enzyme with high enzymatic activity.
The main disadvantage is the possible interference of other ions, and special attention should be paid in maintaining the correct ionic strength and pH conditions, in order to prevent their easy detachment.

3.3.3 COVALENT BINDING

The covalent binding method uses covalent bonds to tie enzymes to a support or matrix. The bond is normally formed between functional groups present on the surfaces of both the carrier (a specific functional group) and the enzyme (amino acid residues); the most common groups involved in covalent bonds are the amino (NH_2) group of lysine or arginine, carboxyl (CO_2H) group of aspartic acid or glutamic acid, hydroxyl (OH) group of serine or threonine and sulphydryl (SH) group of cysteine.

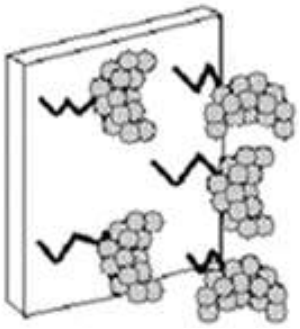


Figure 3.3: Enzyme immobilization via covalent bonds on a suitable carrier.

There are many reaction procedures for joining an enzyme to a material with a covalent bond, and the most frequent are diazotation, amino bond, Schiff's base formation, amidation reactions, thiol-disulfide, peptide bond, and alkylation reactions. The connection between the support and the biocatalyst can be direct, with a straight connection between the enzyme and the support, or undirect, with the use of a linker of variable length, known as *spacer* or *harm*.

The advantage of using a spacer molecule is that it gives a greater degree of mobility to the coupled biocatalysts, so that its activity can, under certain circumstances, be higher than if it is bound directly to the support.

Using this immobilization method, a pretreatment of the carrier is required, since the functional groups on the support material must be activated by specific reagents (e.g., cyanogen bromide, carbodiimide, aminoalkylethoxysilane, isothiocyanate, and epichlorohydrin, etc.).

Thanks to the fact that covalent bonds are very strong linkages, immobilization of enzymes through covalent attachment can be considered one of the strongest as regards enzyme engraftment, and it has also been demonstrated that this immobilization procedure induces higher resistance to temperature, denaturants, and organic solvents in several cases, also on account of the nature of the enzyme and the type of support.

Speaking of the support, many factors may influence its selection, and the first to consider are:

- cost and availability;
- binding capacity (amount of enzyme bound per given weight of matrix);
- hydrophilicity (the ability to incorporate water into the matrix and stability of matrix);
- structural rigidity;
- durability during applications.

Natural polymers, which are very hydrophilic, are popular support materials for enzyme immobilization since the residues in these polymers contain hydroxyl groups, which are ideal functional groups for participating in covalent bonds. A frequent disadvantage of immobilization by covalent binding is that it places great stress on the enzyme, indeed it contemplates harsh operative conditions which in some cases can considerably affect the enzymes, leading to remarkable changes in its conformation, with a resulting loss of catalytic activity.

3.3.4 CROSSLINKING

The crosslinking method is based on the formation of covalent bonds between the enzyme and the support through bi- or multifunctional reagents, such as glutardialdehyde, glutaraldehyde, glyoxal, diisocyanates, hexamethylene diisocyanate, toluene diisocyanate and other similar compounds.

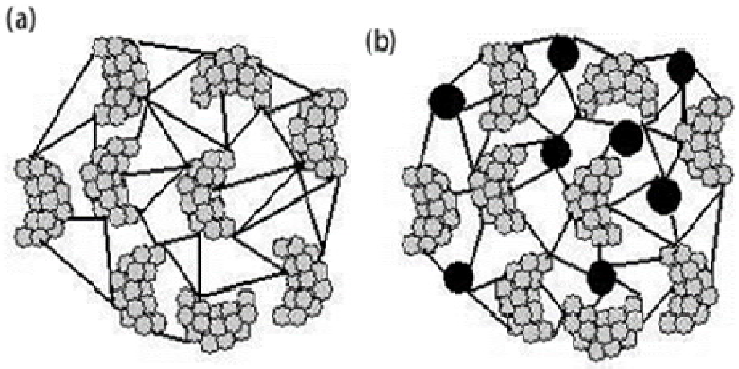


Figure 3.4: Enzyme immobilization via crosslinking (a) and co-crosslinking (b) on suitable carriers.

Enzyme crosslinking on a support normally involves the amino groups of the lysine but, in occasional cases, the sulfhydryl groups of cysteine, phenolic OH groups of tyrosine, or the imidazol group of histidine can also be used for binding. An advantage is the simplicity of the process.

The main disadvantages are the fragility of the particles produced in some cases and diffusion limitations. Furthermore, since crosslinking usually involves covalent bonds, immobilized biocatalysts in this way frequently undergo changes in the conformation with a resultant loss of activity.

3.3.5 ENTRAPMENT

The entrapment method for immobilization consists of the physical trapping of an enzyme into a film, gel, fiber, coating, or microencapsulation. In particular enzymes can be immobilized in three-dimensional matrices such as an electropolymerized film, an amphiphilic network composed of polydimethylsiloxane (PDMS), a photopolymer, a silica gel, a polysaccharide or a carbon paste.

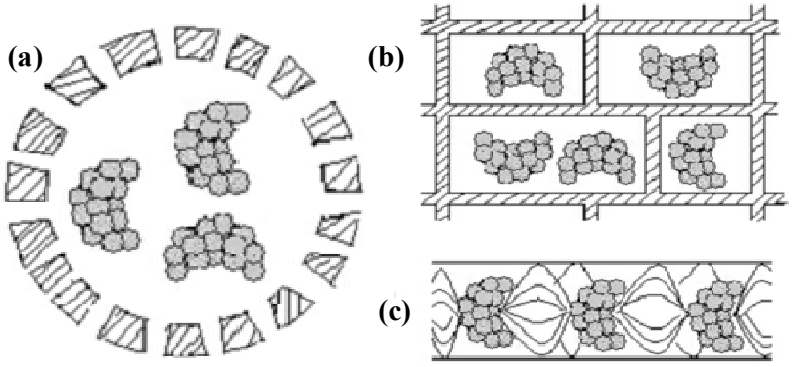


Figure 3.5: Enzyme immobilization via entrapment in pill (a) (encapsulation), in a matrix (b), in a fiber (c).

This method can be achieved by mixing an enzyme or active molecule with a polymer and then crosslinking the polymer to form a lattice structure that traps the enzyme. Microencapsulated enzymes are formed by enclosing enzymes solution within spherical semipermeable polymer membranes with controlled porosity. While the encapsulation of dyes, drugs, and other chemicals has been known for some time, it was not until the mid-1960s that such a method was first applied to enzymes. Since that first report, a number of other enzymes have been successfully immobilized via microencapsulation, using a number of different materials and methods to prepare the microcapsules. Therefore, it can be said that this kind of immobilization is easy to perform and there is no modification of the biological element, so that the activity of the enzyme is preserved during the immobilization process.

The advantages of this immobilization method are the extremely large surface area between the substrate and the enzyme, within a relatively small volume, and the real possibility of simultaneous immobilization of more than one molecule.

However, limitations such as leaching of biocomponent and possible diffusion barriers can restrict the performances of these systems. The major disadvantages of this method include the occasional inactivation of enzyme during microencapsulation and the high enzyme concentration required. In addition, to retain the enzyme, the pore size needs to be very low and these systems tend to be very diffusion limited.

Among all the explored entrapment methods, the sol-gel procedure for the entrapment of enzymes in a matrix is the chosen one for this study.

3.3.5.1 SOL–GEL PROCEDURE

The sol-gel procedure involves hydrolysis of suitable precursors of various nature under acidic or alkaline conditions, followed by condensation of the hydroxylated units, which leads to the formation of a porous gel. It is worthy to say that this technique is based on the ability to form solid metal or semimetal oxides via the aqueous process of hydrolytically labile precursors, such as esters of silicic, polysilicic acid, alkoxides, halide of aluminum.

Generally speaking, the sol-gel procedure can be thought about as a sequence of three main phases: choice of proper precursor(s), hydrolysis and policondensation reactions (reticulation), final treatment to remove the exceeding solvent.

As reported in *Avnir, 2005*, this technique is not so recent, and in fact the incipience of sol–gel immobilization of whole cells coincided with the large wave of development of organic–inorganic materials and applications in the early eighties, and, since then on, a very large number of enzymes have been trapped within materials obtained by sol-gel routes, showing that they usually retain their catalytic activity and can even be protected against degradation, but the investigation about this procedure is not fully developed yet, and in fact it is still a prominent object of research.

During encapsulation, enzymes remain trapped within a – generally silica – cage tailored to their size, and, as in all immobilization procedure, mobility within this confined space is restricted; on the other hand, unfolding is prevented, thus avoiding denaturation even when exposed to harsh operative conditions. Alkaline phosphatase (AlP) for instance, for which the natural -optimum pH is 9.5, appears to be active also at a pH as low as 0.9. This might be due to the fact that within the pores, because of space limitation, proteins are only surrounded by one or two layers of water molecules. Furthermore, the release of alcohol during the hydrolysis–condensation of silicon alkoxides represents an obstacle, due to its potential denaturing activity on the entrapped biological moiety. Tetramethoxysilane (TMOS, $Si(OMe)_4$), is therefore currently used instead of tetraethoxysilane (TEOS, $Si(OEt)_4$), as methanol is less harmful than ethanol. However some enzymes are especially sensitive to traces of alcohol so that the usual two-step alkoxide route has to be modified.

One way to overcome this drawback would be to remove the alcohol via evaporation under vacuum, in order to get a fully hydrolyzed solution before adding enzymes. Aqueous sol–gel processes have been developed in order to avoid any trace of alcohol. They use

aqueous solutions of sodium silicate as precursors. Silicic acid $Si(OH)_4$ is first formed via acidification followed by condensation in the presence of biomolecules at neutral pH. Another way to avoid denaturation by alcohol is to use biocompatible alcohols, such as polyol-based silanes. In this way, biocompatible compounds, like glycerol, are produced so that the catalytic efficiency and long-term stability of enzymes are significantly improved. This route was extensively developed during the past few years.

The silica matrix forms around the trapped biomolecule, but some shrinkage always occurs during the condensation process and the drying of the gel, and stresses can then lead to some partial denaturation of the enzymes. Polymers have been often used as additives to form hybrid organic–inorganic gels, in order to reduce shrinkage via a 'pore filling' effect.

Focusing the attention on practical procedure, at first, a low-molecular weight metal alkoxide precursor molecule, such as TMOS or TEOS, is hydrolyzed in the presence of water at acid (or alkaline) pH, resulting in the formation of Si–OH groups. In the second step, a condensation reaction between silanol moieties at alkaline (or acidic) pH results in the formation of siloxane (Si–O–Si) polymers, thus creating a matrix in which an enzyme can be successfully entrapped *(Campas and Marty, 2006; Kandimalla et al., 2006; Gupta and Chaudhury, 2007; Jeronimo et al., 2007)*.

The result is a matrix built-up around the enzyme particles, and – once removed the exceeding solvent – it can be used in the relative reagent system, allowing the contact between the substrates and the trapped enzyme.

In recent times, silica precursors are the preferred one because of various advantages; when used in a sol-gel procedure, the obtained silica gels are highly porous, showing physical rigidity, chemical and biological inertness and thermal stability.

However, as already outlined, these matrices suffer from considerable shrinkage during the drying process, which leads to fracture of the material and pore collapse. To provide for this setback, several reports suggest that the use of additives might be useful, by decreasing internal stress and shrinkage of the materials. For example, the use of trehalose or glycerol as a drying control chemical additive is considered as an interesting possibility in the sol–gel process (*Desimone et al., 2008*). In the same way, some polymers such as the natural polymer chitosan, poly(ethyleneglycol) or Nafion can also be used to prevent the shrinkage (*Choi et al., 2005*).

However, in spite of the possible inconveniences about shrinkage or similar problems, sol-gel process has been used to immobilize molecules employed in various industrial fields

since several years; in particular, it is used classically to immobilize enzymes in order to develop biosensors, as reported in *Gill and Ballesteros, 2000* and *Kandimalla et al., 2006*.
The sol–gel technology is also highly employed for the development of biosensors, optical (*Jeronimo et al., 2007*), electrochemiluminescent (ECL) (*Zhu et al., 2002a*), as well as fluorescent (*Salinas-Castillo et al., 2008*), or electrochemical, all of them sol-gel based.
Silica gels have also been used in association with nanostructures such as carbon nanotubes (CNTs) or gold nanoparticles (NPs) (*Shi et al., 2005*).

3.4 STATE OF ART

3.4.1 LIPASE IMMOBILIZATION

Lipases are the most employed enzymes in the industrial field, thanks to their properties and the huge range of reactions they catalyse. As a matter of facts their versatility leads to multiple industrial applications in foods, flavours, pharmaceuticals, cosmetics and environmental protection, and so on, that are possible through a wide variety of reaction in which the protein acts as a catalysts, i.e. in hydrolysis, esterification, transesterification and aminolysis. In addition, it has been widely used in bio-transformations such as resolution of racemic acids and resolution of secondary alcohols due to the high enantioselectivity. If on one side lipases are so interesting and offer such a huge range of industrial applications, on the other they are often easily inactivated and difficult to be separated from the reaction system for reuse. Consequently, by an appropriate choice of the immobilization process, operational costs for lipase industrial processes can be reduced and its use in industry can be simultaneously enhanced.

Upon time, different immobilization methods have been adopted, and some of them are reported below:

KIMURA ET AL., 1983, exploited the lipase from *Candida cylindracea* immobilization by entrapment with photo-crosslinkable resin prepolymers or urethane prepolymers, and by covalent binding or by adsorption to different types of porous inorganic or organic supports. All of the immobilized lipase preparations, obtained following the diverse procedures, were tested for the hydrolysis of olive oil and revealed some activity for it. The highest activity was found out for lipase entrapped with a hydrophobic photo-crosslinkable resin prepolymer, which was about 30% with respect to the free enzyme. Furthermore, immobilization conferred to lipase a better operational stability. Semicontinuous hydrolysis of olive oil using immobilized lipase was also accomplished in a packed-bed reactor with a recycling system. In this reactor, immobilized lipase was observed to have the sufficient activity and stability.

BOSLEY AND CLAYTON, 1994, selected the hydrophobic controlled-pore glasses as model system for the immobilization of *Rhizomucor miehei* lipase via adsorption, on the basis that the effect of pore diameter and surface chemistry on enzyme efficiency in a typical

esterification reaction under essentially non-aqueous conditions were of paramount concern.

They found out that pore diameters of at least 35 nm were essential for the lipase to be able to utilize the internal volume of the support particles in the immobilization process. Despite the small size of the substrates in the esterification reaction, even larger pores (>100 nm) were required for the lipase efficiency to become independent of pore diameter; below 100 nm lipase activity and efficiency were markedly reduced. They also proved that the chemical nature of the hydrophobic surface were an essential issue to consider in the catalyst design.

REETZ, 1997, examined the entrapment of lipases in hydrophobic organic-inorganic matrices derived from R-Si(OCH_3)$_3$ or R-Si(OCH_3)$_3$/Si(OCH_3)$_4$, resulting in chemically and thermally stable heterogeneous catalysts with very high recorded catalytic activities, thus being applied in chemo-, regio- and stereoselective organic transformations as heterogeneous catalysts. In addition, the author proved that its use in aqueous media also showed a great deal of promise.

REETZ ET AL., 2003, improved the original procedure for the encapsulation of lipases in sol-gel materials produced by the fluoride-catalyzed hydrolysis of mixtures of RSi(OCH_3)$_3$ and Si(OCH_3)$_4$, through higher enzyme loading, variation of the alkylsilane precursor, and the use of additives such as isopropyl alcohol, Tween 80 or other similar compounds, recording a considerable increase in enzyme activity. This immobilization method revealed to be useful also in the kinetic resolution of chiral alcohols and amines, recycling without any substantial loss in enantioselectivity and a residual activity of 70% being possible even after 20 reaction cycles.

NAWANI ET AL., 2006, carried out an investigation on the improvement of a thermostable lipase, partially purified from the culture supernatant of a thermophilic *Bacillus* sp., via the use of different immobilization techniques and reaction conditions. The lipase was immobilized on different solid supports and their enzyme activity and stability was compared. The authors found out that the enzyme adsorbed on silica and HP-20 beads, followed by cross-linking with gluteraldehyde on HP-20, registered an improvement in thermostability. The optimum pH (pH 8.5) was nearly same for aqueous and immobilized enzyme while optimum temperature was nearly 5ºC higher in case of immobilized enzyme.

The immobilized/cross linked enzyme was more thermostable at 70 and 80ºC in comparison to aqueous and surface adsorbed lipase on silica and HP-20. The optimum temperature for esterification reactions was determined to be 60-65ºC.

CHANG ET AL., 2007, performed the immobilization of *Candida rugosa* lipase (lipase AY-30) by adsorption on Celite, with a markedly improved performance of the enzyme. An optimization of the immobilization conditions was accomplished, and the highest specific activity obtained was 18.16 U/mg protein, with activity yield of 34.1%.

ALLOUE ET AL., 2008, aimed to immobilize lipase from *Yarrowia lipolytica* using three methods, i.e. inclusion, adsorption, and covalent bond, to study enzyme leaching, storage, and catalytic properties.
Sodium alginate and chitosan were the polymers selected to immobilize lipase by inclusion. The beads of each polymer were properly pre-treated to allow them to be more adapted to the inclusion of the enzyme.
Silica gel and celite were selected to immobilize the lipase by adsorption; maximum immobilization yield of 76% was obtained with celite followed by 43% in silica gel. The enzyme adsorbed on the two supports exhibited greater stability at 50 °C and in no polar solvents (Isooctane, n-heptane, and n-hexane).
The lipase immobilized by covalent bond retained residual activity equitable to 70%. It was demonstrated that the enzyme immobilized by covalent bond showed greater activity (80%) after 5 months of storage.

YANG ET AL., 2010, studied the bioimprinting and sol–gel encapsulation of lipases by silane precursors as efficient methods of enhancing lipase performance in non-aqueous medium. The correlation between bioimprinting, the alkyl-chain length of silane precursors, and the catalytic activity of gel-encapsulated lipase was investigated using a series of silane precursors: methyltrimethoxysilane (MTMS), vinyltrimethoxysilane (VTMOS), vinyltriethoxysilane (VTEOS), and n-octyltrimethoxysilane (OTMOS), thus also determining the optimal parameters for lipase immobilization. Both bioimprinting and increasing the chain-length of alkyl groups, apparently by increasing hydrophobicity, significantly improved the specific activity and the total activity of the immobilized lipase. Compared to a non-imprinted MTMS/TMOS gel, the specific activity of an imprinted OTMOS/TMOS gel improved 14.4-fold, and the total activity improved 6.8-fold.

Structural analyses demonstrated that the bioimprinting molecule and the hydrophobic alkyl groups of silane triggered lipase to change from the closed to the open conformation, and contributed to creating sol–gel matrices that were more porous and with less mass transfer resistance structure, apparently improving the activity of encapsulated lipase.

ZDARTA ET AL., 2016, carried out a study on the possible use of a silica–lignin hybrid as a novel support for the immobilization of lipase B from *Candida Antarctica* via adsorption, based on the formation of hydrogen bonds. Results obtained by elemental analysis, Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM), as well as the determination of changes in porous structure parameters, confirmed the effective immobilization of the enzyme on the surface of the composite matrix. Based on a hydrolysis reaction, a determination was made of the retention of activity of the immobilized lipase, found to be 92% of that of the native enzyme, and of its stability. Immobilization on a silica–lignin matrix produced systems with maximum activity at pH = 8 and at a temperature of 40°C, while for the native lipase the corresponding values were pH = 7 and 30°C. The immobilized enzyme exhibited increased thermal and chemical stability and retained more than 80% of its activity after 20 reaction cycles.

3.4.2 XYLANASE IMMOBILIZATION

As seen for lipase, also xylanases were immobilized since the immobilization techniques started to spread out in the industrial world, bringing their advantages into processes they were applied into.

Only few cases of immobilized xylanases are reported below, but much more are present in literature, since the end of last century, when xylanase immobilization established itself as an interesting solution to many issues from an industrial point of view.

GWANDE AND KAMAT (1998A) immobilized *Aspergillus sp.* strain 5 and *Aspergillus sp.* strain 44 on 400-mesh nylon bolting cloth in shake flask culture. They reported a 1.68-fold higher xylanase yield in immobilized *Aspergillus* sp. strain 5 than that freely suspended cells. The xylanase from the same organism was non-covalently immobilized on Eudragit S-100 for saccharification, which enabled its recovery and reuse for a longer period (*Gwande and Kamat 1998b*).

TOKUDA ET AL. (1997) also reported that maximum xylanase yield can be obtained by immobilizing *Aspergillus niger* on silk, rayon, and polyester fibres, which have several advantages over free enzyme.

In *BEG ET AL. 2000C*, the effectiveness of polyurethane foam (PUF) and three non-woven fabrics, namely cotton, silk, and polyester, as support materials for *Streptomyces sp.* QG-11-3 mycelia immobilization was investigated. The xylanase yields were enhanced by 2.5-fold, 1.91-fold, 1.54-fold, and 1.47-fold using PUF, polyester, silk, and cotton, respectively, compared with the xylanase yield in liquid-batch fermentation.

These results also indicated that the type of fibre material has a significant role in providing a favourable environment for enzyme production, thus having an influence on xylan hydrolysis activity of the immobilized mycelia of *Streptomyces sp.* QG-11-3. Mycelia grew inside the pores of fabric material and PUF particles. Unlike other techniques involving active immobilization, the use of PUF particles does not require the growth of cells prior to immobilization. The inert particles are simply placed in the fermenter before sterilization and the fermenter is inoculated in the normal way. Mycelia/cells become immobilized within the PUF pores as a natural consequence of growth, during an initial growth period. This technique has also been applied successfully to a wide variety of microbial cell systems for immobilization.

EDWARD ET AL., 2002, studied a xylanase from *T. lanuginosus* SSBP and immobilized it on the polymers alginate, chitosan, and Eudragit S-100, and between them they selected Eudragit S-100 as the best carrier, on the basis on the collected data regarding the activity. Xylanase was non-covalently bound to Eudragit S-100, and confirmed an enhanced thermal stability than the free enzyme at 70°C and a good response to operative conditions in general; furthermore, the trapped enzyme had a showed an adequate activity in prolonged cycles. Therefore, the authors suggested that that kind of trapped xylanase, due to its improved characteristics, could be usefully applied in particular for the bleaching of pulp in the pulp and paper industry.

KAPOOR ET KUHAD, 2007 chose a xylanase from *Bacillus pumilus* (strain MK001) to investigate its immobilization on different matrices, according to various immobilization methods (Entrapment in polyacrylamide, Ca-alginate, Gelatin; physical adsorption onto silica, alumina, and chitosan; ionic binding on DEAE-sepharose, Q-S, CM-sepharose, Amberlite IR-120, and Amberlite IR-440; covalent binding on Chitin and HP-20). Entrapment using gelatin (GE) (40.0%), physical adsorption on chitin (CH) (35.0%), ionic binding with Q-sepharose (Q-S) (45.0%), and covalent binding with HP-20 beads (42.0%) showed the maximum xylanase immobilization efficiency.

