INSTITUTO SUPERIOR TÉCNICO
Universidade Técnica de Lisboa

High Throughput in Biocatalysis

Steroid Bioconversions

Vera Sequeira Ribeiro Guerra

Dissertação para obtenção do Grau de Mestre em

Biotecnologia

Júri

Presidente: Professora Doutora Maria Raquel Murias dos Santos Aires Barros

Orientação: Doutor Pedro Carlos de Barros Fernandes

Doutor Marco Paulo Cardoso Marques

Vogal: Doutora Carla da Conceição Caramujo Rocha de Carvalho

Novembro, 2011

Acknowledgements

I would like to thank to my supervisors at IST, Dr. Pedro Fernandes and Dr. Marco Marques, for the extended support on the elaboration of the laboratorial and written work, to my colleagues at BERG/CEBQ/IBB and to all my Professors. Also, a huge thank to all my friends and family for supporting me through this long journey.

Resumo

Os microreactors têm vindo a ser amplamente utilizados em processos biocatalíticos devido às suas propriedades intrínsecas, nomeadamente, fácil manuseamento, menos limitações à transferência de massa e necessidade de menores volumes. Além disso, beneficia a economia geral do processo pois diminui a quantidade necessária de reagentes e mão de obra. O aumento observado na transferência de massa neste tipo de sistemas pode eventualmente aumentar o rendimento total do processo.

O objectivo deste trabalho era obter uma plataforma micro-industrial para a produção continua de esteróides. A reacção modelo usada foi a oxidação do colesterol usando a enzima colesterol oxidase, produzindo 4-colesten-3-ona. Esta reacção forma como subproduto peróxido de hidrogénio, o que dificulta a aplicação industrial. De modo a aumentar o rendimento e reduzir os custos do processo, as reacções realizaram-se em microreactores. Todo o processo for experimentalmente optimizado tendo em conta ambas as condições operacionais e biocatalíticas e o processo contínuo foi montado de acordo com todos os dados experimentais reunidos.

O processo de optimização demonstrou que as enzimas deveriam ser colocadas em reactores diferentes (Packed-bed reactor para o colesterol oxidase e Fluidized-bed reactor para a catalase) e imobilizadas num suporte de PVA-PEG, que possibilita a reutilização das enzimas. Também foi provada a eficácia na redução do peróxido de hidrogénio da fase aquosa usando um dispositivo de monitorização on-line. Apesar disso, quando se usou o sistema contínuo, a produção de colestenona foi baixa, o que demonstra que novas modificações são necessárias para obter uma quantidade de produto satisfatória. As capacidades dos reactores usados eram de 2 ml e 3 ml para o PBR e FBR, respectivamente e que o caudal usado foi de 0,5 ml.min-1. Este caudal deu origem a tempos de residência de 4 minutos (PBR) e 6 minutos (FBR). A conversão máxima de colesterol foi cerca de 50% mas a degradação do peróxido foi estabilizada durante a operação, o que deu origem a um ambiente livre de peróxido de hidrogénio dentro do sistema.

Estes resultados mostram que, apesar de ser necessária a optimização do processo, foram dadas boas indicações em relação ao uso das duas enzimas dentro do mesmo sistema reaccional, acerca da eliminação do subproduto e também acerca da reutilização das enzimas, o que providenciou uma estratégia base adequada para facilitar a implementação industrial deste processo multienzimático.

Key-words: colesterol oxidase, catalase, microreactores, sistemas reaccionais contínuos, processos multienzimáticos.

Abstract

Microreactors had been widely used in biocatalytic processes due to their intrinsic characteristics, namely, easier handling, lower mass transfer limitations and smaller volume requirements. Additionally, it benefits overall process economics, lowering cost by reducing quantity of reagents and manpower. The increase observed in mass transfer in these system can eventually increase the overall process yield.

The aim of this work was to obtain a micro-industrial platform for the continuous production of steroid molecules. The chosen model system was the oxidation of cholesterol performed by cholesterol oxidase, yielding 4-cholesten-3-one. This system forms as a by-product hydrogen peroxide which hinders industrial application. To overcome this limitation and avoiding cholesterol oxidase deactivation, an extra enzymatic reaction, using as a catalyst catalase, was coupled to reduce this product. Moreover, to increase throughput and reducing overall process cost, the reactions were both performed in microreactors. The overall process was tentatively optimized concerning both operational conditions and biocatalytics and the final process was assembled according to all the experimental data gathered.