As generally expected, the immobilization caused some changes in the responses, indeed for example the optimum pH shifted up to 1.0 unit (pH 7.0) as compared to free enzyme (pH 6.0) and optimum temperature was observed to be 8°C higher (68.0°C) than free enzyme (60.0°C); furthermore the immobilized xylanase exhibited higher pH stability (up to 28.0%) in the alkaline pH range (7.0–10.0) as compared to the free one and comparing the residual activities of free and immobilized enzyme, they were 50.0% for the free and 68.0, 64.0, 58.0, and 57.0% on HP-20, Q-S, CH, and GE, respectively, after 3 h of incubation at 80.0°C.

Moreover, some recycles were accomplished and it was found out that the immobilized xylanase retained up to 70.0% of its initial hydrolysis activity after seven enzyme reaction cycles. The immobilized xylanase was found to produce higher levels of high-quality xylo-oligosaccharides from birchwood xylan, indicating its potential in the nutraceutical industry.

SHAH AND GUPTA, 2008 focused their attention on the possibility to use nanocarbon tubes to entrap the xylanase, and they found out that the hydrophobic surface of the nanotubes enabled the binding, refolding, purification and simultaneous immobilization of the enzyme (via adsorption), observing an immobilization efficiency of 92%, with the commercial preparation of xylanase in 8 M urea.
An FT-IR spectroscopy investigation was also carried out to ensure the preservation of secondary structures, and they revealed that α-helical content decreased from 17% to 14%, β-sheet content increased from 53% to 61% and β-turns decreased from 20% to 15% upon immobilization on the nanotubes, that was an expected behaviour, since immobilization normally generates a refolding of the internal structures as a response to the interactions with the carrier.

NICHELE ET AL., 2011 directed their attention towards the realization of a new pharmaceutical formulation for the treatment of lactose intolerance, trying to increase the stability of β-galactosidase in order to prolong its activity upon time, thus improving its performance as dietary supplement in the digestion of lactose. To do so, they studied a procedure for the immobilization of β-galactosidase in a three-dimensional silica network, via entrapment (so the enzyme was not covalently bound to the support, but physically entrapped in the gel network that has grown around it, realized in a one-step sol–gel process using TEOS as precursor, with 1 TEOS : 6 H_2O molar ratio), properly characterizing the support as regards textural properties of the porous surface, through N_2 physisorption. The activity of β-galactosidase was evaluated *in vitro* in the hydrolysis of o-nitrophenyl-β-d-galactopyranoside (used instead of lactose) at pH 7.4 and 37°C, thus reproducing the conditions of the human intestine.
The enzyme entrapment within the porous matrix allowed to avoid its direct contact with the surrounding medium, thus increasing its stability, without hindering the reagents to reach the catalytic site.

YAN ET AL., 2012, proposed new carrier for xylanase immobilization, employing dealloyed nanoporous gold, a nanostructured metallic sponge with tunable porosity and excellent biocompatibility. Structure analyses revealed that the immobilization of xylanase was realized via chemical bonding of sulphur end-groups with gold surface atoms. The activity and stability for the immobilized enzyme were investigated under various conditions and the immobilized enzymes were found to keep 80% of the activity of free ones, also

showing an impressive stability; as a matter of facts, even after ten reaction cycles, these bio-nanocomposites could still retain ≈60% of the initial activity.

DHIMAN ET AL., 2012, focused their study on covalent immobilization of the crude xylanase preparation onto functionalized silicon oxide (SiO_2) nanoparticles with an average particle size of 80 nm, founding out that 66 % of the loaded enzyme was retained on the particle. Immobilized enzyme gave 45 % higher concentrations of xylooligosaccharides compared to the free enzyme. Also a recycle investigation was realized, and they obtained that after 17 cycles, the immobilized enzyme retained 97 % of the original activity, demonstrating its prospects for the synthesis of xylooligosaccharides in industrial applications.

FIGUEIRA ET AL.,2013, studied the immobilization of β-Glucosidases, an important group of enzymes due to their pivotal role in various biotechnological processes. As commonly known, they can be employed in biomass degradation for the production of fuel ethanol from cellulosic agricultural residues and wastes, and undoubtedly their immobilization may prove to be advantageous. Within such scope, they evaluated the feasibility of entrapping β-glucosidase in either sol−gel or in Lentikats supports for application in cellobiose hydrolysis, and performed the characterization of the resulting bioconversion systems. The activity and stability of the immobilized biocatalyst over given ranges of temperature and pH values were assessed, as well as kinetic data, and compared to the free form, and the operational stability was evaluated.
As desired, the immobilization increased the thermal stability of the enzyme, with a 10°C shift to an optimal temperature in the case of sol−gel support. Mass transfer hindrances as a result of immobilization were not significant, for sol−gel support. Lentikats-entrapped glucosidase was used in 19 consecutive batch runs for cellobiose hydrolysis, without noticeable decrease in product yield. Moreover, encouraging results were obtained for continuous operation. In the overall, the feasibility of using immobilized biocatalysts for cellobiose hydrolysis was established.

ZHANG ET AL., 2014, investigated the production of resveratrol (an herbal medicine) via enzymatic hydrolysis of polydatin, using β-glucosidase immobilized on cross-linked chitosan microspheres, modified by l-lysine. The enzyme immobilization conditions were optimized and the characterization of the immobilized enzyme was carried out. Under optimized operative conditions (45°C and pH 6.0, initial polydatin concentration of 2.0%,

flow rate of 1.0 mL/min), the overall hydrolysis yield could reach ≈86%, and the activity of the immobilized enzyme remained ≈92% after continuous hydrolysis for 8 h. Therefore, this was considered as a promising method to increase the supply of resveratrol in pharmaceutical markets worldwide, which also showed a new application of immobilized β-glucosidase in biotechnological industry.

JIANG ET AL., 2015, assumed alumina hydroxide as carrier material to examine xylanase immobilization on it, at first because of its high surface area, the advantages of simple and low-cost preparation and resistance to biodegradation. In particular, they chose a xylanase from *Thermomyces lanuginosus* and immobilized it onto two types of aluminum hydroxide particles (gibbsite and amorphous $Al(OH)_3$) through adsorption, analyzing in depth the properties of the adsorbed enzymes. They found out that both particles had considerable adsorptive capacity and affinity for xylanase, indeed xylanase retained 75% and 64% of the original catalytic activities after adsorption to gibbsite and amorphous $Al(OH)_3$. Both the adsorptions improved pH and thermal stability, lowered activation energy, and extended lifespan of the immobilized enzyme, as compared with the free enzyme. Xylanase adsorbed on gibbsite and amorphous Al(OH)3 retained 71% and 64% of its initial activity, respectively, after being recycled five times. These results indicated that aluminum hydroxides served as good supports for xylanase immobilization. Therefore, they opened a new possibility in xylanases immobilization field, with very interesting perspectives for practical production.

BIRÒ ET AL., 2015, studied the β-d-Galactosidase from *Kluyveromyces lactis* and for the first time it was immobilized by entrapment in hybrid organic-inorganic sol–gel materials with microporous structure, obtained from alkoxy silanes and alkylsubstituted alkoxy silanes, in different combinations. The immobilization matrix was tailored by fine tuning of several parameters (nature of alkyl group of silane precursors, molar ratio of silane precursors, nature of additives, protein concentration). Unlike other enzymes, β-d-galactosidase showed the best catalytic activity at low alkyl group content in the sol–gel matrix, at a molar ratio of 7:1 between the various silane precursors. The immobilized enzyme demonstrated enhanced storage, pH and thermal stability compared to the soluble enzyme, and it was characterized through various analyses (TEM, SEM, fluorescence confocal microscopy, porosity measurement). The biocatalyst was successfully reused in five reaction cycles, maintaining more than 60% of the initial activity. On the basis of the

collected data, the authors achieved their aim, which was to prepare highly stable solid phase biocatalysts by entrapment in nanostructured sol–gel matrices, obtained from alkoxysilanes and silane precursors with covalently linked alkyl groups.

VINOGRADOV AND AVNIR, 2015, developed an alumina sol–gel matrix based on boehmite nanorods as a superior carrier for enzyme immobilization. Proteinase and xylanase were chosen for this study, interesting representatives of industrially applied enzymes. For these two enzymes they registered exceptional thermal stability by entrapment within the alumina, revealing that the activity of xylanase entrapped within alumina increases with temperature up to 80°C, that it did not bear if in solution or entrapped in silica. These data confirmed the potential new applications of these enzymes for high temperature organic syntheses.

LIU ET AL., 2015, reported the realization of novel carbon nanoparticle-based supports, prepared by layer-by-layer self-assemble approach. The enzyme ATXX (chimeric and bifunctional xylanase) was successfully immobilized onto the supports by covalent bonds. The prepared carbon-coated chitosan nanoparticles showed high binding capacity.
The immobilized ATXX showed improved thermostability and storage stability compared with the free enzyme; it was also characterized by transmission electron microscopy (TEM, JEM 2100), Fourier transform infrared spectroscopy (FTIR, Bruker Tensor 27), and thermogravimetric analysis (TGA, TA Q5000). The immobilized ATXX retained 82.6% xylanase activity after seven successive reactions. High performance liquid chromatography (HPLC) analysis revealed that xylobiose (X_2) was the main hydrolysis product released from beechwood xylan, birchwood xylan, and oat spelt xylan by immobilized ATXX. Wheat bran and wheat bran insoluble xylan could be directly hydrolyzed by immobilized ATXX, which demonstrated a potential use for xylan bioconversion to xylooligosaccharides by the immobilized ATXX.

3.4.3 D-PHGAT IMMOBILIZATION

As reported above, aminotransferases PLP dependent enzymes and are responsible for transferring amino groups, so they are absolutely relevant enzymes as regards biotransformations, as a matter of facts they play a central role in the biocatalytic preparation of enantiopure amines and amino acids, challenging reactions to achieve by conventional synthesis. They are gaining attention because of their application in the manufacture of pharmaceutical intermediates on a large scale, and in particular ω-transaminases are gaining increasing attention due to their potential for the preparation of chiral amines, needed in optically pure form by the pharmaceutical industry.

Furthermore, it is nowadays compulsory the recycling for expensive enzyme preparation, thus running economic viable processes, and this could be obtained by immobilization of the enzyme through appropriate techniques, which – as commonly known – confer higher stability to the enzyme itself, with wide potential applications ranging from chemical synthesis to biotechnology and medicines.

Until now, it is known that transaminases have only been immobilized few times, reported below.

YI ET AL., 2007, tried to covalently attach an ω-transaminase of *Vibrio fluvialis* on chitosan beads (treated with glutaraldehyde) at pH 6.0, prepared by emulsion method. The yield of enzyme immobilization was satisfying, 54.3%, and together with its residual activity (17.8%), they were higher than those obtained with other commercial beads. From the collected data, the optimal pH of the immobilized enzyme was pH 9.0, which was the same as that of the free enzyme; moreover the immobilized enzyme on chitosan beads retained ≈77% of its conversion after five consecutive reactions with the 25 mM substrate, while the immobilized enzyme on Eupergit1 C retained 12%.

Addition of the cofactor, pyridoxal 5-phosphate (PLP), was needed to maintain the stability of the immobilized ω-transaminase.

KOSZELEWSKI ET AL., 2010, proposed a sol-gel entrapment for an ω-transaminase, due to the great characteristics of the techniques, especially for its simplicity and effectiveness as immobilization method, but also because sol–gel materials offer unique intrinsic properties, such as high surface to volume ratio, large surface area and porosity. Furthermore, nanoporous materials are usually nontoxic, inert, chemically and thermally stable, so they are applicable where biocompatibility and thermal stability is required,

which are all features required for a pharmaceutical application. Due to the low temperature processing, the sol–gel technology represents a useful methods to immobilize sensitive biomolecules. For these reasons, commercially available ω-transaminases were immobilized in a sol–gel matrix, also employing Celite 545 as additive to gain an enhanced result. The immobilized ω-transaminases were tested in the kinetic resolution of α-chiral primary amines and the results were great, with very high activity even at pH 11, in contrast to the free enzyme. Furthermore, the sol-gel/celite immobilized AT was also performed over five reaction cycles without any substantial loss in enantioselectivity and conversion.

PLANKESTAINER ET AL., 2017, successfully gained the covalent immobilisation of the amine transaminase from *Halomonas elongata* (HEWT) onto metal-derivatised epoxy Sepabeads. The immobilised enzyme retained 30% to 40% of the original activity and registered an increased organic solvent tolerance. Furthermore, immobilization allowed for an easy catalyst recovery and activity was unchanged when assessed over several cycles. In addition, continuous production of amines was performed for the first time by exploiting the combination of an immobilized, cell-free, biocatalyst and continuous flow reactors. Thanks to the high efficiency of heat and especially mass transfer in the continuous flow system, the bioconversion rate and yield was enhanced compared to the batch system, in addition to the fact that also the reuse of the biocatalyst was guaranteed, much more than in a batch system, since it was not strained by mechanical stirring or agitation; hence, the authors confirmed with their research that integration of flow chemistry and biocatalysis can be considered as a key technology intrinsically compatible with the principle of green chemistry.

4.MICROSTRUCTURED REACTORS

Enzyme immobilization seems to be the solution to many industrial issues, as reported in the previous section, thanks to the enhancement of the characteristics of the immobilized biocatalysts, which improve by far the processes they are employed into.

An additional way to improve process intensification is to reduce the size of the units, so the subsequent step could only be the employment of such immobilized enzymes in continuous mode processes, by applying appropriate reactor designs, that can be called immobilized enzyme reactors, or simply microreactors.

Indeed, this concept of miniaturize the size is proper of microstructured reactors, that are realized using microtechnology and precision engineering, with channels or chambers with internal characteristic dimensions < 1mm, often in the range of a few hundred microns.

As reported in *Anuar et al., 2013,* a microreactor is a device made up of microstructured components, in which chemical reactions can take place in continuous flow.

They were initially designed for integrating different analytical or chemical processes, including sample preparation, derivatization, separation and detection into a single platform, while upon time they have been optimized and applied into the most varied fields, especially as regards micro- and sometimes nano-liter process volumes, hence the terms *microfluidic* and *nanofluidic*. Therefore, their employment allows to use small reagent volumes, thereby reducing waste and costs of expensive compounds, and to achieve high separation efficiencies, making them well suited to be reused multiple times with no considerable loss of enzyme activity.

Furthermore, in the small spaces of microreactors thin fluid layers form and the surface to volume ratio is large. These key features significantly enhance the heat and mass transfer rates.

In addition, they are well-suited for chemical and biochemical synthesis and analysis, and a noticeable parte of them use silica as a support for enzyme immobilization, for its good properties, even if the choice of support is anyway dependent upon the nature and mechanism of the enzyme, the properties of the support and the final application and conditions of use of the obtained microreactor.

Moreover, the resulting immobilized enzyme reactors can also be integrated directly to further analytical methods, such as liquid chromatography or mass spectrometry.

Anyway, attention must be paid to the fact that commercially available microreactors can be expensive due to the complex methods required for their manufacture. These fabrication methods depends on various factors, such as end purpose of the microreactor, choice of the support (e.g. silica, quartz, metal, glass or polymer supports) and the catalyst that is immobilized.

As reported in *Pirozzi et al., 2016,* immobilized enzymes are increasingly used in microreactors, due to the advantages offered by microfluidic systems in terms of higher efficiency and repeatability. Indeed, the choice itself to have an enzyme instead of a synthetic catalysts already ensures an improved efficiency of the overall process, thanks to the specificity of the enzyme towards the substrates, besides the mild operative conditions generally required; in addition, when using immobilized biocatalysts in a continuous process, the amounts of required reagents is reduced, and this fact promotes environmentally friendly reactions, and also the immobilization gives the possibility to exploit the same enzyme several time, avoiding time and money wasting, thus resulting in an overall favourable procedure.

Enzymatic microreactors are currently applied in different fields, such as medical diagnostics, organic synthesis, drug discovery or biosensors.
Different biocatalyst configurations can be developed in microreactors, selecting time by time the best immobilization method, as entrapment on microchannel surfaces, in microbeads, in patterning structures, or in monoliths.
In particular, the use of enzyme-immobilized monolithic microreactors offer several benefits, some of which are listed below:
– simpler fabrication, with no need of specialized techniques or skills;
– reduced void volume in comparison to the packed columns, and increased S/V ratio, providing a larger surface area for enzyme immobilization;
– flexibility in fabrication into various geometries;
– controllable porosity, allowing convective flow through the micro-interstices with reduced backpressure in comparison to packed-bed reactors;
– reduced diffusion paths, producing increased mass and heat transfer;
– flexibility as regards the choice of functional groups for surface chemistry;

– feasibility of multi-enzyme systems by separate immobilization of enzymes in different compartments of the monolith, so that intermediates produced in a compartment can be used as substrates in the subsequent compartments (microreactors in series!);
– higher mechanical stability in comparison to conventional packed-bed bioreactors.

Among the various techniques to realize a microreactor, the sol–gel route represents a valid choice to have powderless processing of shaped materials such as films, micro-spheres or fibers, as also described in *Avnir et al., 2015*, with many examples already available in literature, as regards sol–gel based bio-reactors for the enzymatic conversion of CO_2 into methanol, or a sol–gel immobilized trypsin bioreactor integrated in microtiter plates, or trapped enzymes for making biosensors, used till 1990s.

5.MATERIALS AND METHODS

5.1 GENERAL

Lipase (triacylglycerol ester hydrolase) *Candida Rugosa* Lipase (CRL) AY30 is purchased from ACROS ORGANICS and used without further purification; *Thermomyces lanuginosus* xylanase (TLX, 2500 U/g), Birch-wood Xylan, Tetramethoxysilane, $Si(OCH_3)_4$ (TMOS), methyltrimethoxysilane, $Si(OCH_3)_3CH_3$ (MTMS), n-propyltrimethoxysilane, $Si(OCH_3)_3C_3H_7$ (PTMS), and p-nitrophenyl butyrate, $C_{10}H_{11}NO_4$, were of reagent grade and were obtained from ALDRICH Chemistry (Sigma-Aldrich) and used without further purification.

As regards the D-phenylglycine aminotransferase (D-PhgAT) enzymatic system, all the standard chemical reagent (Pyridoxal-5'-phosphate PLP, D-phenylglycine D-Phg, L-Glutamic acid L-Glu, benzoylformate BZF and all the buffers used in the enzyme purification steps) were from Sigma, VWR, Alfa Aesar and Acros Organics.

EziG carriers were kindly offered by the EnginZyme society.

Silica gel and re-verse-phase silica gel and TLC silica gel plates were from E. Merck (Darmstadt, Germany). Compounds on TLC plates were visualized by charring with α-naphthol reagent. Chemical determination of reducing sugar amount was determined by the Bernfeld method (3,5 dinitrosalicylic acid-DNS essay), using a xylose based calibration curve.

TLC solvent systems: (A): n-BuOH/AcOH/H_2O 6:2:2 by vol.; (B): EtOAc/AcOH/2-propanol/HCOOH/H_2O, 25:10:5:1:15 by vol.

Syringe driven filter units (0.22 mm PVDF hydrophilic) were acquired by Millipore; the equipment required for continuous tests with the microstructured reactors, i.e. PEEK (poly(ether ether ketone)) tubes (ID 1/16''), connectors and frits (A-710 frit PEEK 2µm, .125*.065*.200 (in)), was purchased from IDEX Health and Science.

Spectrophotometric analyses were realized using a PharmaSpec UV-1700, UV-Visible Spectrophotometer SHIMADZU, HPLC analyses were performed on a Shimadzu LC10Ai instrument equipped with a UV/VIS photodiode array detector SPD-M20A prominence; detection at λ 220 nm. For all the enzymatic systems, protein concentration was determined by the method of Bradford, using bovine serum albumin as standard.

As already said, the used lipase and xylanase are commercial enzymes, while transaminase is obtained by a purification route, analysed below.

5.2 PRELIMINARY OPERATIONS FOR TRANSAMINASES

As regards in particular D-Phenylglicine AminoTransferase:

5.2.1 CLONING, PROTEIN EXPRESSION

The full-length, codon optimized dpgA gene (Pseudomonas stutzeri ST-201, UNIPROT code: Q6VY99) was purchased from GenScript and cloned into pET 15b plasmid to give a recombinant D-PhgAT with a non-cleavable N-terminal His6-tag.

The construct was used to transform E. coli BL21 (DE3) competent cells and selection was carried out on agar plates containing ampicillin (100 μg/mL; LB/Amp100). A single colony was used to inoculate 250 mL of LB/Amp100 broth and the overnight culture was grown at 37°C with shaking at 250 rpm. The overnight culture was used to inoculate 1 L of fresh LB/Amp100 broth and grown to an A600 of 0.6–0.8. Protein expression was induced by addition of IPTG to a final concentration of 0.1 mM and growth was continued for 16 hours h at 16°C.

Cells were harvested by centrifugation (Thermo Scientific Multicentrifuge X3R) at 4000g for 30 min at 4°C.

5.2.2 D-PHGAT PURIFICATION

All purification steps were carried out at 4°C. Cells were resuspended in lysis buffer (0.1 M CAPS pH 9.5, 150 mM NaCl, 20 mM imidazole, 50 μM PLP with the addition of complete EDTA-free protease inhibitor tablets (Roche), DNAse (0.2 mg per 10 mL buffer) and lysed by sonication for 15 cycles (30 sec on, 30 sec off).

The lysed cell suspension was cleared by centrifugation (Thermo Scientific Multicentrifuge X3R) at 18000 for 30 min at 4°C. The cell-free extract was loaded onto a 1 mL HisTrap nickel affinity column (GE Healthcare). The column was washed with binding buffer for 20 column volumes, then the protein eluted with an imidazole gradient (10 to 500 mM) over 30 column volumes. Protein containing fractions were analyzed by SDS-PAGE and concentrated down to 1 mL using the Vivaspin 20 MWCO 30000. The concentrated D-PhgAT was loaded onto a pre-equilibrated (0.1 M CAPS, 150 mM NaCl, 50 μM PLP) HiPrep™ 16/600 Superdex™ S-200 size exclusion column (120 mL). Recombinant protein was eluted at a flow rate of 1 mL min^{-1} in buffer. The purity of the

recombinant proteins was analyzed by SDS-PAGE and the concentration was determined by the Bradford assay.

5.2.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) BASED ASSAY

For the HPLC method, a calibration curve with a standard D-Phenylglycine was realized; reactions were then analyzed by chiral HPLC using a Chirobiotic T column (Astec, chiral phase Teicoplanin, 5 μm, 250 mm × 4.6 mm) with these operative conditions: mobile phase: 0.025% TEAA:MeOH (50:50, v/v), flow rate: 0.5 mL min^{-1}, detection λ: 210 nm, temperature: 25°C, run time: 40 min.

The conversion was used to measure enzyme activity. Lab solution software (Shimadzu) was used to measure the area under product peaks.

5.3 D-PHGAT IMMOBILIZATION VIA IONIC BONDS: THE ENGINZYME TECHNOLOGY

This enzyme was selected to study two different immobilization procedure: ionic bonds and entrapment in a sol-gel matrix.

As regards the ionic bonds, a commercial carrier proposed by the Swedish *EnginZyme* company was used. EnginZyme was founded only in 2014, but its offer is very interesting, especially in a green vision for the future of chemical industry, due to the fact that it aims to obtain products that simplify the employment of enzymes in industrial processes while also reducing costs. Combining productivity and economic costs is of paramount concern, as the motivation of reducing the environmental impact alone is not sufficient to grow the biocatalysis market.

Scaling the process, it is known that enzymes are normally used as crude preparations of cell extracts or whole cells from overexpression in recombinant bacterial protein production strains, and they are used in considerable volumes, i.e. high costs to obtain huge amount of purified enzyme. Necessary cost reduction is therefore required.

Furthermore, the enzyme is only a fraction of the crude preparations, in fact most often the majority is native protein and other cellular components from the production strain which has no catalytic properties. For this reason, enzyme isolation becomes tedious and expensive and, as already said, enzyme purification frequently escalates the cost beyond the scope of cost-efficiency. As such, cost efficient and pure enzyme catalysts suitable for use in industrial settings are inadequate.

Summarizing, the use of biocatalysis in a single process can result in a significantly reduced environmental impact, but the main barrier for increased use of biocatalysis in chemical production is the high cost of the enzymes and their high development costs compared to the employment of chemical catalysts.

The solution should be found in the immobilization, which can allow reuse of the same enzyme, enhancing its properties too.

EnginZyme has chosen to firstly focus on the pharmaceutical industry, where the need for enantiopure products and the capability to produce complex molecular entities at as low a cost as possible gives an obvious benefit in favour of biocatalysis.

As universally admitted, enzyme immobilisation is not a standard technology in the industry because the available methods are effective only for a few verified enzymes, lack

general applicability and/or require screening and development work to function in a process for a given enzyme.

Arguably, if 90% activity is lost, more than ten-time reusability is required to ensure that a company sees a return on investment (ROI) for any method. Such high activity loss is not uncommon, yet ten-time reuse is a considerable challenge. (*Karim Engelmark Cassimjee and Hans-Jürgen Federsel*).

EziG is a material based on a rational design approach. It was necessary to identify a carrier material that could function as closely as possible to a *magic anchor*. The choice was a specific controlled-pore glass (CPG), a material that has interconnecting pores, which enables more efficient mass transfer to the interior than other porous materials. The compromise required from a 'magic' anchor in space is a porous silica skeleton that allows a solvent to effectively reach any given surface point. An important factor is the ability for reactants to reach the enzyme, *i.e.* efficient mass transfer into the porous material. Poor mass transfer is a common cause for low catalytic activity in general; a problem that is specifically pronounced for immobilised enzyme preparations.

The CPG concept has been used for enzyme immobilisation for decades, but is generally not regarded as a viable option since its performance does not outweigh its high cost. The main issue has been the nature of its silica-like surface, which is not stable to hydrolysis and may also cause denaturation of the enzymes themselves. Although the mass transfer is favourable, activity loss is observed and the number of cycles of reuse are limited. Therefore, the solution to this issue was to cover the porous surface of the CPG with an organic polymer layer, thus forming hybrid CPG , which is stable and not harmful to enzymes. Now, a suitable micro-environment for the enzyme is achieved in an accessible pore, and utilisation of a favourable mass transfer is thereby made possible while maintaining an active enzyme. The organic polymer completely covers the interior and exterior surfaces of the particle, and it is stable towards aqueous environments and most organic solvents.

Thereby, EziG immobilisation is claimed to be general for any enzyme produced with an accessible His-tag. The distortion of the enzyme is minimal, though reversible interactions of the carrier's surface with the enzyme still occur.

By utilising the specificity of the affinity tag, immobilisation on EziG can be performed without prior purification of the enzyme, in contrast to other techniques in which purification may be required to reach a workable enzyme loading on a carrier. The

frequently used crude preparations, *i.e.* cell extracts, can be directly applied to EziG to achieve a reusable preparation. Additionally, different enzymes can easily be co-immobilised on the same carrier for cascade reactions.

Commonly, porous materials used for enzyme immobilisation can be loaded to carry up to around 10% w/w enzyme. Since a large fraction of this is often deactivated, and other proteins are also bound (unless the immobilisation is performed from a purified preparation), the loading of active enzyme is usually limited to single digit percentages. With EziG, however, examples of up to 30% w/w active enzyme are produced.

For slower reactions, the EziG-immobilised enzyme can reach the same activity as the dissolved enzyme, or extend even higher when stabilising effects come into play.

By using the affinity tag technique for binding to a tailored material suitable for biocatalysis, immobilisation can be performed by a standardised procedure and active reusable enzyme can generally be expected. Thereby, a great deal of uncertainty is removed and motivation to plan the development of a biocatalytic process with immobilised enzyme is enhanced.

Accordingly, EziG is currently produced in three varieties with varying surface hydrophobicity. The binding of enzyme in active form is very rarely an issue, in contrast to common resins, and the focus can now shift to optimising the target reaction to accommodate for the introduction of the porous material.

Since the immobilisation can be done directly from a crude extract, few or no additional steps need be added to the process. Additionally, since the binding with the His-tag is reversible, the same carrier material can often be reloaded with fresh enzyme after exhausting the first batch of bound material and then recycled within the given process, thus avoiding any risk of cross-contamination. When this scenario is possible, the cost contribution from the carrier becomes insignificant.

Therefore, thanks to the presence of the non cleavable N-terminal His_6-tag, purified (P) and lysed (crude, C) D-PhgAT was immobilized through ionic bonds between the His-tag and the Fe^{3+} ions on the particles surface of the EziG carriers, according to the official EnginZyme standardized procedure. These carriers were made up of particles based on the controlled porosity glass (CPG) technology.

Figure 5.1: EziG carriers offered by EnginZyme for enzyme immobilization via ionic bonds.

All the immobilization steps were realized at room temperature.

At first, the best EziG carrier was chosen among the three offered by EnginZyme, as follow: after the preparation of cell free extract and purified D-PhgAT solutions, an estimation of the activity was realized by applying the Bradford assay; then four samples were prepared, one for each EziG carrier (EziG1, EziG2, EziG3) and one as control; the three EziG carriers were prepared, weighing in an eppendorf the same amount of 9 mg, aiming to load on each one 1 mg of enzyme (lysed, C, or purified, P), so to have a 10% w/w loading (later, a wider percentage range of loading was considered, from 10 to 50%).

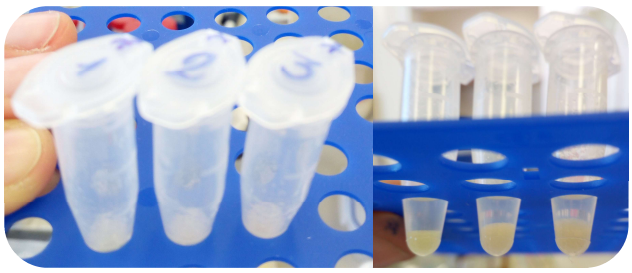


Figure 5.2: EziG-immobilized D-PhgAT.

For this reason, 200µL (later, different binding volume were explored, up to 1 mL, but this turned out to be the best) of an enzymatic solution containing 1 mg of enzyme was poured on each EziG carrier, and mixed on a wheel for 30min (also 60 min was explored). This step was realized in triplicate.

After the contact time considered, a 10 min for the sedimentation of the CPG particles occurred, and then the enzyme binding was confirmed by the Bradford assay on the supernatant. Afterwards, a washing step in the same buffer used for the enzyme preparation was required, for 30 min on the wheel, to ensure the release of both the enzyme only adsorbed on the external surface of the carrier and of other potential impurities, followed by pipetting the supernatant after 10 min of sedimentation. So the carriers were used in target reactions and from the collected data on catalytic activity, the best carrier was chosen. Ezig2 proved to be the best among them.

5.4 ENZYME ENTRAPMENT IN A SOL-GEL MONOLITH

Two precursor solutions of alkoxysilane were used for the preparation of four different – but very similar – enzymatic matrices. The explored procedure are defined *Single Step* (SS) and *Double Step* (DS) routes: in the SS route, the enzyme was immediately loaded within the precursors solutions, while in the DS, it was added later, aiming to explore the effects of a more hydrophobic microenvironment for the enzyme. The sol-gel enzymatic matrices were prepared at room temperature and under laminar flow hood. The samples obtained from these SS and DS routes, were subjected to two different dehydration post-treatments: drying (heating to constant weight in oven ventilated at 30°C, D) and lyophilisation (L).
For both CRL and TLX, four matrices were prepared for both batch and continuous tests (SS_D, SS_L, DS_D, DS_L), while for D-PhgAT only a single step route for batch tests was prepared (SS_D).

5.4.1 SINGLE STEP SOL-GEL ROUTE (SS) - MATRIX A

To prepare the microstructured reactors, at room temperature, a proper amount of each enzyme (10 mg of commercial CRL-AY30, 10 mg of commercial TLX, a volume corresponding to 10 mg of purified D-PhgAT), 0.290 mL of dH_2O, 0.950 mL of MTMS, 0.247 mL of TMOS (molar ratio of MTMS:TMOS = 4:1), 2.7 mL of 100 mM phosphate buffer (pH 7.5) and 0.0125 mL of 40 mM HCl were mixed (200 rpm) in a flask under a laminar flow hood. When required, in order to obtain higher amounts, the volumes have been proportionally increased by a chosen factor.
A fixed volume of the mixture (5 mL) was rapidly poured into the narrow PEEK tubing (ID 1/16'') of different lengths (from 8 to 10 cm), properly connected to a syringe, to channel the precursors mixture. From this solution, milk-like gels were obtained as a result of hydrolysis and policondensation reactions (gelation), occurring in no later than 5 min. The gelled systems were kept at room temperature under laminar flow hood for 24 h, recorded as the ageing time, and subsequently lyophilized for 24 h, in order to remove the exceeding solvent from the system and thus obtaining a porous structure.
Powders with similar composition were used for batch reaction experiments and for structural analysis. To this scope, the milk-like gel obtained by 5-min gelation was kept for 24 h at room temperature, and subsequently lyophilized or dried in an incubator shaker (Infors HT Minitron) at 30°C, obtaining a milk-like hardened dry gel. The hardened gel

was ground, washed three times with deionized water and fully desiccated (up to constant weight).

5.4.2 DOUBLE STEP SOL-GEL ROUTE (DS) - MATRIX C

This route was only used for CRL and TLX. An enzyme-free mixture was prepared according to the preparation procedure of the matrix A.

For continuous tests, after the 24h lyophilisation step, a second mixture containing the enzyme was prepared. At room temperature 10 mg of CRL-AY30 or 10 mg TLX, 0.041 mL of bidistilled water, 0.091 mL of PTMS, 0.0177 mL of TMOS (molar ratio of PTMS:TMOS = 4.43:1), 3.17 mL of 100 mM phosphate buffer pH 7.5 and 0.0055 mL of 40 mM HCl were mixed (200 rpm) in a flask under a laminar flow hood. A fixed volume of this mixture (5 mL) was rapidly poured into the PEEK tubing containing the enzyme-free matrix. The gelled system was kept for 24 h at room temperature, and subsequently lyophilized (L) or desiccated (D) for 24 h.

Again, following a procedure similar to that adopted for the SS sol-gel route, powders with similar composition were used for batch reaction experiments and for structural analysis for both enzymatic systems.

In all cases, hardened gels were obtained and they were finely grounded to perform the subsequent characterization.

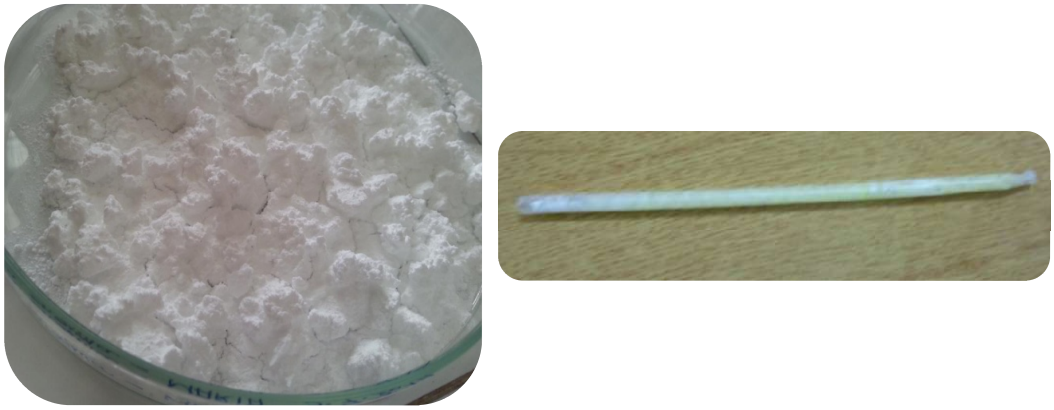


Figure 5.3: Enzymatic sol-gel matrix, ready to use in batch (on the left, trapped enzyme prepared in Petri dish) or continuous tests (on the right, trapped enzyme prepared in a Teflon tube, ID=0.19cm, L=8.0cm).

5.5 SET-UP OF MICROSTRUCTURED BIOREACTORS FOR CONTINUOUS OPERATION

As already said, immobilized lipase and xylanase were used in both batch and continuous experiments. For both of them, a similar equipment was arranged: Lipase-immobilized monoliths (CRL-CSS_D, CRL-CDS_D, CRL-CSS_L, CRL-CDS_L) and xylanase-immobilized monoliths (TLX-CSS_L) were prepared in PEEK or Teflon tubes (length 8 cm, ID 0.19 cm, internal volume 0.23 mL) according to the procedure reported above.

The lipase-immobilized microstructured silica bioreactor (CRL-CSS_D, CRL-CDS_D, CRL-CSS_L, CRL-CDS_L), consisted of a PEEK tubing of 10 cm of length, containing originally 5 mg of CRL, as reported in Figure 5.4.

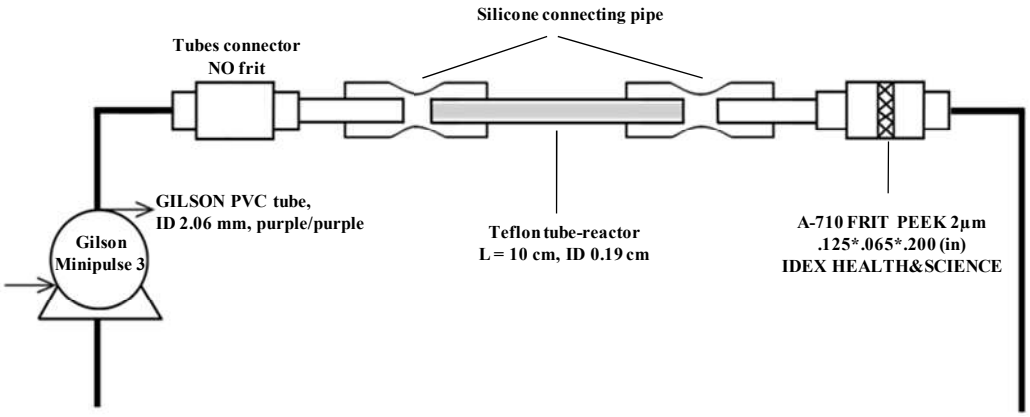


Figure 5.4: Structure of the continuous microstructured bioreactor for CRL enzymatic tests.

This tubing was linked with the other components of the system by silicone joints and also connected to a peristaltic pump (Minipuls 3-Gilson) to feed the reagent pNPB solution at constant volumetric flow rate. Downstream the tubing, a connector containing a frit filter (pore size = 2 µm) was placed to prevent the leakage of the gel-derived material.

Before each continuous experiment, a preliminary washing of the monoliths was performed by 100 mM phosphate buffer pH 7.5 for a chosen time (generally 1h). In order to evaluate the extent of the enzyme leaching, samples were collected from the effluent stream and tested for the amount of released protein via the Bradford assay *(Bradford, 1976).*

The xylanase-immobilized microstructured silica bioreactor (TLX-CSS_L) consisted of two Teflon tubings of 8 cm of length, each one containing originally 5 mg of TLX, as showed in Figure 5.5.

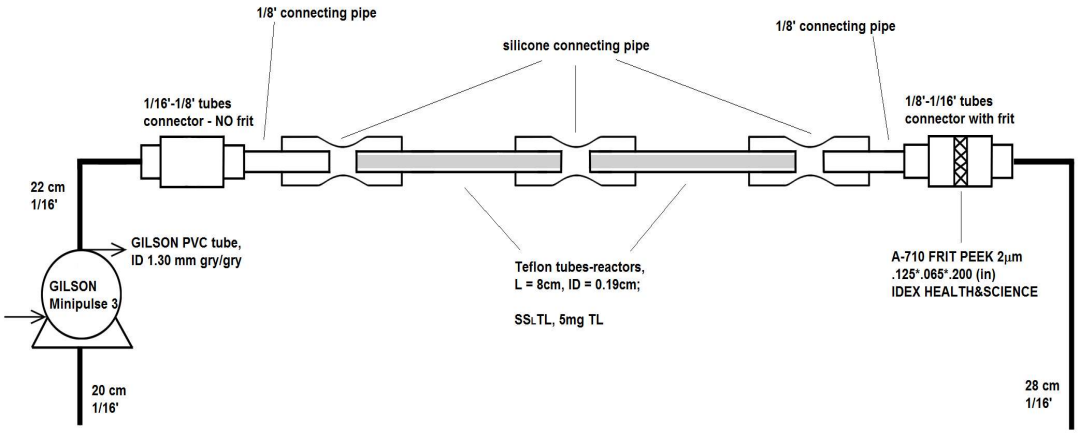


Figure 5.5: Structure of the continuous microstructured bioreactor for TLX enzymatic tests.

These two tubings were linked in series by silicone joints and also connected to a peristaltic pump (Minipuls 3-Gilson) to feed the reagent xylan solution at constant volumetric flow rate. The apparatus was put at 34°C by a sand bath. Downstream the second tubing, a connector containing a frit filter (pore size = 2 µm) was placed to avoid the leakage of the gel-derived material.

Before each continuous experiment, a preliminary washing of the monoliths was performed by 100 mM phosphate buffer pH 7 for 40 min. In order to evaluate the extent of the enzyme leaching, samples were collected from the effluent stream and tested for the amount of released protein by Bradford assay *(Bradford, 1976).*

5.6 DIFFUSE REFLECTANCE FTIR DATA ANALYSIS

Fourier-transform infrared (FTIR) spectroscopy is a powerful technique for the determination of the secondary structure of proteins.

For all the three enzymatic systems, Diffuse Reflectance FTIR (DRIFT) spectra were carried out at room temperature by a Nicolet system, Nexus model, equipped with a DTGS KBr (deuterated triglycine sulfate with potassium bromide windows) detector. The spectral resolution was of 2 cm^{-1} using 1% wt of sample properly diluted in KBr (about 400 mg x 1 cm^2). The spectrum of each sample represents an average of 64 scans, which were corrected for the spectrum of the blank KBr.

In each spectrum, the Amide I band was analyzed as a linear combination of spectral components. In the fitting, the number of Gaussian components and the initial values were acquired from the second derivative spectrum and these spectra were gained applying the Savinsky–Golay method (2^{nd} grade polynomial, five smoothing points, half-bandwidth (HW = 14 cm^{-1}), after a binomial 4 points smoothing of the spectrum. Curve fitting was performed by GRAMS/32 (Galactic Industries Corporation, Salem, NH, U.S.A.). Taking the above values as initial parameters, fitting curves of the Amide I band were performed by leaving the parameters free to adjust iteratively.

5.7 DIONEX ANALYSES

Only as regards the xylanase system, a dionex analysis was realized: it is an high-performance anion-exchange chromatography (HPAE-PAD Dionex ICS 5000+ DC) with a CARBOPAC PA1 column (250 × 4.60 mm), by which selected xylan hydrolyses were analysed at a solvent flow of 0.25 mL min^{-1}. The samples were centrifuged at 16000 rpm for 10 min and properly diluted with water before each injection. The separation of saccharides obtained from enzymatic digestions was achieved with 333 mM NaOH as the isocratic elution system and at 25°C. The retention times of the hydrolytic products (XOSs) were compared with known standards, purchased by Sigma. Under these elution conditions, calibration curves of xylose (rt: 2.42 min), xylobiose (rt: 3.41 min), xylotriose (rt: 5.18 min), xylotetraose (rt: 8.48 min), xylopentaose (rt: 15.3 min) and xyloexaose (rt: 28.01 min) were elaborated by using solutions of pure compounds in the concentration interval of 0.0016:0.016 mg mL^{-1}.

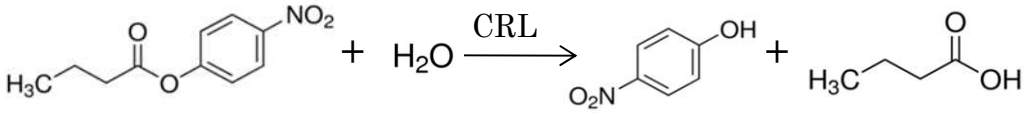
5.8 ENZYMATIC PROCESSES

5.8.1 BATCH EXPERIMENTS

5.8.1.1 LIPASE

For the three enzymatic systems, the catalytic performances of all the enzymatic matrices were explored in the hydrolysis of para-nitrophenylbutyrate for CRL, in the hydrolysis of Birchwood xylan for TLX and in the transamination of benzoylformate using L-Glutamic acid as amino donor for D-PhgAT, referring the results to the respective free enzymes.

The hydrolysis of 4-nitrophenyl butyrate, (5.a), was selected as test reaction to explore the catalytic activity of CRL:

(5.a) 

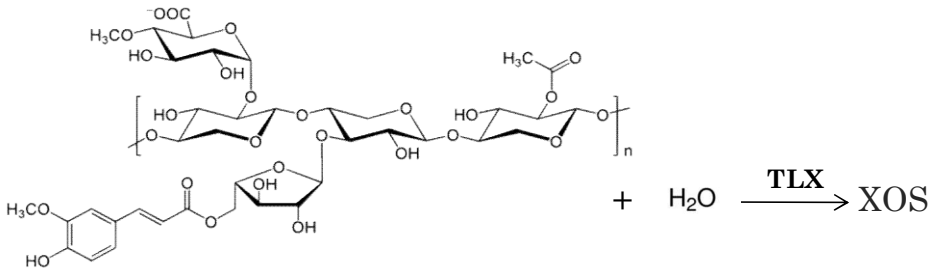
In a typical experiment, 1.0 mL of a 5 mM pNPB solution in acetonitrile was mixed with 9.0 mL of 100 mM pH 7.0 phosphate buffer in a test tube; then 20 mg of biocatalyst were added to start the reaction. A stirring speed was produced by an orbital shaker (717+ multi mixer). At given times, samples were collected and filtered through a syringe driven filter unit to remove the residual catalyst powder and subsequently the absorbance was recorded at 400 nm. The specific activity of the biocatalyst in the batch tests was determined as reported on formula (5.b):

(5.b) $$a = \frac{[pNP] - [pNP]_{blank}}{t_{test} \cdot W_{enz}} \cdot V$$

where [pNP] and $[pNP]_{blank}$ are the concentrations of product (mmol L^{-1}) in the reacting sample and in the blank, respectively, V is the volume of the sample (L), t_{test} (min) is the reaction time, W_{enz} (mg) is the total enzyme loading in the sol-gel matrix.
Two controls were carried out at the same time: one with all the reagents but the enzyme, one with all the reagents and with the free enzyme.