The optimization process demonstrated that the enzymes should work in separate reactors (Packed-bed reactor for cholesterol oxidase and Fluidized-bed reactor for catalase) immobilized in a PVA-PEG support, providing the reuse of the enzymes. It has also proved effective the reduction of the hydrogen peroxide from the aqueous phase using of an on-line monitoring device. Nevertheless, when using the continuous system, the cholestenone production was low, showing that further modifications are needed to achieve a satisfactory product amount. The reactor capacities were 2 ml and 3 ml for PBR and FBR, respectively and the flow used was 0.5 $ml.min^{-1}$. This flow provided a residence time of 4 minutes (PBR) and 6 minutes (FBR). The maximum cholesterol conversion obtained was near 50% but hydrogen peroxide degradation was stabilized during the operation, providing a hydrogen peroxide free environment inside the system.

These results show that despite the required process optimization, indications were given concerning the use of both enzymes within the same system, the by-product elimination and the reuse of the enzymes, which provided a suitable base line strategy in order to facilitate industrial implementation of this multienzyme process.

Key-words: cholesterol oxidase, catalase, microreactors, continuous-mode systems, multienzyme processes.

Table of Contents

Figure Index

Table Index

Abbreviations and Terms

HPLC – High Performance Liquid Chromatography

MS – Mass Spectroscopy

STR – Stirred-tank reactor

PFR – Plug-flow reactor

PBR – Packed-bed reactor

FBR – Fluidized-bed reactor

MBR – Membrane bioreactor

CSTR – Continuous stirred-tank reactor

CLEA – Cross-linked enzyme aggregates

FAD – Flavin adenine dinucleotide

PVA – Poly vinyl-alcohol

PEG – Polyethylene glycol

AOT – Sodium bis(2-ethylhexyl) sulfosiccinate

TMOS – Tetramethylorthosilicate

PTFE – Polytetrafluoroethylene

BCA – Bicinchonic acid

SDS – Sodium dodecyl sulfate

ADH – Alcohol dehydrogenase

OTR – Oxygen transfer rate

Introduction

1. Introduction

1.1 Biocatalysis

Catalysis is the acceleration of the rate of a process or reaction, brought by a catalyst. The original definition of a catalyst, that remains suitable nowadays, was stated by Ostwald in 1985, in which “a catalyst accelerates a chemical reaction without affecting the position of the equilibrium”. Catalytic reactions were used in antiquity, although it was not recognized at the time. For instance the fermentation of sugar to ethanol and the conversion of ethanol to acetic acid are catalyzed by enzymes (biocatalysis). In catalysis the reactants bind to the catalyst and then the products are released, generating the initial stage, as pictured in Figure 1.

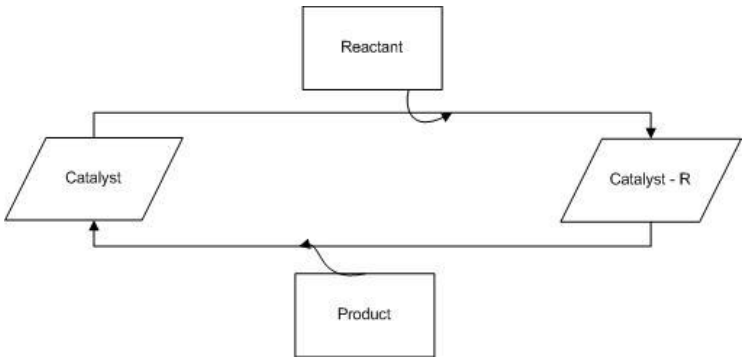


Figure 1 – Catalytic cycle (Adapted from Buchholz et al., 2005)

Biocatalysis is the use of natural occurring compounds as catalysts. Those can be proteins (enzymes) or, rarely, nucleic acids (called ribozymes) which catalyze the hydrolysis of RNA. In enzyme technology, the *ex-vivo* properties of enzymes as catalysts are explored, using them in several chemical reactions to efficiently produce a wide variety of substances (Buchholz et al., 2005).