5.8.1.2 XYLANASE

As regards xylanase, the hydrolysis of xylan was chosen as test reaction, 5.c):

(5.c) 

The kinetic batch tests were performed starting from Birchwood xylan at 1% (w/v) (1.5 mL) under magnetic stirring, in 50 mM phosphate buffer at pH 7, at temperatures of 34°C and 70°C, and adopting two xylan/protein weight ratios (donor/enzyme, d/e) values of 3.3 and 6.6. Samples were withdrawn at given times to be analysed by TLC (solvent systems A and B) and by measurement of produced reducing sugars as xylose equivalent.

In particular, in a first set of reactions, the xylanase activity of enzymatic matrices was qualitatively analyzed by TLC investigation of reactions products. In a second set of hydrolysis reactions, the enzymatic matrices activity was quantitatively investigated by evaluating the percentage of xylan hydrolysis over the time as production of xylo-oligosaccharides, which were measured as equivalent reducing xylose. In this case, aliquots of 0.3 mL of each reaction were withdrawn at 0 min, 1, 4, 5, 6 and 24 h; they were denatured at 100°C for 2 min, then stored at 0°C overnight and centrifuged at 13000 rpm for 20 min at 4°C. The supernatants were collected and 0.05 mL of each one was essayed for xylose equivalent production (DNS test). The tests were conducted in triplicate and the calibration curve of xylose was elaborated in the concentration interval of 0.05:0.25 mg mL^{-1}. Reactions were also monitored by TLC (system solvent A and B).

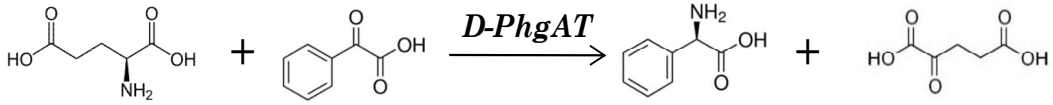
Processes with two different amount of the free TLX protein (4.54 mg or 2.27 mg) were carried out as reference.

In addition, hydrolysis of xylopentaose was performed by using either TLX or TLX-SS_L. In this case, 1.5 mg of xylopentaose in 1.5 ml buffer phosphate 50 mM pH 7 and at 34°C was hydrolysed by 0.45 mg of TLX or 16 mg of TLX-SS_L (xylan/protein weight ratios, d/e: 3.3).

Each case-study was compared to two controls at the same time: one with all the reagents but the enzyme, one with all the reagents and with the free enzyme.

5.8.1.3 D-PHENYLGLYCINEAMINOTRANSFERASE

For D-Phenylglycine aminotransferase, the chosen test reaction was the transamination of Benzoylformate, using L-Glutamic acid as amino donor, (5.d):

(5.d) 

Biotransformations were performed in 3 mL eppendorfs. For each component of the reaction the pH was adjusted to 9.5 before being added to the reaction mixture. A proper amount of purified (P) or lysed (crude, C) immobilized D-PhgAT was added at a final concentration 1 mg/ml in 0.1 M CAPS pH 9.5, 150 mM NaCl, 50 μM PLP containing 10 mM BZF and 300 mM L-Glu. The biotransformations were carried out at different temperatures (15°C, 37°C, 40°C, 50°C, 60°C) and pHs (5, 7, 9.5, 11), at a vigorous magnetic stirring (≈ 1200 rpm), to ensure an appropriate contact between the substrates and the immobilized enzyme. Time points were collected at time 0, 15 min, 30 min, 45min, 1h, 2h, 4h, 7h, 24h, 36h, 72h. Each time point was collected in triplicate and reactions were stopped by diluting 40 fold in 0.025% triethylammonium acetate (TEAA):MeOH (50:50). Samples were analyzed by Chiral HPLC.

For each case-study, two controls were performed at the same time: one with all the reagents but the enzyme, one with all the reagents and with the free enzyme.

5.8.2 REUSABILITY OF ENTRAPPED ENZYMES IN BATCH EXPERIMENTS

The reusability of immobilized enzymes in batch systems was essayed for several cycles, each of them of a chosen period of time related to the enzyme explored (CRL 4h, TLX 24 h, D-PhgAT from 5min up to 24h).

For each enzymatic system, a similar procedure was adopted: after each cycle, the immobilized enzyme was recovered by centrifugation (13000 rpm for 20/30 min at 4°C, D-PhgAT) then washed with the proper buffer, re-centrifuged and re-employed in the next reaction using a new aliquot of fresh reagents, in the same conditions of the previous test.1% xylan for further 24 h. The recycle of TLX-SS_L was performed seven times. At the end of each recycle, the residual catalytic activity was evaluated; all runs were performed in triplicate.

5.8.3 CONTINUOUS EXPERIMENTS

The lipase-immobilized silica monolith microstructured bioreactor consisted of one tube (10 cm long) kept at constant room temperature and connected to a peristaltic pump (Minipuls 3-Gilson), as reported in Figure 5.4. The reagent solution was made up of 10% v/v of p-nitrophenyl butyrate (pNPB) 5 mM in acetonitrile, and 90% v/v of 100 mM phosphate buffer pH 7.0 and this was fed to the microstructured bioreactor at constant volumetric flow rate, at T = 30°C. At given times, samples were collected from both the upstream and the effluent stream to measure absorbance at λ = 400 nm, that is the wavelength at which the product, pNP, is optically active. The specific activity of the biocatalyst in the continuous tests was determined as:

$$a = \frac{[pNP]_{out} - [pNP]_{in}}{W_{enz}} \cdot Q \qquad \text{(5.e)}$$

where $[pNPH]_{in}$ and $[pNPH]_{out}$ are the upstream and the effluent concentrations of product (mmol L^{-1}), respectively, Q is the volumetric flow-rate (L min^{-1}), W_{enz} (mg) is the total enzyme loading in the sol-gel matrix.

As concern xylanase, hydrolysis experiments were carried out in continuous mode by using the microstructured bioreactor system depicted above (Figure 5.5) and fed by a Birchwood xylan solution of 0.26 % (w/v) in buffer phosphate 50 Mm at pH 7; the flow rate was kept at 0.012 mL min^{-1} (0.72 mL h^{-1}). 0.5 mL of the eluate solution were collected over the time according the following time-scheduling: 340, 590, 930, 1440, 1620, 1980,

2340 minutes. These collected fractions were analyzed by TLC and were essayed for the reducing xylose production *(P. Bernfeld, 1955).*

5.8.3.1 Evaluation of enzyme leaching in continuous experiments

For both lipase and xylanase, each sol-gel matrix obtained in the tube was tested under the conditions adopted for the continuous activity assay. At given times, samples were collected from the effluent stream to evaluate the enzyme concentration by the Lowry *(Lowry)* or the Bradford method *(Bradford)*, to evaluate the extent of the enzyme leaching.

5.8.3.2 Stability of immobilized enzymes for continuous operations

The stability of the free and immobilized enzymes was assessed by repeated incubation cycles, each one of them carried out in the same operative conditions of the corresponding test reaction, for a chosen period of time. After each test, the monolith was washed with the proper buffer (the same used in the test reaction) and the activity was measured again, to evaluate the reusability of the immobilized enzyme.

6.RESULTS AND DISCUSSION

6.1 GENERAL

As stated above, and as also reported in *Pirozzi et al., 2016*, the immobilization of enzymes is nowadays necessary to develop continuous reactors whose industrial use let them join a green eco-friendly route.

Indeed, there are several reasons why enzyme immobilization has gained so much attention from an industrial point of view, first of all because immobilized enzymes can be easily removed from the effluent stream, thus allowing their regeneration and re-use: this is a very struggling issue in enzymatic processes, because biocatalysts are often very expensive, and so the possibility to reuse them allows to have a positive economic balance. Furthermore, using immobilized biocatalysts gives the possibility to avoid uncontrolled transformation of the products, as well as their contamination or low purity degree, due to the fact that when using a free biocatalyst, this latter is present in the product stream together with other substances; this question is of particular importance for food or pharmaceutical preparations.

Furthermore, immobilization may significantly increase the enzymatic stability against inactivating factors such as organic solvents, extreme temperature and pH. However, the enzyme catalytic properties can be strongly affected by the physico-chemical and morphological properties of the solid supports used for their immobilization: as reported in the immobilization section, the chosen immobilization method can modify the internal structures organisation of the trapped biomolecule, due to the bindings between the residues on the support surface and the enzyme itself, thus compromising its activity; for this reason, the selection of a proper immobilization technique is of paramount concern.

Anyhow, besides all the probable disadvantages that can be listed, the advantages are much more interesting, and as a matter of facts immobilized enzymes are increasingly used in industry, especially as regards microreactors, due to the advantages offered by microfluidic systems in terms of higher efficiency and repeatability. In addition, in the continuous mode there is the possibility to use a reduced amount of reagents: this promotes environmentally friendly reactions, in addition to a lower economic investment for expensive reagents.

As already reported, immobilized enzymes also allow a greater variety of bioreactor designs, thus allowing their applications in different fields, such as medical diagnostics, organic synthesis, drug discovery or biosensors.

Biocatalyst configurations for continuous microreactors can be the most varied: immobilized enzymes on microchannel surfaces, in microbeads, trapped in patterning structures, in monoliths, and so on.

In this study, an investigation of three trapped enzymes in some sol-gel silica monoliths was carried out, and in particular, as reported in the Materials and Methods sections, different sol-gel enzymatic matrices were built for each enzymatic system, following the SS and DS methods, with diverse combinations of sol-gel precursors (MTMS, TMOS, PTMS).

Every enzymatic matrix was tested regarding protein leaching by the Bradford assay, as well as the monolith shrinkage and detachment by visual examination, the rearrangements of the internal structures of both the carriers and the proteins, by IR comparison between trapped and pure enzyme and void and enzymatic carrier.

Focusing the attention on a FTIR analysis, the most important absorption bands due to the peptide groups belonging to the enzyme are classified as Amide I, Amide II and Amide III bands and they generally occur in the 1230–1900 cm^{-1} spectral region (*Barth A, 2007*).

As a matter of facts, the Amide I band, ranging in ≈1600–1700 cm^{-1}, offers a greater conformational sensitivity, due to the frequency of the C=O stretching mode (ν_{CO}) depending on the strength of hydrogen bonding interaction involving the Amide group and dipole–dipole interaction between the carboxyl groups. ν_{CO} vibrations are coupled with an out-of-phase C–N stretching (ν_{CN}) and C–C–N deformation of the peptide backbone.

The Amide II (≈1500–1580 cm^{-1}) is due to the N–H bending (δ_{NH}) with a contribution of the C–N stretching vibrations (ν_{CN}) and the Amide III (≈1230–1400 cm^{-1}) is related to the N–H bending (δ_{NH}), C–Cα ($\nu_{CC\alpha}$) and C–N (ν_{CN}) stretching vibrations.

Again, the limits of the spectral range in which these bands occur are very sensitive to the secondary structures of the enzyme.

6.2 CRLIPASE

As regards lipase from *Candida rugosa*, three enzymatic matrices were realized, *matrices A* (MTMS-based) and *B* (PTMS-based) according to the SS procedure (the enzyme was forthwith introduced) and *matrix C* (MTMS&PTMS-based) following the DS route (the enzyme was trapped halfway through the method).

The matrix A was selected for further experiments, as no shrinkage was observed, and the enzyme leaching was less than 1%. Subsequently, the sol-gel matrices offering better stability were compared as regards the catalytic activity of the biocatalyst.

Method	Matrix	a_{cat}[a] mmol $(mg_{cat}\ min)^{-1}$	a_{CRL}[b] mmol $(mg_{CRL}\ min)^{-1}$
SS	A	$2.08 \cdot 10^{-6}$	$8.87 \cdot 10^{-4}$
SS	B	$1.98 \cdot 10^{-5}$	$6.72 \cdot 10^{-3}$
DS	C	$1.04 \cdot 10^{-5}$	$7.06 \cdot 10^{-3}$

Table 6.1: Specific activities of immobilised CRL, measured in batch tests.
[a] Specific activity referred to the whole mass of the sol-gel matrix.
[b] Specific activity referred to the mass of the immobilized lipases.

Firstly, specific activities referred to the whole mass of both the enzymatic matrix (a_{cat}) and the trapped enzyme (a_{CRL}) were evaluated, measured in batch tests, as reported in Table 6.1: it is obvious that it results $a_{CRL} > a_{cat}$, as the mass of immobilized lipase represents only a small fraction of the matrix. Focusing the attention on the data related to SS method, it is clear that the *matrix A*, though being the best as regards shrinkage and enzyme leaching, displayed a relatively low lipase activity, with the highest catalytic activity observed for the *matrix B*. This result is due to the characteristics of the utilized carriers, indeed, as reported above, lipases need a water-oil interface to accomplish the interfacial activation, switch from close to open structure and allow catalysis: the same behaviour can be obtained in a hydrophobic environment, and between the two employed precursors, PTMS confers a more hydrophobic nature to the matrix, thus enabling the open-lid configuration of lipases.

On the base of this result, a DS sol-gel route – resulting in the *matrix C* – was accomplished: in this way, the properties of both the MTMS-based and PTMS-based carriers were exploited, thus gaining the best adhesion properties and the most suitable microenvironment for the enzyme.

Comparing the data in Table 6.1 as regards *matrices B* and *C*, the higher specific activity a_{CRL} is the one related to *matrix C*, confirming the efficiency of the DS method.

This result is not observed considering the catalytic activity based on the biocatalyst weight (a_{cat}), due to the fact that the entrapped enzyme is added to matrix C only in the second step of the DS procedure, thereby gaining a final matrix constituted by a relative lower percentage of enzyme on the whole weight in relation to the SS procedure matrix.

6.2.1 FTIR ANALYSIS

Matrices A and *C* were selected for IR studies, due to their good performance in terms of shrinkage and hydrophobicity. For this reason, FTIR spectra of free and immobilized (in *A* and *C*) CRL were recorded and overlayed as reported in Figure 6.1A, to compare their trends and better understand the effects of the matrix properties on the secondary structures of the enzyme itself (Figure 6.1B), i.e. on its catalytic activity.

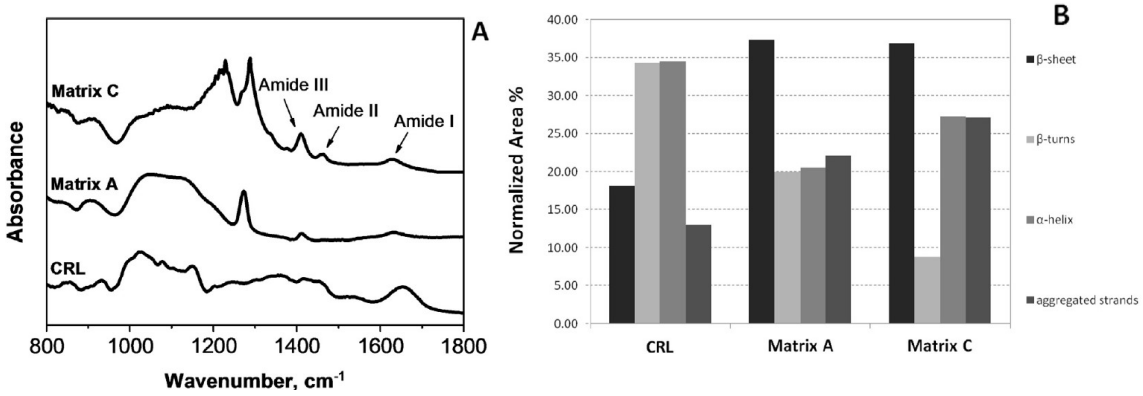


Figure 6.1: Results of FTIR analysis.
(A) FTIR spectra recorded at T = 37°C of free lipase (CRL), Matrix A and Matrix C.
(B) Distribution of the different secondary structures in the free (CRL) and trapped lipase (Matrix A and C).

Besides the Amide I, II and III bands described above, the FTIR spectrum of the native CRL exhibits different absorption bands in the 900–1200 cm^{-1} range, due to the vibrations of amino acid side chains (*Barth A, 2007*).

The Amide I, the Amide II, and the Amide III bands are clearly visible in both FTIR spectra of matrices *A* and *C*, even if they appear slightly downshifted compared to the free CRL, indicating that during the entrapment procedure the lipase secondary structures rearranged – but essentially preserved – their configuration.

These spectra clearly show also the main envelope in the 1000–1300 cm^{-1} region, where some of the vibration modes of Si–CH_3 and Si–$CH_2CH_2CH_3$ bonds (1273 and 1228 cm^{-1}) (*Okawara R., 1958; Aronne A. et al., 2007*) overlap with the ones typical of a siloxane network (1080 and 1250 cm^{-1}). Moreover, a weak and broad band lying in the 870–970 cm^{-1} range is present, due to overlap of the stretching modes of Si–OH bonds ($\nu_{Si\text{-}OH}$) with those of Si–CH_3 ($\nu_{Si\text{-}CH3}$) bonds. Therefore both matrices exhibit a suitable hydrophobic microenvironment allowing to successfully host the enzyme, with the matrix C showing a greater hydrophobicity with respect to the matrix A.

In order to investigate the influence of the sol-gel route on the conformational changes of the immobilized lipase, a curve fitting analysis of the Amide I absorption band (≈1600-

1700 cm^{-1}) was carried out (*Natalello A. et al., 2005; Aronne A. et al., 2014)*, using GRAMS/32 as the dedicated software for the FTIR data elaboration.

The quantitative fitting analysis was performed as a linear combination of the components identified in the second-derivative spectra, reported in Figure 6.2:

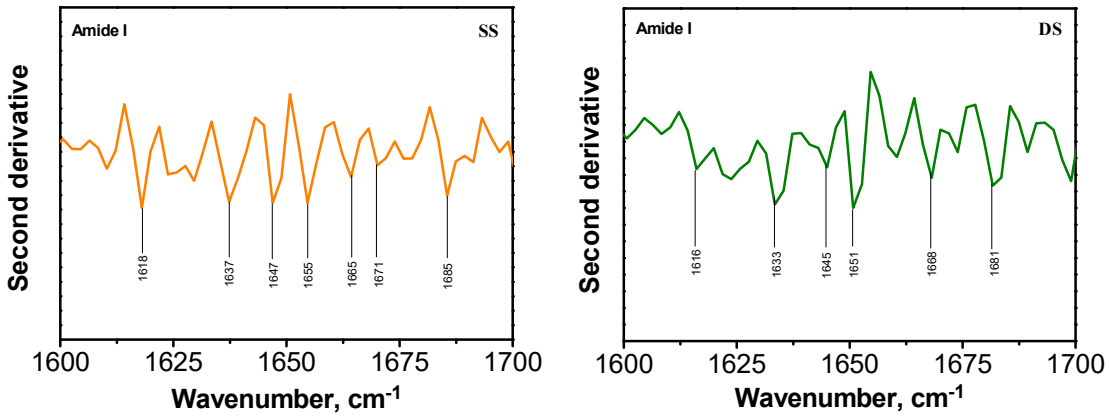


Figure 6.2: Second-derivate spectra for CRL-SS and CRL-DS trapped CRL.

Once considered the main peaks of the second derivative, these components were used as first attempt values and approximated by Gaussian functions whose peak positions, widths and heights were left free to adjust iteratively in the curve-fitting procedure, as suggested by Arrondo and Goñi (*Arrondo J.L.R. and Goñi F.M., 1999*).

In this way, the fitting curves showed in Figure 6.3 were obtained, with each curve ascribable to a defined secondary structure:

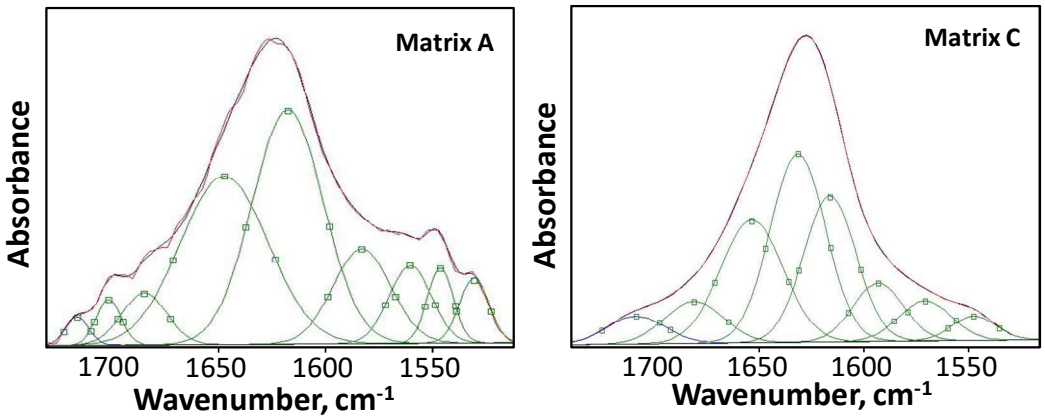


Figure 6.3: Amide I fitting curve of Matrices A and C: the blue line is the real Amide I band trend, the green ones are the deconvolution curves, related to the secondary structures, the pink one is the elaboration of the green fitting curves.

The better the pink deconvoluted line (in Figure 6.3) fit the blue one, the more the deconvolution was well done: the reiteration was stopped only when the fitting converged.

From fitting curves the contribution of each component to the Amide I band was evaluated by integrating the area under the curve, and then normalizing it to the total area of the

Amide I band. These data were reported in Table 6.2, with the relative assignment for each band, according to literature *(Barth A., 2007; Natalello A. et al., 2005; Foresti M.L. et al., 2010*):

Best-fit Gaussian components cm^{-1}	Normalized areas (%)			Spectral Range cm^{-1}	Assignment
	Pure CRL-AY30	Matrix A	Matrix C		
1694	6.3	–	–	1672–1694	β-sheet β-turns
1685	11.2	8.2	8.7	1653–1691	β-turns
1672	23.2	11.8			
1656	34.5	20.6	27.3	1642–1660	α-helices
1635	11.8	37.4	36.9	1615–1638	β-sheet
1621	13.0	22.1	27.1	~1620	Aggregated strands

Table 6.2: Component band position and assignment of the best-fit Gaussian components of the Amide I band for pure CRL (χ^2=0.00007; R^2=0.99986), Matrix A (χ^2=0.00003; R^2=1.0001) and Matrix C (χ^2=0.0000001; R^2 = 1.00000).

Comparing these data with literature, it is worthy to notice that the conformational analysis predicted by FTIR is in very good agreement with that obtained by XRD, reported in *Grouchulski P. et al., 1994,* and in *Chronopoulou L. et al., 2011.* The percentages evaluated by two XRD studies for α-helical structures were 33.3% (*Grouchulski*) and 30% (*Chronopoulou*) in comparison with the value (34.5%) evaluated in this study. The same occurred for the β-sheet structures. The values predicted by XRD were about the same 12.4% (*Grouchulski*) and 12% (*Chronopoulou*), very close to 11.8% predicted by FTIR.

As regards β-sheet structure, there are usually two components: the main one at 1635 cm^{-1} and the second one at 1694 cm^{-1}, partially overlapped with vibration related to the β-turns structures. Anyway, even considering the component contribution at 1694 cm^{-1}, the overall value was 18.1%. Considering this assumption, the histogram reported in Figure 6.1B was built, to compare the relative amount of the different secondary structures of native and trapped lipase.

From those results, it was clear that during the immobilisation route, the protein was influenced by the presence of the support itself; as a matter of facts the relative amount of different secondary structures varied, if compared to that of the native lipases, pointing out some kind of modification of lipase structures upon contact with the siloxane hybrid structure. In particular, focusing the attention on Figure 6.1B and considering the free

lipase as reference, Matrix C suffered a lower decrease of α-helices percentage in comparison to that of the Matrix A, whereas an opposite trend was observed for β-turns structures. Both entrapment routes caused a rearrangement of the enzyme secondary structures, thus inducing – as predictable – the relative increase of aggregated strands and β-sheet structures and, at the same time, the decrease of β-turns and α-helical structures, because the molecule needed to fold itself in a new way, being affected by the support.

Nevertheless, although these conformational changes in the structure of the immobilized enzyme brought to a partial denaturation of the enzyme itself, the catalytic activity was preserved in both Matrices A and C, much more in the second one, due to its higher hydrophobicity, as described above. This result was a further demonstration of literature (*Noureddini H. and Gao X., 2007)* that one of the main aims in an entrapment procedure is the achievement of a suitable microenvironment for the enzyme: the more suitable is the environment, the more the enzyme remains catalytically active.

6.2.2 CONTINUOUS CATALYTIC TESTS

Regarding continuous tests with trapped CRL, the procedure reported in the Material and Methods section was applied, in order to study the hydrolysis of para-nitrophenylbutyrate, monitoring the production of para-nitrophenol, that was an optically active compound, very easy to detect in a spectrophotometric way (400 nm).

Typical activity-time profiles obtained with monolith continuous reactors were shown in Figure 6.4.

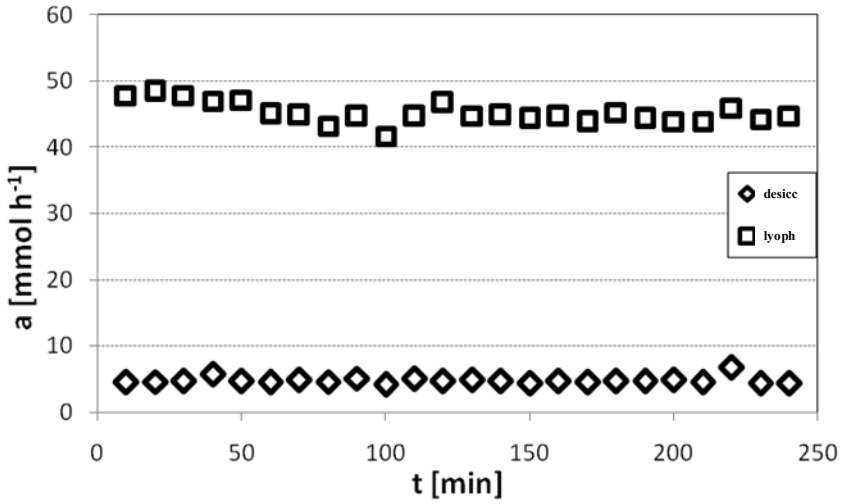


Figure 6.4: Activity-time profiles of a monolith continuous reactor for the hydrolysis of pNPB. T = 30°C. Buffer: 100 mM phosphate buffer pH 7.0.

As reported in Materials and Methods section, two different final treatment on the sol-gel enzymatic matrices were explored – desiccation (◊) and lyophilisation (□) – in order to remove the exceeding solvent mainly produced by the policondensation reactions and obtain a porous structure, through which the reagent solution could pass through.

These curves were obtained using a DS-CRL biocatalyst, but a similar – even a bit worse – behaviour was recorded with the SS-CRL.

The expected trend was the Michaelis-Menten one, with an initial increase and a plateau after a certain time; rather, these curves demonstrated that a very short transient time (less than 10 min) was required to achieve a steady-state condition in the reactor in both cases of desiccated and lyophilized samples. The reason of this behaviour was confirmed by the steady state values of the catalyst activity reported in Figure 6.5A (for the first step) and 6.5B (for the second step), which elucidated the effect of different aging times after the gelation in each step of the DS procedure.

Figure 6.5A reported the results regarding the effect of the ageing time after the gelation in the first step of the procedure. Among the considered time points, the best results were those related to the microbioreactor with 72 h of ageing after gelation, with the highest

specific activity value; assuming this result, the effect of the second step ageing time after gelation was also studied, and in this case the best results were obtained with an ageing time after gelation of 24 h.

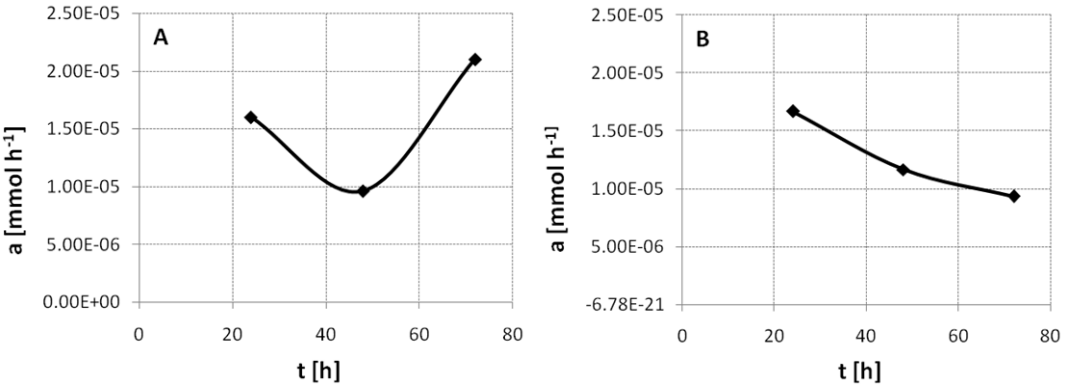


Figure 6.5: Specific activity of the immobilized lipase as a function of ageing time (explored ageing time: 24h-48h-72h) after gelation. T = 30°C. Buffer: 100 mM phosphate buffer pH 7.0.
(A) Effect of the first step aging time after gelation. (B) Effect of the second-step aging time after gelation.

As reported in literature (*Noureddini H. and Gao X., 2007)* and also stated above, biocatalysts prepared via the sol-gel procedure showed long term stability after several reuses. This was confirmed also by the results obtained in the course of this study, and indeed the Figure 6.6 described the stability of the free and the immobilized lipase in repeated continuous tests on the same microbioreactor, all of them carried out in the identical operating conditions of temperature, time of each single test, time of the whole test per day, ensuring the same volumetric flow rate through a peristaltic pump, as described for continuous activity assay in the Materials and Methods section.

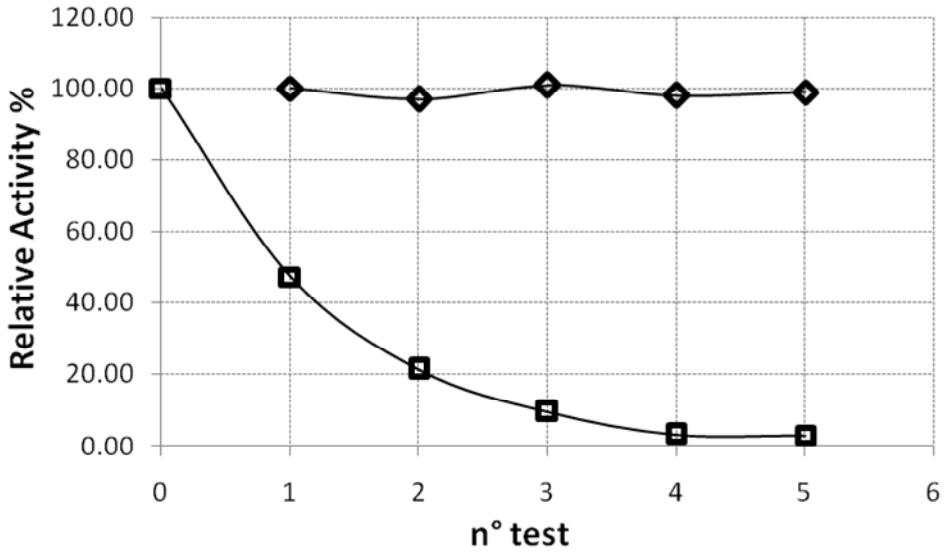


Figure 6.6: Stability of free (□) and immobilized (◊) CRL. T = 30°C. Buffer: 100 mM phosph. buffer pH 7.0.

It was clear from Figure 6.6 that the stability of the immobilized CRL was much higher than the one of the free enzyme. The activity of the immobilized catalyst was kept substantially constant per 5 tests, whereas that of the free enzyme reduced more than a half after the first reuse, decreasing more and more until 13% after five recycles.

These data demonstrated that no appreciable reduction of the intrinsic activity occurred, as well as no leak of entrapped enzyme.

So, ideally, according to these collected data, a trapped lipase could be reuse several times without activity loss, thus also confirming an economic benefit on the overall process, avoiding the continuous purchase of fresh enzyme. In order to enable a comparison between the productivities of the free and the immobilized CRL, additional tests were carried out to measure the specific activity and the half life ($t_{1/2}$) of both the free and the immobilized lipase. The specific activity observed for the free lipase ($8.22 \cdot 10^{-3}$ mmol/(mg min)) was only slightly higher than that pertaining to the immobilized enzyme, reported in Table 6.1. The values of $t_{1/2}$, obtained from suitable long-term stability tests, were $t_{1/2}$ = 8,49 h for free CRL and $t_{1/2}$ = 91,6 h for trapped CRL: according to these data, the productivity of the immobilized enzyme (g of nitrophenol produced/g enzyme applied, as reported in *Weiss N. et al., 2013*, is much higher than that of the free enzyme. This is even more evident when considering that, using free enzymes in industrial applications, multiple reuse is usually not allowed.

6.3 TLXYLANASE

Thermomyces lanuginosus xylanase (TLX) was entrapped in silica matrices by two sol-gel routes, SS and DS procedures, as reported in the Materials and Methods section. In particular, as done for the lipase system, a precise combination of alkoxysilane precursors (MTMS/TMOS and PTMS/TMOS), and two different dehydration post-treatments (D=desiccation, L=lyophilization) were explored for the preparation of four silica sol-gel enzymatic matrices, namely: TLX-SS_D, TLX-DS_D, TLX-SS_L and TLX-DS_L, used for batch catalytic tests and for structural analyses. Furthermore, in order to develop a continuous enzymatic microreactor, the SS procedure, coupled with lyophilization, was selected for the realization of xylanase-immobilized monoliths in proper Teflon tubes for continuous operations (TLX-CSS_L). The Teflon was chosen among other kinds of materials due to its good chemical and mechanical properties, favouring the sol-gel enzymatic adhesion and its mechanical performance, as also reported in *Pirozzi D. et al., 2016*.

6.3.1 FTIR ANALYSIS

The structural properties of the five matrices, TLX-SS_D, TLX-DS_D, TLX-SS_L, TLX-DS_L and TLX-CSS_L were examined through FTIR spectroscopy.

The FTIR spectra of the studied materials were displayed in both 400-4000 (Figure 6.7) or enlarged (Figures 6.8, 6.9, 6.10) wavenumber ranges.

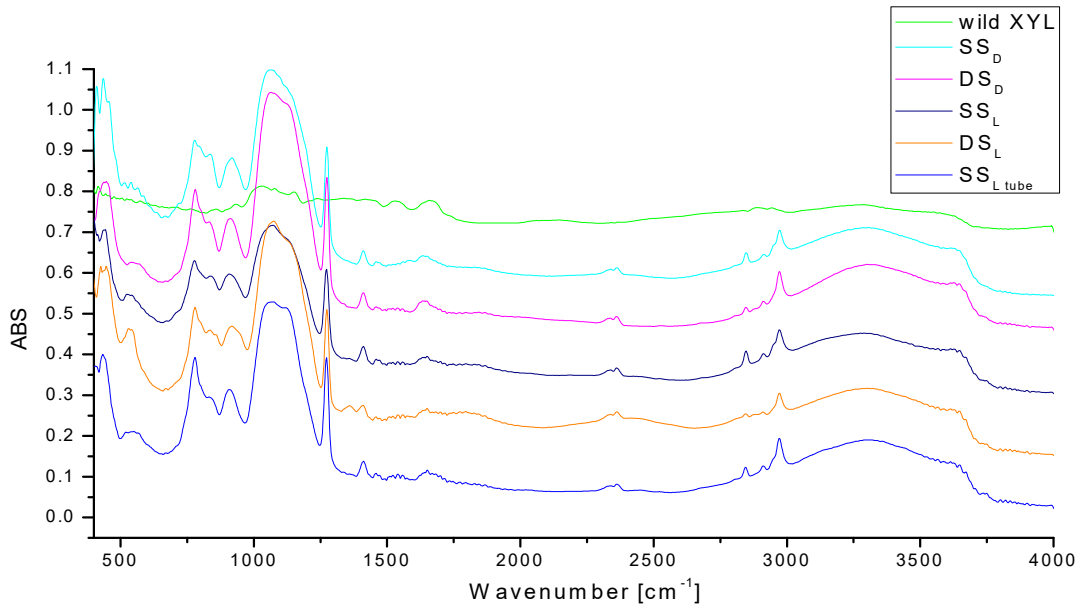


Figure 6.7: FTIR spectra of native TLX and of the studied matrices TLX-SS_D, TLX-DS_D, TLX-SS_L, TLX-DS_L and TLX-CSS_L, recorded at room temperature in the whole spectral range 400-4000 cm^{-1}.

Changes in the procedure (single or double step) and in the dehydration method (heat-treatment or lyophilisation) did not strongly affect the microstructural characteristics of the diverse matrices.

Analyzing in detail the various parts of these spectra, all matrices exhibited in the 400-1350 cm^{-1} region the characteristic features of a hybrid siloxane matrix still containing partially hydrolysed MTMS and PTMS molecules, reported in Figure 6.8.

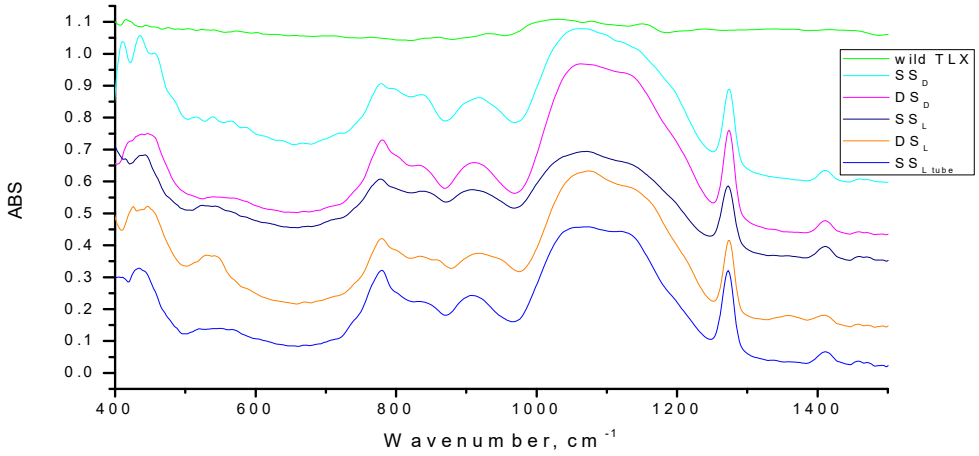


Figure 6.8: FTIR spectra in the 400-1500 cm^{-1} enlarged wavenumbers range.

As a matter of facts, besides to absorption bands related to vibrational modes of Si—O bonds at about 430 cm^{-1} ($\delta_{Si—O—Si}$), 840 cm^{-1} ($\nu_{sym\ Si—O—Si}$), 1060 and 1126 cm^{-1} ($\nu_{asym\ Si—O—Si}$), the sharp band at 1270 cm^{-1} together with the one at 780 cm^{-1} related to vibrational modes of Si—CH_3 bonds, as well as the bands at 2970, 2910 and 2830 cm^{-1}, due to the C—H stretching of CH_2 and CH_3 groups and the related bending modes (features in the 1350-1410 cm^{-1} range) were also seen (*Okawara R., 1958*).

Moreover, a broad band lying in the 3000-3800 cm^{-1} range was recorded in the FTIR spectra of all prepared matrices with a maximum at about 3300 cm^{-1} and a shoulder at about 3620 cm^{-1}, reported in Figure 6.9.

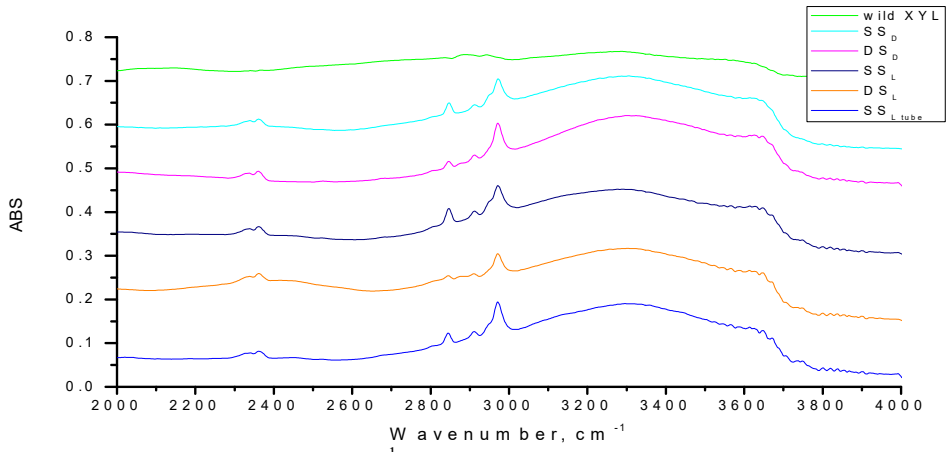


Figure 6.9: FTIR spectra in the 2000-4000 cm^{-1} enlarged wavenumbers range.

In this wavenumber range, protein backbone N—H vibrations (amide A) could overlap to the O—H bonds stretching modes of silanol groups. As the electronegativity of oxygen is greater than that of nitrogen, OH groups form stronger hydrogen bonds, shifting toward lower wavenumbers the stretching mode of O-H bonds. For the same reason, the isolated OH groups stretched at higher wavenumbers than the isolated N-H groups.

Anyway, as reported above, the most important enzyme absorption bands due to the peptide group vibrations were expected to occur in the 1230–1900 cm^{-1} spectral region, where the amide I band (~1600-1700 cm^{-1}), the amide II (~1500-1580 cm^{-1}) and the amide III (~1230-1400 cm^{-1}) can be located.

All these bands were clearly visible in the FTIR spectra reported in Figure 6.7, as a matter of facts in all cases the three bands were easily recognizable.

For all matrices only a small downshift of the amide I band (~1640 cm^{-1}) with respect to the native TLX (~1660 cm^{-1}) could be detected, indicating that during the entrapment procedure the secondary structures of xylanase were somehow altered.

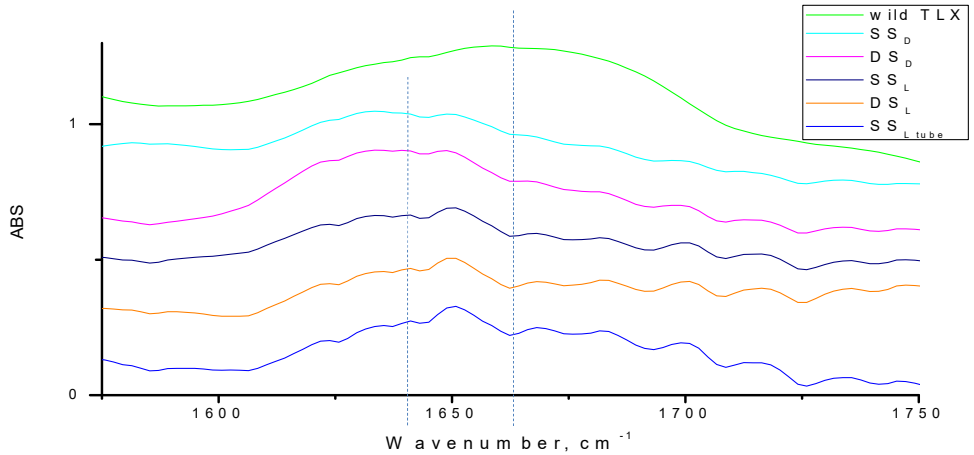


Figure 6.10: FTIR spectra in the 1575-1750 cm^{-1} enlarged wavenumbers range.

To investigate the extent of conformational changes occurring in the xylanase during the sol-gel entrapment procedure, as done for lipase, a curve fitting of the amide I absorption band was realized. As a matter of facts, the stretching modes of C=O bonds depend on the strength of hydrogen bonding interaction involving the amide group and dipole–dipole interaction between the carboxyl groups and, therefore, the shape and the position of this band result very sensitive to changes ad secondary structure of the enzyme (*Natalello A. et al., 2005)*. Also in this case, the quantitative fitting analysis was performed by a linear combination of the components identified in the second derivative spectrum, approximated by Gaussian functions, following the same procedure applied in the lipase system.

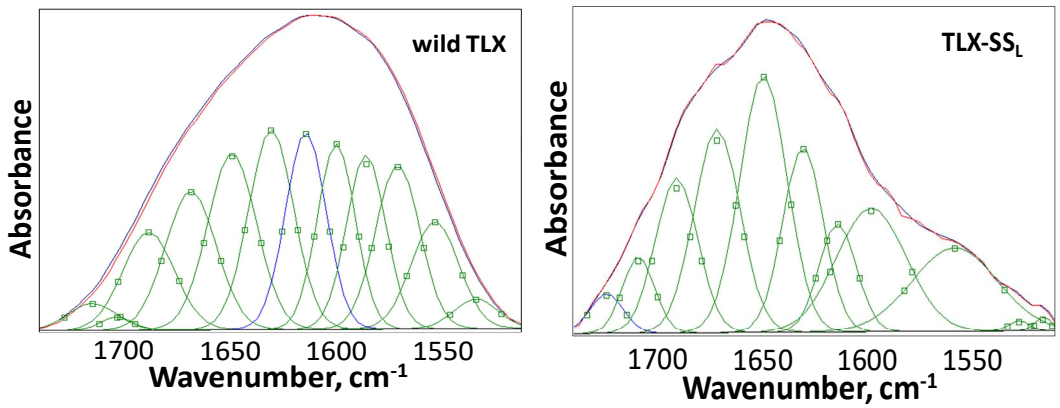


Figure 6.11: Comparison of the deconvoluted Amide I band (1600-1700 cm^{-1}) of free and trapped TLX: the green curves are related to the various secondary structures of the enzyme.

The elaboration of these data was reported in Table 6.3, where the assignment to each secondary structure was also reported, on the basis of the literature data given by *Steiner G. et al., 2007,* and *Arrondo J.L.R. et al., 1993.*

Best-fit Gaussian components cm^{-1}	Normalized Areas %						Spectral Range cm^{-1}	Assignment
	TLX	TLX-SS_D	TLX-DS_D	TLX-SS_L	TLX-CSS_L	TLX-DS_L		
1698	7.9	4.8	1.3	0.4	8.3	0.3	1672 - 1694	β-sheet & β-turns
1675	45.2	22.9	25.0	27.1	39.6	27.2	1653 - 1691	β-turns
1649	27.4	26.3	27.7	20.0	19.8	30.9	1642 - 1660	α-helices unordered
1632	11.0	24.2	31.9	37.0	15.7	29.1	1615 - 1638	β-sheet
1621	8.5	21.8	14.1	15.5	16.6	12.5	1620	Aggregated Strands/Side chain

Table 6.3: Component band position and assignment of the best-fit Gaussian components of the Amide I band for TLX (R^2=0.9999), TLX SS_D (R^2=0.9999), TLX DS_D (R^2=0.9995), TLX SS_L (R^2=0.9998), TLX CSS_L (R^2=0.9999), TLX-DS_L (R^2=0.9991).

The crystal structure of xylanase from T. lanuginosus was determined by *Gruber et al.*, using single-crystal X-ray diffraction.

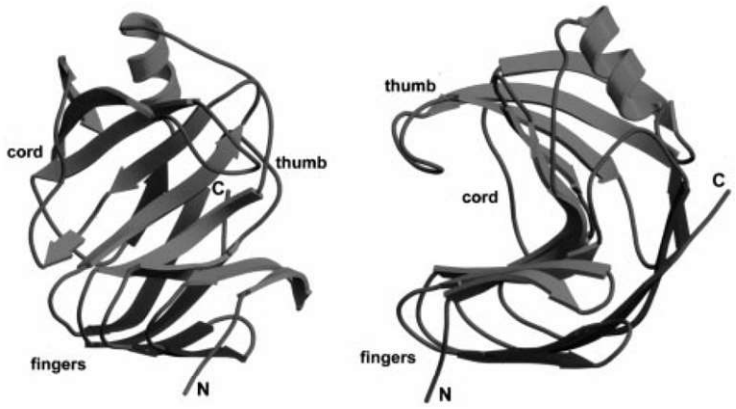


Figure 6.12: Two perpendicular views of the structure of the xylanase from *T. Lanuginosus*, generated with programs MOLSCRIPT and Raster3D (*Gruber et al., 1998*).

It resulted formed by two heavily twisted β-sheets, the first of which formed the hydrophilic outer surface of the enzyme and consisted of five antiparallel β-strands. The second β-sheet was formed by nine mostly antiparallel β-strands, one face of which formed the active site of the enzyme, while the other one was packed against the first β-sheet to form the hydrophobic core of the protein. This structure exhibited only one α-helix, formed by 10 residues, that was packed against the hydrophobic face of the second sheet (*Gruber K. et al., 1998)*. The conformational analysis predicted by FTIR seemed to overestimate the component due to β-turns with respect to the β-sheets, as already reported in Table 6.3. This discrepancy could be due to the higher extent of distortion of the two β-sheets forming this globular protein in the lyophilized sample used in this study with respect to that of the single-crystal. However, the conformational analysis predicted by FTIR showed a clear prevalence of β-strands (about 64 %) allowing to classify the TLX as an all β-protein, in agreement with literature (*M. Levitt, 1976)*.

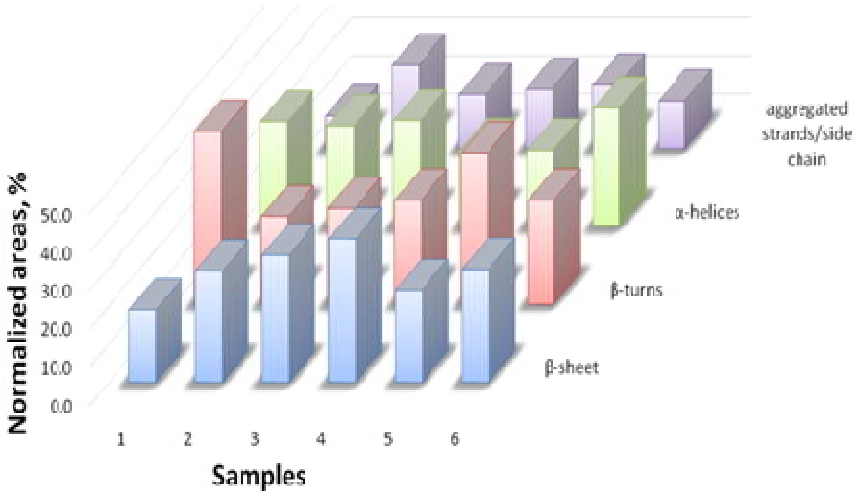


Figure 6.13: Distribution of the main secondary structures in the free (1–TLX) and entrapped (2–TLX-SS_D, 3–TLX-DS_D, 4–TLX-SS_L, 5–TLX-CSS_L, 6–TLX-DS_L) xylanase.

In Figure 6.13 the relative amounts of the different secondary structures of the native and the entrapped xylanases were compared. There was made the assumption that the component at 1698 cm^{-1} was entirely assigned to β-sheet, neglecting the contribution due to β-turns. The analysis of these data showed that during the immobilization the relative amounts of different secondary structures changed with respect to that of the native enzyme, evidencing some kind of modification of TLX structure upon contact with the siloxane hybrid structure. Particularly, changes concerning both β-turns and β-sheets were observed when adopting a different kind of matrix, as well as when adopting a different type of dehydration process. Among them all, only TLX-SS_L and TLX-CSS_L samples showed a more similar distribution of secondary structures compared to the native enzyme. Particularly, in these samples the total amount of β-strands is almost preserved, in contrast to the other matrices, suggesting that a more efficient catalytic behaviour can be expected for these biocatalysts.

6.3.2 SEM IMAGES AND B.E.T. EVALUATION

The obtained sol-gel enzymatic matrices were used for further structural analyses. In particular they were analysed by Scanning Electron Microscopy technique, collecting some very interesting images about the physic relative arrangement of the sol-gel particles within a particular structure, and then each one of them was also subjected to a measurement of surface area, according to the B.E.T. theory (*Brunauer S. et al., 1938)*.

Xylanase entrapment in a sol-gel matrix as reported in this work was an innovative suggestion, especially as regards its possible industrial applications; for this novelty, very few documents could be found out, to compare these results regarding SEM and BET to literature ones.

As clearly visible from Figure 6.14, all the matrices tended to form a monolithic structure constituted by aggregated particles, organised in a more or less porous framework. The particles shape – i.e. the overall structure of the considered matrix – which in some cases seemed to be smother and better defined (Figure 6.14, TLX-SS_D and TLX-DS_D), while in others were smaller and more compact, depended on the sol-gel procedure itself, and in particular on the relative rate between hydrolysis and policondensation reactions, the *core* of reticulation during the enzymatic matrix synthesis procedure; it could be understood even more from Figure 6.15 and Figure 6.16, in which different magnitude and size are recorded:

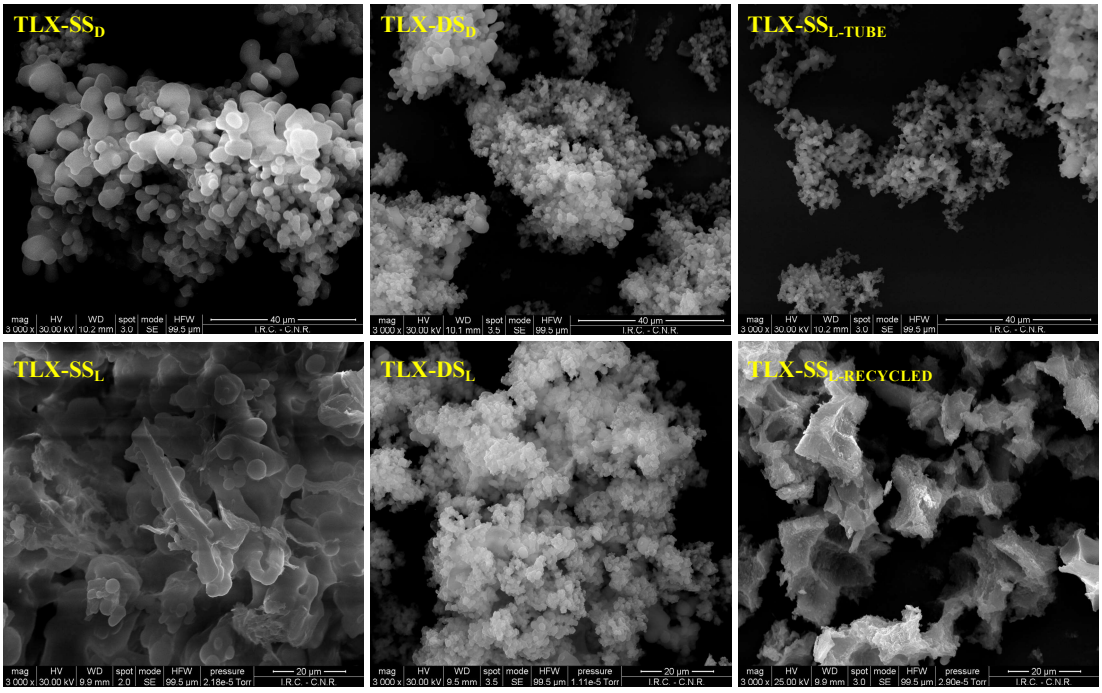


Figure 6.14: comparison of SEM images for TLX-SS$_D$, TLX-DS$_D$, TLX-SS$_{L\text{-}TUBE}$, TLX-SS$_L$, TLX-DS$_L$, TLX-SS$_{L\text{-}RECYCLED}$; mag 3000 x, 40 and 20 µm.

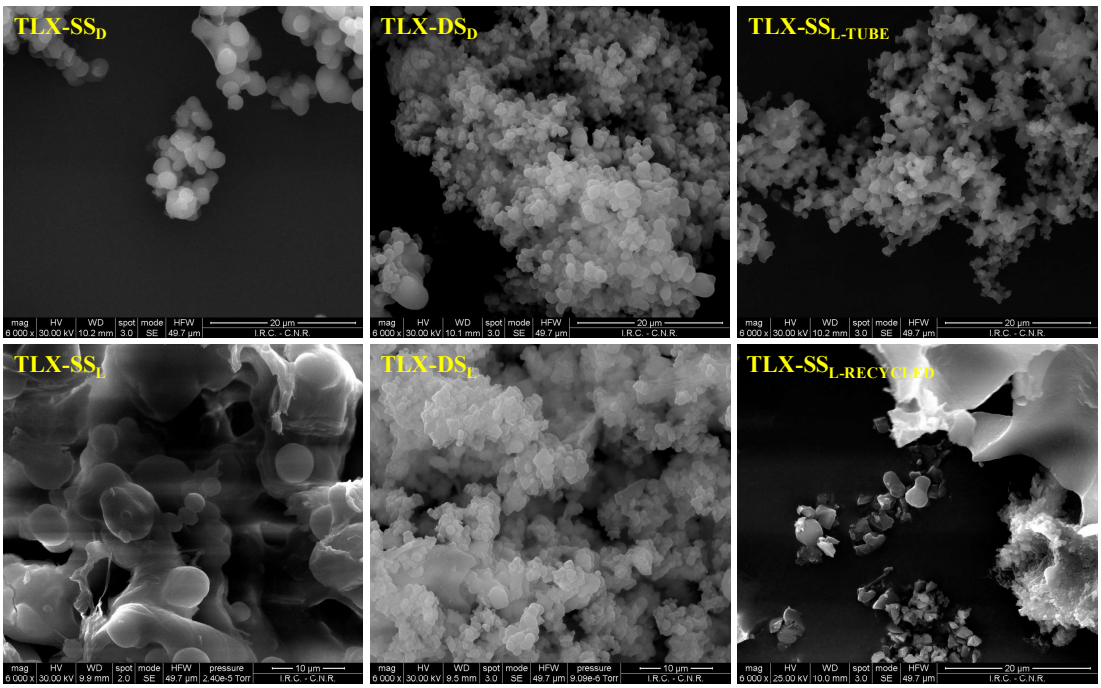


Figure 6.15: comparison of SEM images for TLX-SS$_D$, TLX-DS$_D$, TLX-SS$_{L\text{-}TUBE}$, TLX-SS$_L$, TLX-DS$_L$, TLX-SS$_{L\text{-}RECYCLED}$; mag 6000 x, 20 and 10 µm

Particular attention might be paid to the SS$_{L\text{-}RECYCLED}$ sample: from the three figures, the consumption of the matrix after several re-uses was totally evident, and it was potentially due to the continuous stresses of magnetic stirring, temperature and pH, fresh substrate concentration.

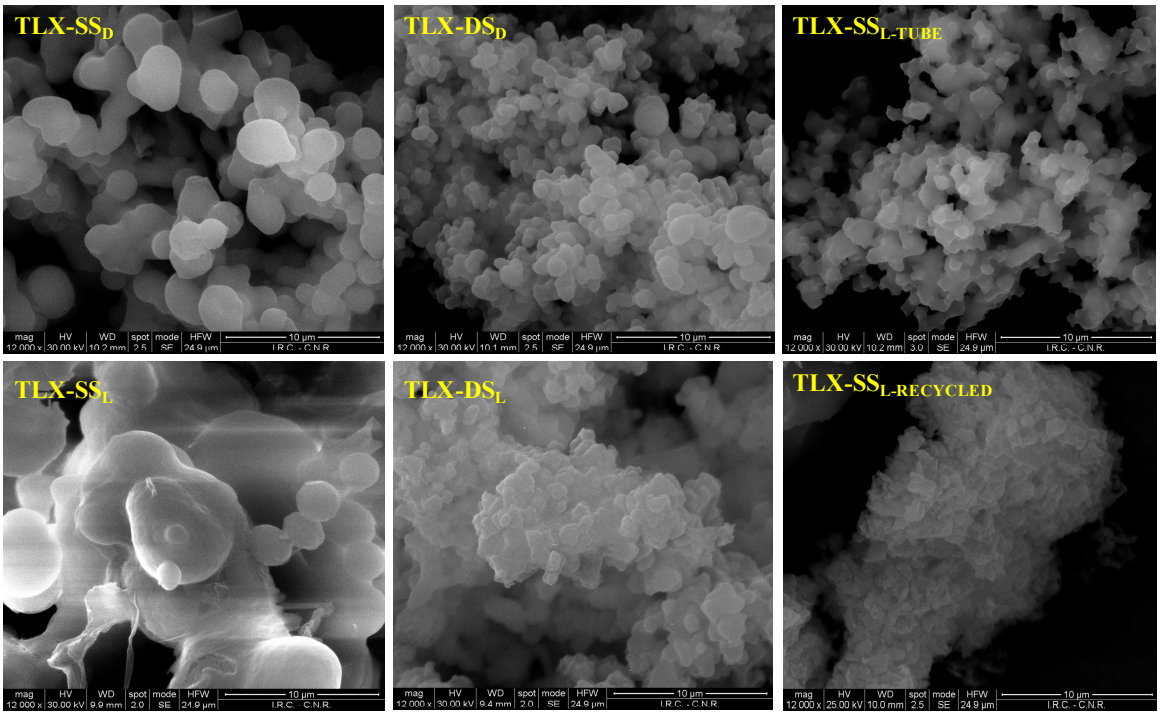


Figure 6.16: comparison of SEM images for TLX-SS$_D$, TLX-DS$_D$, TLX-SS$_{L\text{-}TUBE}$, TLX-SS$_L$, TLX-DS$_L$, TLX-SS$_{L\text{-}RECYCLED}$; mag 12000 x, 10 µm.

A study of the surface area was also done, according to the BET procedure (*Brunauer S. et al., 1938)*. Analyses were accomplished using the software Quantachrome® ASiQwin™-Automated Gas Sorption Data Acquisition and Reduction © 1994-2013, Quantachrome Instruments, version 3.01. From literature, a common expected BET value for silica sol-gel porous materials was of few hundred m^2/g, but surprisingly in this case the values obtained for the selected samples (TLX-SS$_L$, TLX-DS$_E$, TLX-DS$_L$) were surprisingly not in agreement with the expected one, but in perfect compliance with each other and also with *Yang et al.,* who made a study on surface area and porosity of silica carriers for enzymes (*Yang J. et al., 2010)*:

Enzymatic matrix	**Surface area, m^2/g**
TLX-SS$_L$	43.454
TLX-DS$_D$	24.757
TLX-DS$_L$	35.442

Table 6.4: BET surface area values for TLX-SS$_L$, TLX-DS$_D$, TLX-DS$_L$ samples.

These were the very first data collected for trapped xylanase; further investigation might be done as regards surface area and porosity studies.

6.3.3 CATALYTIC TESTS: BATCH & CONTINUOUS

6.3.3.1 STABILITY OF THE IMMOBILIZED TLX AND CATALYTIC TESTS IN BATCH REACTORS

Before every other kind of test, all prepared enzymatic matrices were studied as regards the degree of their protein leaching by testing the amount of protein released at room temperature in water.

In particular, the TLX-SS_L,TLX-SS_D and TLX-DS_L,TLX-DS_D enzymatic matrices were investigated. A given amount (11 mg) of each xylanase-containing powder was suspended in 0.5 mL of MilliQ H_2O under magnetic stirring and at room temperature for 1 h. The systems were centrifuged for 13 min at 13000 rpm at 4°C and the supernatant was essayed (*Bradford*) in triplicate for the evaluation of the released protein, and the data were collected in Table 6.5.

Enzymatic matrix	Xylanase entrapment yield (%)
TLX-SS_L	95.8
TLX-SS_D	94.7
TLX-DS_L	93.2
TLX-DS_D	96.2

Table 6.5: Xylanase entrapment yields in the studied sol-gel matrices.

Very high entrapment yields were found in all cases, demonstrating the high efficiency of the proposed sol-gel procedures.

The xylanase activity of the studied biocatalysts was then verified by evaluating the hydrolysis of Birchwood xylan and the corresponding production of xylo-oligosaccharides (XOS), using the free *T. lanuginosus* xylanase (TLX) as enzymatic system reference. Xylan depolymerisations were performed at two xylan/protein weight ratios (donor/enzyme ratio, d/e) of 3.3 and 6.6 values, on the basis of previous studies (*Lama L. et al., 2014)*. The reactions were performed at 34°C and 70°C, as reported in Materials and Methods section, being 70 °C the optimal temperature for *T. lanuginosus* xylanase activity and taking into account its wide thermostability range, acknowledged by literature (*Cesar T. and Mrša V., 1996)*.

Process	Biocatalyst	d/e ratio	Temperature (°C)	Time (h)
A	TLX-SS_D	6.6	34	24
B	TLX-SS_D	3.3	70	72
C	TLX-SS_L	6.6	34	72
D	TLX-SS_L	3.3	70	72
E	TLX-DS_D	6.6	34	24
F	TLX-DS_D	3.3	70	24
G	TL-DS_L	6.6	34	24
H	TL-DS_L	3.3	70	24
I	TLX	3.3	34	24
J	TLX	6.6	34	24
K	TLX-SS_L	3.3	34	24
L	TLX-DS_L	3.3	34	24
M	TLX-SS_D	3.3	34	24
N	TLX-DS_D	3.3	34	24

Table 6.6: Enzymatic digestion of xylan from Birchwood by TLX, TLX-SS_D, TLX-DS_D, TLX-SS_L, TLX-DS_L enzymatic matrices in various operative conditions.

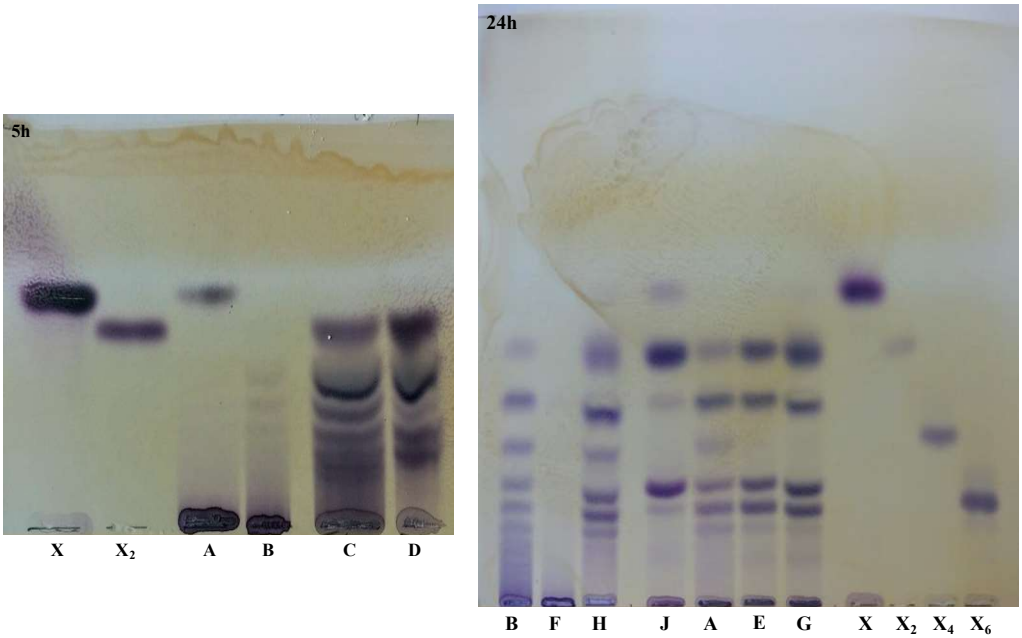


Figure 6.17: TLC analysis related to hydrolysis of Birchwood xylan catalysed by different enzymatic sol-gel matrices. Reactions are listed in Table 6.6; TLC standards: X: xylose; X_2: xylobiose; X_4: xylotetraose; X_6: xylohexaose.

In Figure 6.17 the results of a TLC qualitative monitoring of xylan digestion with TLX-SS_D,TLX-DS_D, TLX-SS_L, TLX-DS_L matrices were reported and both TLC analyses clearly indicated that the xylanase activity was in all cases preserved. However, the enzymatic

matrices obtained by lyophilization (TLX-SS_L and TLX-DS_L) showed a kinetic behaviour similar to that of the free enzyme: TLC spots corresponding to X_2 and xylooligosaccharides with up to 5-6 units were recorded. Differently, the samples dehydrated by desiccation (TLX-SS_D and TLX-DS_D) exhibited slower reaction kinetics, corresponding to the presence on TLC plates of longer xylooligosaccharides spots. The observed decrease of enzymatic activity after desiccation could be correlated to an enzyme conformational change as described by FTIR measurements above. The lyophilized biocatalyst TLX-SS_L worked better than the desiccated matrix TLX-SS_D, even after 5 h, regardless of reaction temperature. For TLX-SS_L matrix, the mixture of produced xylooligosaccharides resulted more similar to that obtained by the free TLX at all investigated times. When testing TLX-DS_L at 70°C (reaction H) a wider fraction of smaller oligosaccharides was obtained, in comparison to the case of desiccated TLX-SS_D and TLX-DS_D matrices (B and F reactions), as clearly visible in Figure 6.17. Furthermore, it was evident that the SS entrapment procedure improved the thermostability of xylanase allowing the exploitation of xylanase activity up to 24 h at 70 °C . In a previous study concerning the pure *T. lanuginosus* xylanase it was found that at 70°C more than 90% of activity was lost in 40 min (*Cesar T. and Mrša V., 1996)*.

As regards the reason why SS route seemed to be the preferred one, it has been already observed by *Birò et al., 2016,* that hydrophilic matrices may produce a beneficial effect on β-D-galactosidase from *K. lactis* due to a minor water hindrance to the catalytic site. Therefore, also in the reported case-study, this effect was probably the prevailing factor when operating at 70°C, as shown by the better performance of the SS matrices.

Aiming to exploit the unusual thermostability range of the selected xylanase as free and entrapped protein, a quantitative analysis of xylan saccharification by entrapped xylanases was performed at 34°C; enzymatic digestions were analyzed for the production of reducing xylose equivalent and reported in Figure 6.18.

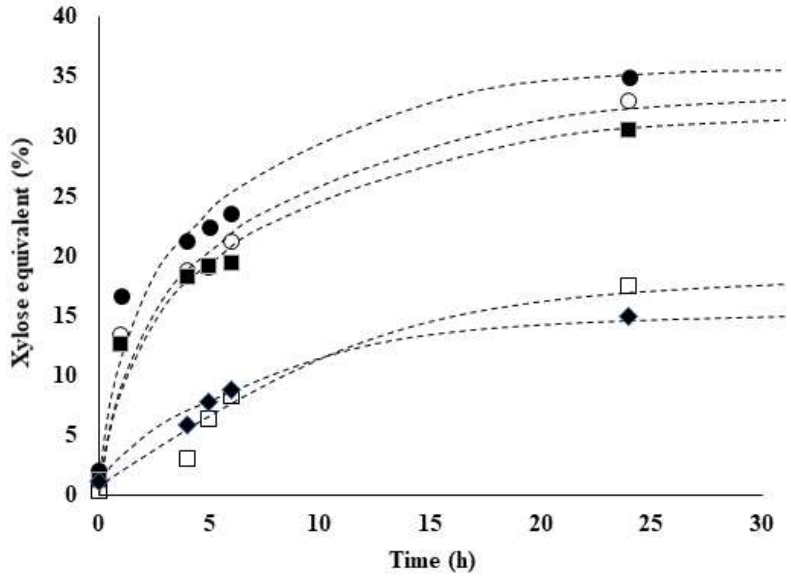


Figure 6.18: Xylose equivalent *vs* time profiles of samples during hydrolysis of Birchwood xylan. Samples: **TLX (**4.54 mg of pure protein**) (●)**, **TLX-SS$_L$ (○)**, **TLX-DS$_L$ (■), TLX-SS$_D$ (□)**, **TLX-DS$_D$ (◆)**. Each point was the average of three determinations (SD < 0.05). The percentage values of xylose equivalent were normalized for the 2.4% which corresponded to the initial value of xylose equivalent observed before the enzymatic treatment, never exceeding of 0.24 mg ml^{-1}.

According to this data, it was evident that, for the xylan hydrolysis after 24 h of reaction, the lyophilized TLX-SS$_L$ **(○)** and TLX-DS$_L$ **(■)** matrices offered the highest amount of XOS, which were evaluated as amount of produced xylose equivalent (33% and 30.6%, respectively), in agreement with the 35% value recorded with the free TLX. Differently, in the presence of the desiccated TLX-SS$_D$ and TLX-DS$_D$ biocatalysts, the conversion was significantly lower (17.5% and 14.9%, respectively).

The hydrolysis rate calculated in the first hour of reactions, when the accumulation of products increased linearly with the time, showed that TLX retained a large part of its original activity when it was entrapped in TLX-SS$_L$ (72%) and in TLX-DS$_L$ (67%), whereas reduced activities were seen for the enzymatic matrices TLX-SS$_D$ (6.6%) and TLX-DS$_D$ (7.7%), in agreement with other studies reported in literature (*Immerzeel P. et al., 2014; Edward V. A. et al., 2002; Yan X. et al., 2012)*.

The best performances exhibited by the TLX-SS$_L$ biocatalyst were in agreement with the results of FTIR analysis. For this reason, its catalytic activity was also tested in the hydrolysis of pure Xylopentaose (X_5) and compared with that of the free xylanase. The reaction was monitored by TLC, as shown in Figure 6.19.

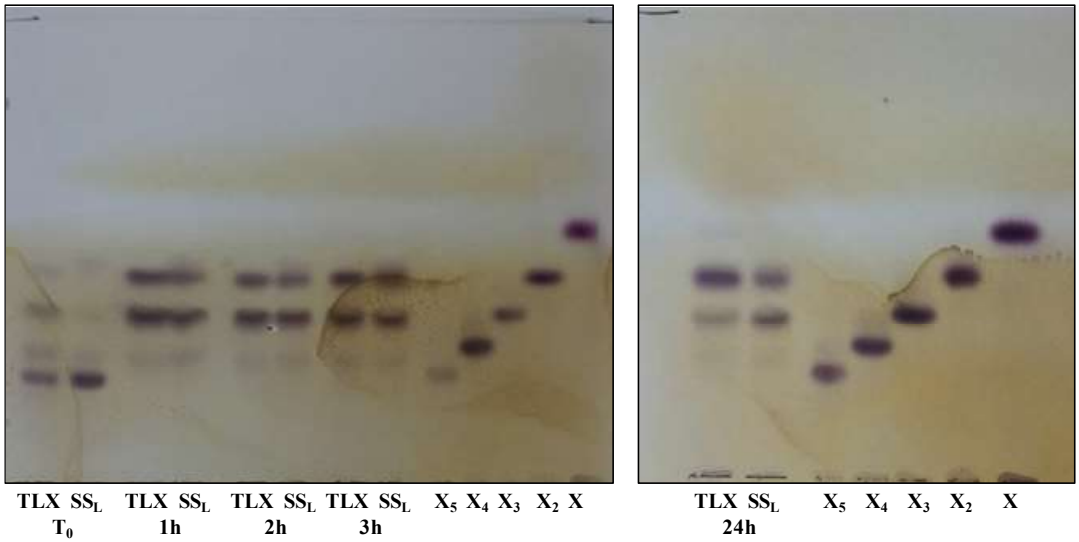


Figure 6.19: TLC results of pure X_5 hydrolysis by free (TLX) and trapped (SS_L, i.e. TLX-SS_L) xylanase at different time points.

A total conversion of X_5 in X_2 and X_3 occurred in about 1 h for both entrapped and free enzyme, showing that the entrapment procedure did affect neither the reaction rate nor the end products, even after 24h, and this was a great achievement, because in the same operative conditions, by using the immobilized enzyme, it was possible to achieve the same results as in the case of the free enzyme, apparently without any kind of loss. It is worthy to underline, anyway, that – even if very clear – this was only a qualitative examination, therefore before asserting such a strong conclusions, a quantitative countercheck had to be done.

6.3.3.2 RECYCLE EXPERIMENTS WITH ENTRAPPED XYLANASE IN BATCH SYSTEM

TLX-SS_L matrix was selected to be tested in recycle trials, to record its hydrolytic activity after various re-uses. Each cycle corresponded to 24 h of enzymatic reaction, and in particular the reaction K (Table 6.6) was chosen as test reaction. After 24 h, the TLX-SS_L was recovered by centrifugation at 13000 rpm for 20 min at 4°C, then washed with buffer phosphate, re-centrifuged and re-employed in the same conditions, with fresh reagents. The recycle was performed seven times. At the end of each 24h-recycle, the residual catalytic activity was investigated for the production of reducing sugars, as previously reported. All runs were performed in triplicate and results were reported in Figure 6.20:

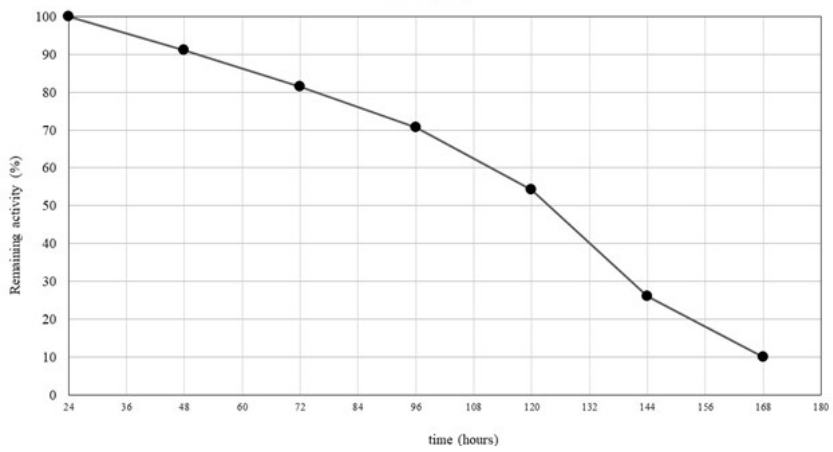


Figure 6.20: TLX-SSL recycle experiments. Each point was the average of three determinations (SD < 0.05).

In the first cycle the produced xylose equivalent was about 33 %. The biocatalyst worked well up to the fifth recycle with 54.2 % of residual activity; it decreased to 10% only after 168 h of reaction, corresponding to the seventh cycle.

As depicted in Figure 6.20, the residual hydrolytic activity of TLX-SS_L was 70.6 % after 96 hours (four cycles). The prevailing products after each 24h-cycle were analysed by TLC (data not shown) finding a similar composition of products mixture: X, X_2, X_3 and XOS with 5 or 6 units. The reaction mixture obtained after the first cycle was also investigated by HPAE-PAD chromatography, as shown in Figure 6.23A.

In this case, X_2 and X_3 resulted to be the most abundant end-products with a concentration of 53.3 % and 22 % of total sugars; xylose was recovered for the 10.4 % and traces of xylotetraose (2.2 %) were detected. Finally, starting from a 1% Birchwood xylan solution at the end of seventh cycle 14.3 mg ml^{-1} of xylose and XOS were produced. This prevalent production of X_2 was extremely interesting by considering its prebiotic properties, having a stimulatory effect on the selective growth of human intestinal *Bifidobacteria* (*Broekaert W. F. et al., 2011; Aachary A. A. and Prapulla S. G., 2011)*.

6.3.3.3 CONTINUOUS CATALYTIC TESTS

Xylanase entrapped in microreactors (TLX-CSS_L) was employed in continuous reactions for Birchwood xylan hydrolysis, as displayed in Figure 6.21.

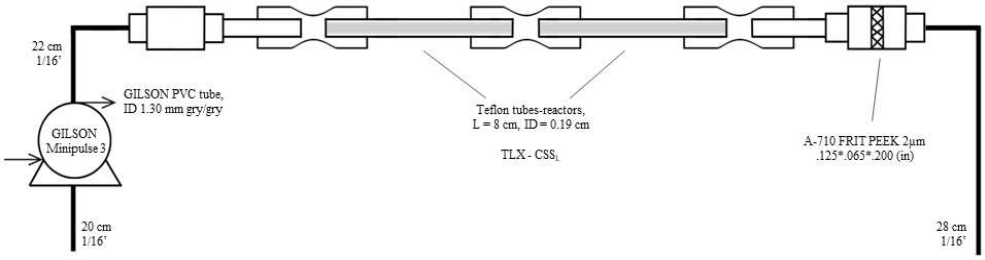


Figure 6.21: Continuous operation scheme for TLX enzymatic processes.

In this case the xylanase leakage was negligible, as shown by the reduced amount of released protein detected at room temperature in the washing solution, never exceeding 1.47%.

The xylan degradation collected data were shown in Figure 6.22: the experimental data suggested that the TLX-CSS_L was able to work with constant activity for 39 h, producing about 18% of xylose equivalent (0.0442 mg/mL of total reducing sugars).

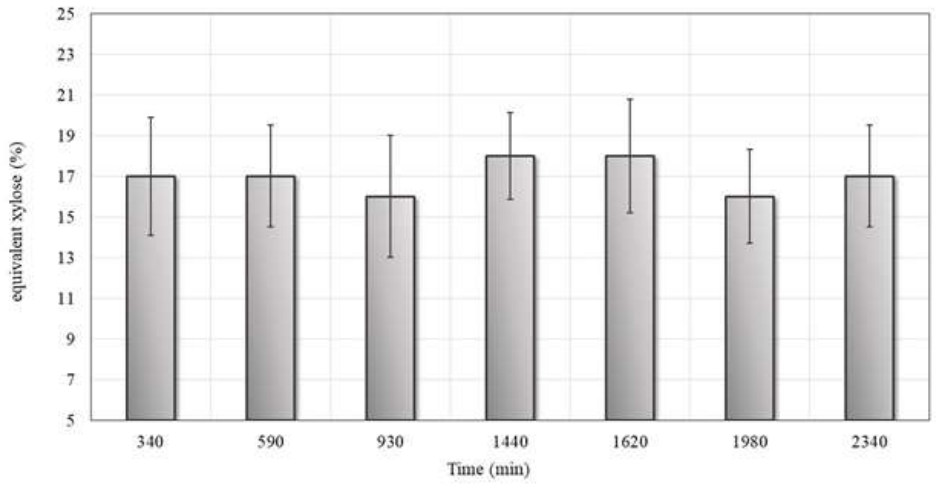


Figure 6.22: Continuous operation for hydrolysis of a 0.26 mg/ml xylan solution (pH 7, 34 °C) in Teflon tubes packed with TLX- CSS_L. Q=0.012 ml/min.
(The percentage values of xylose equivalent were normalized for the 3%, corresponded to the initial value of xylose equivalent observed before the enzymatic treatment, never exceeding of 0.078 mg/ml).

TLC analysis of collected fractions (data not shown) indicated that the reaction mixtures contained xylose, xylobiose, xylotriose, xylotetraose and traces of xylo-oligosaccharides with 5 or 6 units. A comparison among batch and continuous experiments showed a different distribution of produced XOS, being more extensive the xylan degradation in microreactors.

For the continuous experiment, Dionex analysis of the collected fraction at 24 h reported in Figure 6.23 indicated that xylose, xylobiose, xylotriose were produced for the 32, 31 and 29% of total sugars, respectively and xylo-tetraose was present as minor product. Differently, the mixture products obtained by the batch experiment with TLX-SS_L in recycle mode were made of xylobiose and xylotriose for the 75%.

It is interesting to note that, changing the reactor configuration from batch to continuous, the selectivity of the biocatalyst can be shifted towards a different product distribution. On this basis, new application fields for xylanases could be envisaged.

According to literature data, this enzymatic microreactor represents the first example of apparatus containing an entrapped enzyme for the polysaccharides continuous degradation. *Figueira et al.* described the only previous example of a continuous microreactor for the cellobiose degradation (*Figueira J. A. et al., 2013)*, but the studied system was anyway different.

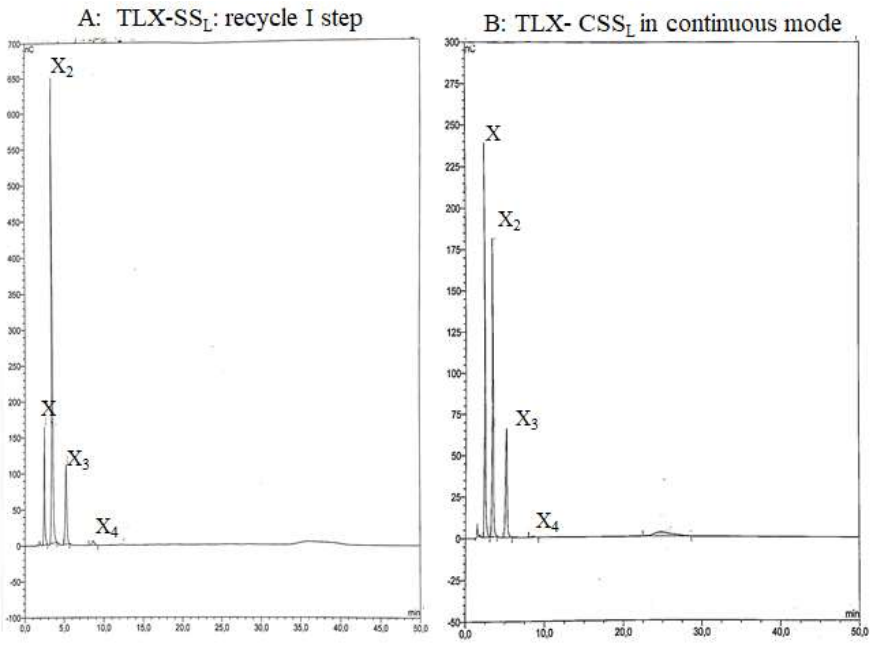


Figure 6.23: HPAE-PAD analyses of Birchwood xylan hydrolysis in batch and continuous experiments.
A: chromatographic profile of TLX-SS_L used in recycle experiment (I cycle); X, X_2, X_3, X_4 with a concentration of 0.345, 1.759, 0.717 and 0.0727 mg/ml, respectively.
B: chromatographic profile of TLX-CSS_L in microreactor for xylan hydrolysis after 1440 min; X, X_2, X_3, X_4 were produced with a concentration of 0.0140, 0.0138, 0.0129 and 0.0032 mg/ml, respectively. In addition, an unknown xylo-oligosaccharide with a retention time similar to X_5 and X_6 appeared at 24,8 min.

6.4 D-PHGAMINOTRANSFERASE

This enzyme was selected to study two different immobilization procedure: ionic bonds and entrapment in a sol-gel matrix.

6.4.1 EZIG ENZYMATIC CARRIER- BATCH CATALYTIC TESTS

As regards the ionic bonds, EziG carriers were used, thanks to the presence of their Fe^{3+} ions on the surface, which bound the His-tag of the transaminase, according to the scheme shown in Figure 6.24:

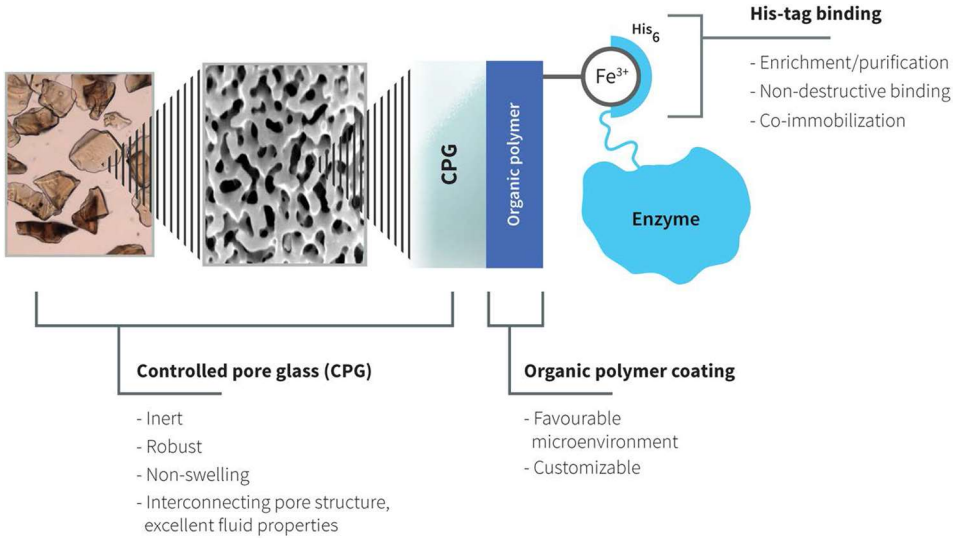


Figure 6.24: Schematic picture of the EziG enzyme carrier. The external and internal porous surface of CPG is covered with an organic polymer layer, which is derivatised to chelate Fe^{3+}, to which His-tagged enzyme can be bound.

Among the three proposed carriers, all of them with similar properties, but different as regards surface, pore diameter and bulk density, the carrier named EziG2 resulted to be the best, following the procedure reported in the Materials and Methods section. The chosen test reaction was the transformation of L-Glutamic (L-Glu) acid and Benzoylformate (BZF) into D-Phenylglycine (D-Phg) and αKetoglutarate (αKet); the formation of D-Phg was analysed via chiral HPLC and the data were collected, assuming the calibration curve of D-Phg as reference.

The first investigation was directed towards the choice of the best carrier among the three proposed, so after the binding – following the procedure reported in Materials and Methods section – a Bradford assay was carried out on the supernatant and the following values were recorded, confirmed by a SDS-PAGE (not shown):

75mM Imid	30min	60min
EziG 1	0.98	1.22
EziG 2	0.05	0.04
EziG 3	0.26	0.09

Table 6.7: Bradford assay data (mg/mL) on the supernatant after the binding of D-PhgAT on the EziG carriers. Triplicate test for each sample was carried out.

Evidently, in the same conditions for the binding, the EziG1 gave the worst response, so it was immediately discarded, focusing the attention on the other two, both of them quite promising; anyway, already from this first result, EziG2 seemed to be the best, due to the fact that in 30 min it managed to bind all the protein, while EziG3 needed more time to obtain the same target. For this reason, a new catalytic test was done, reported in Figure 6.25:

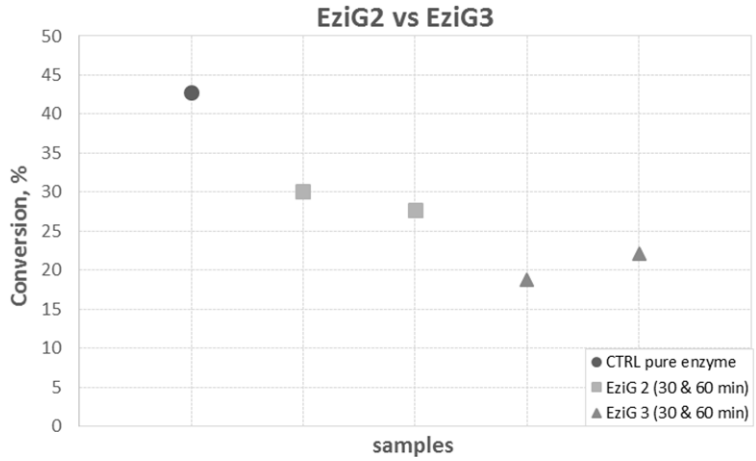


Figure 6.25: Conversion values related to the transamination of L-Glu catalysed by D-PhgAT immobilized into EziG2 and EziG3 carriers. T=37°C, overnight.

A free enzyme catalysed reaction was always run in the same conditions, as control. It was evident that the best carrier for this transaminase was EziG2, and so this carrier was selected for further analyses.

The first in-depth analysis was on the loading capacity: five different enzyme weight percentage – 10%, 20%, 30%, 40%, 50% – were explored, and so proper enzymatic solutions were loaded on the carriers. For all of them, a good degree of immobilization was recorded (average value of ≈83%), but no substantial differences were registered between the samples, as reported in Figure 6.26:

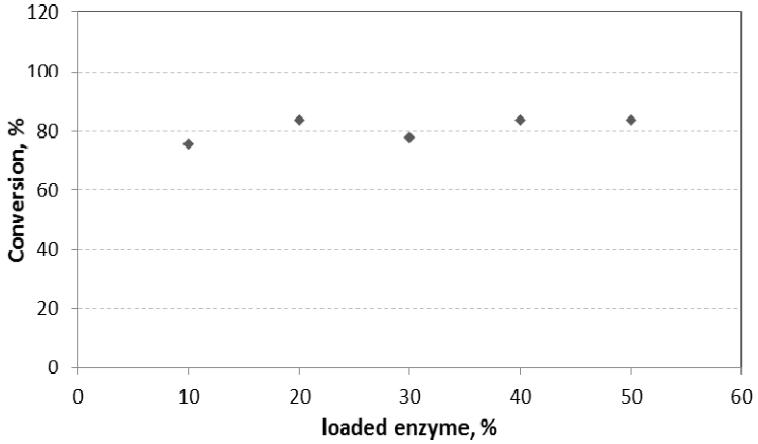


Figure 6.26: Different enzyme w/w % loaded onto the EziG2 carrier (same amount for each sample) and used in transamination reactions. T=37°C, overnight.

As clearly visible from Figure 6.26, it was not necessary to load the carrier more than 10%, because no improvements were recorded as regards the production of D-Phenylglycine. Thus, using a small amount of enzyme, it was possible to obtain a high conversion without wasting precious material.

An important factor to consider was the binding volume: for this reason, various values were assumed and tested, and the Bradford assay confirmed that the best binding volume was 200µL, as reported in Table 6.8; furthermore, at the same time also the possibility to immobilize a crude extract (lysed) enzyme was attempted, with great results.

	% binding	
Binding volume, µL	**Pure D-PhgAT**	**Crude D-PhgAT**
50	89	72
100	100	47
200	100	90
300	100	78
1000	96	-

Table 6.8: % binding of 10% w/w enzyme on EziG2 (9mg EziG & 1 mg enzyme).

This was also confirmed by a SDS-PAGE, reported in Figure 6.27: on the first line, Law Molecular Weight marker (LMW) was put, then the purified enzyme (p.e.) with the relative sample after the binding (in 200µL) (a.b. P) and after the washing (a.w. P), and the

same was done with the crude extract enzyme (c.e.); this SDS-PAGE, qualitative test, totally confirmed the quantitative values obtained by the Bradford assay.

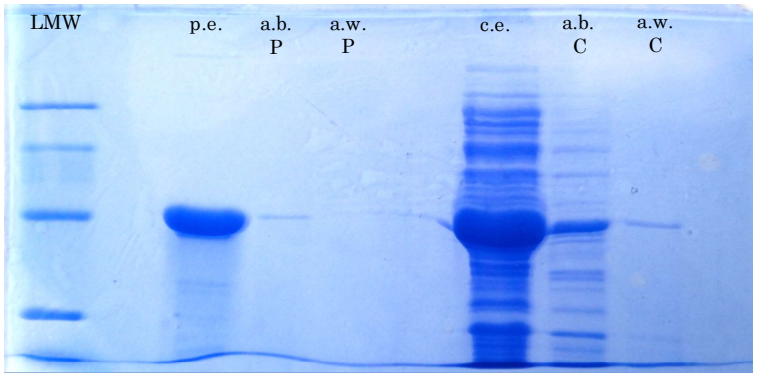


Figure 6.27: SDS-PAGE gel of purified and crude D-PhgAT solutions, before and after the binding.

Lane1: low molecular weight marker,
Lane 2: purified D-PhgAT after purification (before binding),
Lane 3: purified D-PhgAT in the supernatant solution after binding,
Lane 4: purified D-PhgAT in the supernatant solution after washing,
Lane 5: crude extract D-PhgAT (before binding),
Lane 6: crude extract D-PhgAT in the supernatant solution after binding,
Lane 7: crude extract D-PhgAT in the supernatant solution after washing.

A further significant information was obtained: once immobilized, there were no enzyme leakage, and indeed the presence of the protein after the washing was detected neither from the Bradford assay values nor from the gel in both cases of purified and crude enzyme (lanes 4 and 7).

On the basis of these results and being aware of the best operative conditions of the free transaminase (pH 9.5 and 37°C), a first recycle study was carried out, re-using up to nine times both the purified and crude (lysed) immobilized enzyme, providing to properly wash and store the matrices between each cycle, lasting 1h.

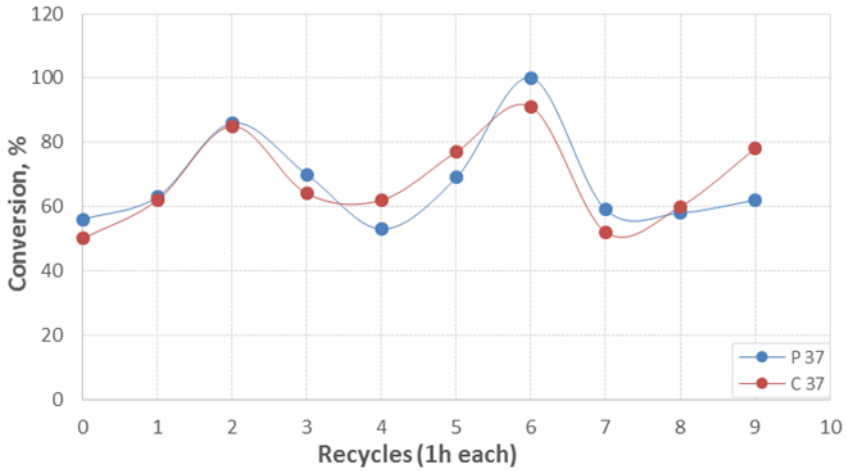


Figure 6.28: EziG2-D-PhgAT batch recycle experiments; purified (blue line) and crude (red line) immobilized enzyme was used in the catalytic reaction. Reactions were carried out at pH 9.5 and 37°C; each cycle lasted 1h. Each point was the average of three determinations.

Surprising results were obtained and reported in Figure 6.28: after nine recycle, the conversion was still very high (62% for the reaction catalyzed by the immobilized purified enzyme; 87% for the one catalyzed by the crude) and no substantial differences were recorded between the purified and the crude sample. This confirmed what previously said, that immobilisation on EziG could be performed without prior purification of the enzyme, by utilising the specificity of the affinity tag.

This gave the possibility to bypass the purification procedure, avoiding time and cost wasting and adding an improved value to the overall process into which the immobilized enzyme itself should be possibly implemented.

The following step was the investigation of wider temperature and pH ranges.

All the operative parameters were fixed, while once the temperature and once the pH was varied, respectively in the ranges 15 - 37 - 50 - 60°C for the temperature and 5 - 7 - 9.5 - 11 for the pH. Collected data were reported in the histograms below:

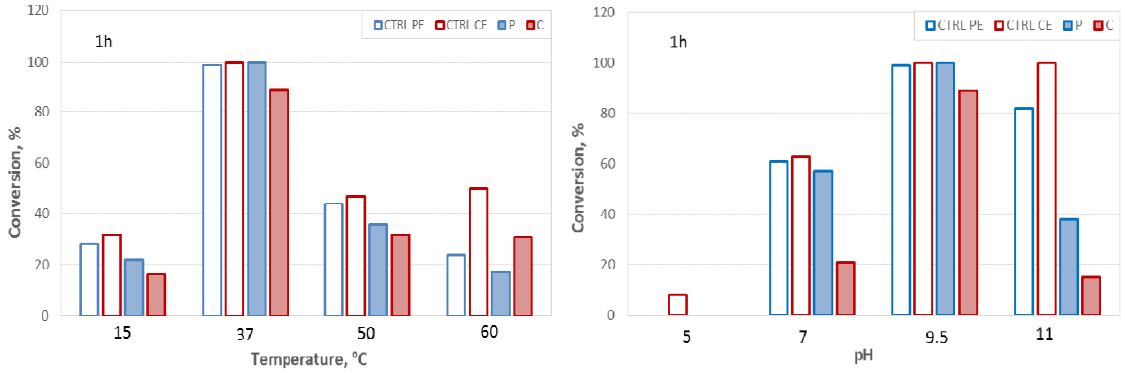


Figure 6.29: Temperature and pH studied ranges for transamination reactions catalyzed by purified and crude immobilized D-PhgAT onto EziG2 carrier; empty bars: free enzyme considered as control (blue: purified enzyme, CTRL PE; red: crude extract enzyme, CTRL CE); filled bars, immobilized enzyme (blue: purified enzyme, P; red: crude extract enzyme, C).

Focusing the attention on the temperature investigation, the immobilization did not widen the range of application, still being – among those explored – 37°C the best operative temperature also for the immobilized enzymes; the same seemed to happen with the pH examination: also in the case of the immobilized enzyme, the best value was 9.5.

These results were not expected, because generally immobilization generates an extension of the operative ranges, enlarging the values within which the immobilized enzyme could work at its best conditions, but it did not happen in this case.

The data collected suggested that the immobilized – purified or crude – enzyme could work at lower or higher values of both temperature and pH, while remaining 37°C and pH 9.5 the best operative conditions.

It is worthy to say that this investigation was at its beginning, and obviously further studies must be carried out on this matter.

By the way, the great advantage brought by the use of an immobilized enzyme in a catalyzed reaction was its recyclability, which is impossible in the case of the use of the corresponding free enzyme.

6.4.2 SOL-GEL ENZYMATIC MONOLITH - BATCH CATALYTIC TESTS

As regards the entrapment in a sol-gel matrix, it must be immediately said that this was the first time that an α-transaminase was immobilized within such a matrix, in order to develop a catalytic system that could be used also in continuous mode. Only the purified enzyme was considered for this immobilization procedure.

An initial analysis strictly related to the synthesis of the matrix was carried out, and later a focus on the operative parameters was accomplished.

Since D-PhgAT properly worked only in presence of its cofactor, the pyridoxal-5'-phosphate (PLP), the first explored parameter was the presence of PLP within the matrix, during the synthesis phase. For this reason, two matrices were synthesized, SGAT M* without PLP and SGAT α with a final concentration of PLP of 50 µM: after 1 h reaction, the best enzymatic carrier was already identified with SGAT α, being its recorded conversion value five times higher than the other one (≈25% SGAT α vs ≈5% SGAT M*). At the same time, eight recycles of both matrices were carried out, and data were compared in the Figure 6.30, taking as control the sole matrix without enzyme (to be sure that it had no intrinsic catalytic properties).

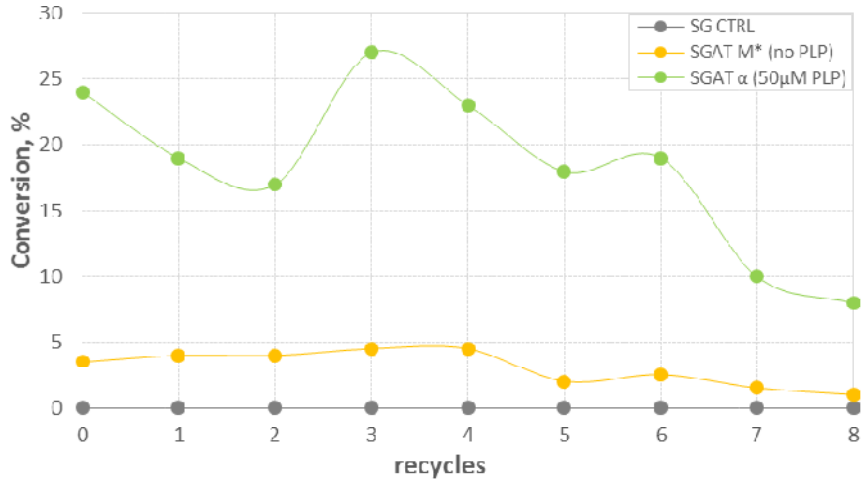


Figure 6.30: Sol-Gel AminoTransferase batch recycle experiments: SGAT M* (NO PLP, yellow line) and SGAT α (50 µM PLP, green line) immobilized enzyme was used in the catalytic transamination. Reactions were carried out at pH 9.5 and 37°C; each cycle lasted 1h. Each point was the average of three determinations. No leakage of enzyme was confirmed by a Bradford assay after each reuse.

The matrix containing PLP resulted the best for all the trials, being the conversion related to its use always higher than the other, for each recycle; only after seven re-uses it dropped down to 10%, which is a predictable result, due to the fact that after several reuses it is plausible that a trapped enzyme lose part of its catalytic activity, as a consequence of deactivation.

Being aware of this result, a new matrix was synthesized, SGAT π, with a final concentration of PLP ten times higher than the previous one, 500 µM, expecting a better conversion percentage, but this way of thinking was misleading; as a matter of facts, lower conversion values were recorded, and compared to those obtained for SGAT α in the Figure 6.31:

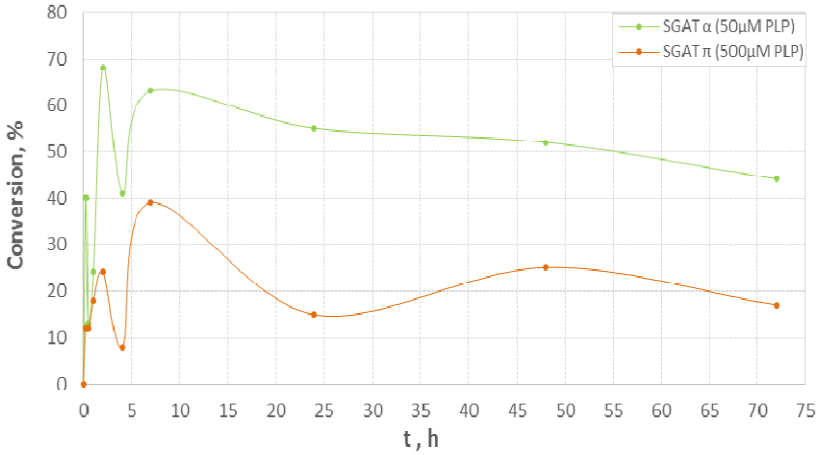


Figure 6.31: Comparison between SGAT α (50 µM PLP, green line) and SGAT π (500 µM PLP, orange line) catalyzed transaminations batch experiments. Reactions were carried out at pH 9.5 and 37°C. Each point was the average of three determinations.

Further analyses with different PLP concentrations in the synthesis of the sol-gel matrix must be realized; by now, according to the collected results, 50 µM PLP was the best PLP concentration to assume in the synthesis of such an enzymatic matrix.

The following variation was done on the buffer used in the synthesis of the matrix; according to the procedure, it was a phosphate buffer 100 mM pH 7.5, but taking into account that the best operative pH for the enzyme was 9.5, a phosphate buffer 100 mM pH 9.5 was employed, and data were collected in Figure 6.32:

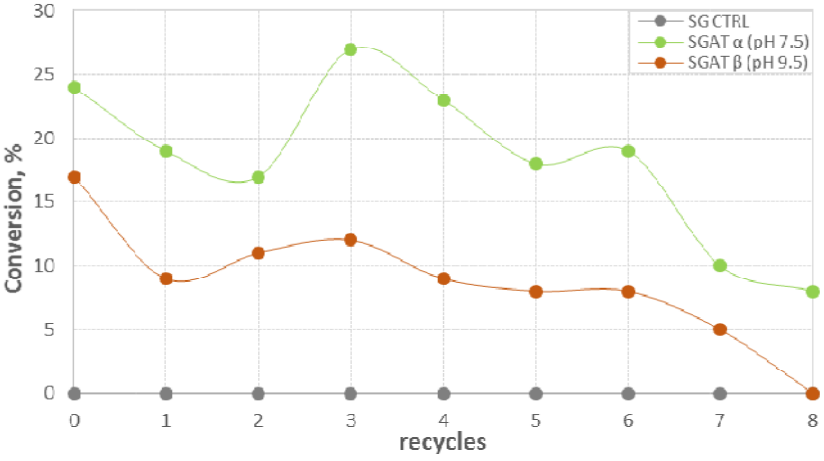


Figure 6.32: Comparison between SGAT α (pH_{matrix} 7.5, green line) and SGAT β (pH_{matrix} 9.5, brownish line) catalyzed transaminations batch recycle experiments. Reactions were carried out at pH 9.5 and 37°C; each cycle lasted 1h. Each point was the average of three determinations.

As clearly visible from Figure 6.32, the choice of a higher pH during the synthesis of the matrix was not convenient, due to a faster condensation reaction rate upon hydrolysis, which brought to a more branched and folded structure, with evidently a worse mass transfer.

Therefore, among the considered parameters, the best carrier seemed to be the SGAT α, with 50 µM

of final concentration of PLP in the matrix and pH 7.5 for the synthesis; comparing the conversion data obtained with this biocatalyst to the results recorded applying the EziG2, it was evident that the sol-gel procedure for the entrapment of this kind of enzyme had to be optimized, as plainly showed in Figure 6.33:

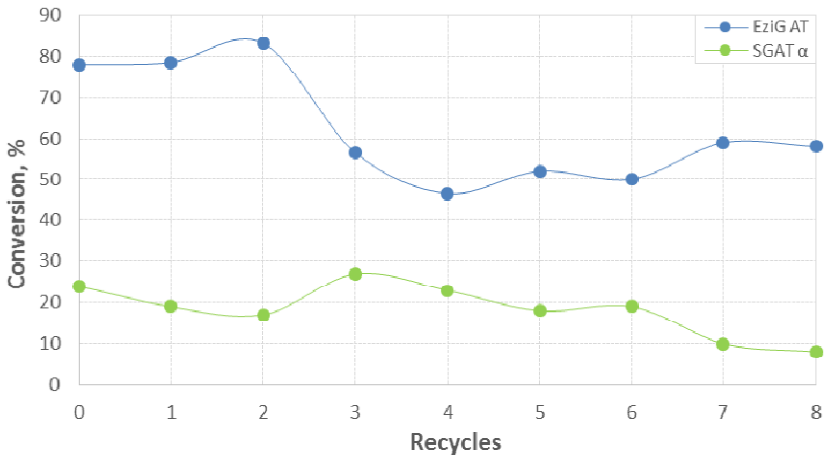


Figure 6.33: Comparison between SGAT α and EziG2AT catalyzed transaminations batch recycles. Reactions were carried out at pH 9.5 and 37°C. Each point was the average of three determinations.

Also for the sol-gel entrapped enzyme, an insight in temperature and pH was achieved, and the results were shown in the histograms below:

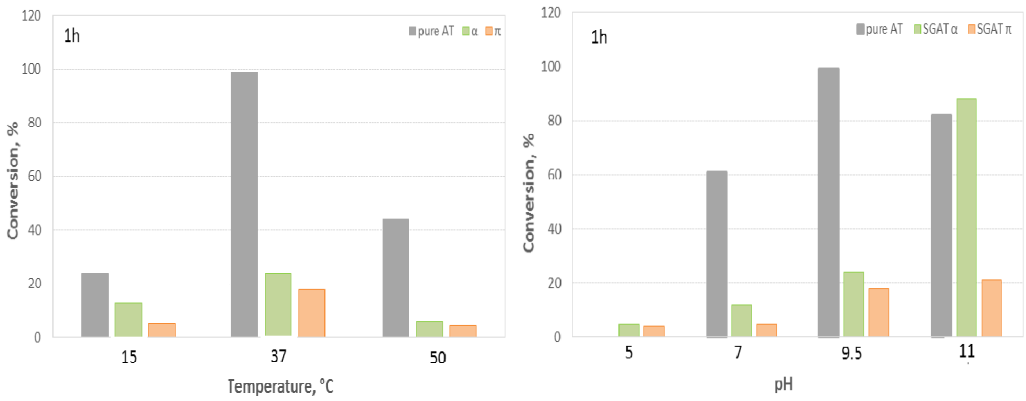


Figure 6.34: Temperature and pH studied ranges for transamination reactions catalyzed by immobilized D-PhgAT into the sol-gel matrices SGAT α (green bars) and SGAT π (orange bars), compared to the free enzyme (grey bars).

These data confirmed the fact that the sol-gel entrapment procedure had to be improved for the immobilisation of this protein, indeed the trapped enzymes showed very low conversion values compared to the free enzyme ones, apart from the case at pH 11: it seemed that the SGAT α worked better at pH 11, but from a recycle analyses (not shown) the carrier at pH 11 lost its activity after the second re-use.

Few literature articles dealt with transaminases immobilization (*Yi et al., 2007; Koszelewski et al., 2010)*, and comparing these collected results to those, it was clear that while EziG technology gave more or less comparable results, for the sol-gel entrapment method many other studies had to be realized to gain interesting data.

7.SUMMARY AND CONCLUSIONS

Without any doubt, enzyme immobilization is gaining growing interest in various industrial fields, due to the great advantages brought by their employment, especially as regards cost saving and green applications, thus taking part to an ecofriendly route which is nowadays of paramount concern.

The purpose of this doctoral research was to evaluate the possibility to provide a tool to ensure a flexible method for enzyme entrapment with an easy-to-use apparatus and without the requirement of highly specific skills.

The optimization of the operative parameters for each selected enzymatic system was carried out, as regards both the preparation of the enzymatic carrier and the reaction tests.

Indeed, this research proved the feasibility of applying the sol-gel method in order to obtain new active biocatalysts, tailoring from case to case the method on the chosen enzyme, to guarantee the formation of the best required microenvironment and to preserve as possible the structure of the wild enzyme itself, i.e. its catalytic properties.

Lipase from *Candida rugosa*, xylanase from *Thermomyces lanuginosus* and D-Phenylglycine AminoTransferase, isolated from *Pseudomonas stutzeri* ST-201, were the three selected enzymes for this study. Their immobilization was realized according to a sol-gel procedure suitably modified for every enzyme, according to their different properties.

As regards CRL and TLX, new and active biocatalysts were achieved, with a very high thermal and mechanical stability if compared to the corresponding free enzymes; in particular, for CRL a double step entrapment procedure with lyophilization as final treatment was found to be more efficient. This result can be explained observing that the employment of the three silan precursors (MTMS, TMOS and PTMS) may generate a hydrophobic microenvironment favourable to the interfacial activation, thus increasing the catalytic activity; for TLX, the most suitable carrier was obtained following a single step procedure, adopting lyophilization as the final treatment.

Structural analyses demonstrated that the presence of the trapped enzyme did not affect the morphological characteristics of the carrier, while on the other side the enzyme was conditioned by the matrix, so that its secondary structures rearranged in a new way, though they were preserved enough to recover the catalytic activity.

For both CRL and TLX, batch and continuous catalytic tests were carried out, implemeting a suitable reactor scheme for each of them and accomplishing also recycle trials, in order to check the stability and reusability of the trapped enzyme.

As regards D-PhgAT only the single step procedure in batch reactor was explored, using desiccation as the final treatment. The enzyme was active though not offering brilliant results, so further investigations must be done. D-PhgAT was also immobilized onto the EziG carrier, proposed by the EnginZyme company: this was a carrier accurately tuned for proteins which bear an His-tag, easy to be immobilized via ionic bonds thanks to the presence of the Fe^{3+} ions on the carrier surface. Therefore, D-PhgAT was a perfect candidate to be immobilised in this way, and as a matter of facts the collected results were excellent.

The obtained results offer in any case a promising basis for further investigations in the field of enzymatic microstructured reactors.

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