The first patented enzymatic process was obtained in 1894 and describes a process that converts starch to sugar, using an enzyme obtained from mold, designated as Takadiastase (Takamine, 1894), which is still present in the market (Illanes, 2008, Liese et al., 2006).

Several processes were patented in which isolated enzymes were used as catalysts. Among these are penicillin acylase, used to hydrolyze penicillin and in the production of antibiotics (Liese et al., 2006, Bruggink et al., 1998), and glucose isomerase which isomerizes glucose into fructose (Foley et al., 1981, Quax et al., 1994,). One of the early multi-enzymatic industrial processes was the conversion of starch into glucose syrup by the use of α-amylase and glucoamylase, obtained from bacteria and fungus, respectively (Illanes, 2008). This base process has been improved by the use immobilization techniques or by genetic and protein engineering tools (Tucker, 1995). The application of enzymes is nowadays widely spread in industrial scale in the manufacture of a large variety of products, such as pharmaceuticals, fine

and bulk chemicals, food, hygiene and environmental technology (Alcade et al., 2006, Thomas et al., 2002). In fact, enzyme applications in organic synthesis represent one of the most challenging areas for enzyme technology development, especially due to the efforts to develop green industrial procedures and products (Illanes, 2008, Woodley, 2008, Wohlgemuth, 2010).

After the isolation of pepsin by classic crystallization experiments (Northrop, 1930), many enzymes were purified and, therefore, available for research (Liese et al., 2006).
According to EZYME database, over 4500 enzymes are presently catalogued (http://www.expasy.org/enzyme, accessed on 30 September 2011). In data from 1994, information relevant to 3546 enzymes was gathered (Bairoch, 1994). Several hundreds of enzymes can nowadays be obtained commercially. The majority of commercialized enzymes has a hydrolytic action and is applied mainly in detergent and dairy industries. Carbohydrases are used in starch, textile and baking industries and represent the second largest group (Liese et al., 2006, Kirk et al., 2002).

Several studies have been done with the goal to improve the role of enzymes in industrial processes and, nowadays, the relevance of enzymes in the industrial market is well established. For instance: fuel ethanol enzymes industry has an average annual growth rate of 15%-20%; China's enzyme industry had a total sales value up to $0.15 billion in 2009 while in 2008 had 700.000$; in India the industrial enzymes market is expected to reach $163 million by the year of 2015 (Comyns, 2010, 2011). This current expansion can be attributed to the progress in molecular biology, high-throughput screening, advanced instrumentation and engineering. As a result, the field of industrial catalysis continues to grow and several literature surveys report the introduction of enzymes into a specific chemical process, but only a few are implemented and have commercial value. Some explanations to that fact are related to the price of the enzyme, price of the scale-up operation or the complexity of the process (Tufvesson et al., 2011).

1.1.1 Novel approaches for biocatalyst selection

The use of enzymes in unnatural conditions in combination with the high specificity that enzymes reveal has been proved useful in the synthesis of compounds that will hardly be obtained with high purity levels if a strictly chemical process was used. The *in silico* directed evolution of specific enzyme libraries has been widely explored (Damborsky et al., 2009, Wedge et al., 2009). Reviews have been recently published that cover the use of genetic algorithms to verify the importance and the most feasible method to obtain the enzymes or the use of programs to design enzymes *de novo* (Golynskiy et al., 2010), like DEZYMER and ORBIT (Fischer et al., 2009). The use of laboratory techniques has been also explored. For instance, only for starch and α-glucan acting enzymes, several improvements are described using directed evolution techniques (error-prone PCR, DNA shuffling and saturated mutagenesis) and different screening methods that improve the success of the identification of the most suitable mutant. These improvements are summarized in another recent review (Kelly et al., 2009). These methods play an important role on the improvement of the stability of the enzymes in the

industrial processes in which they are inserted. The costs related to the maintenance of the enzyme stability without losing the activity and selectivity are often a drawback in the scale up process and have been overcome with the aid of such techniques (Kelly, et al., 2009, Bershtein et al., 2008, Cherry et al., 2003).

For directed evolution to be successful and efficient the improvement of screening processes is needed. These processes are relevant for the assessment of efficient ways to find new or specific enzymes (in libraries), preferably following a high throughput approach. The tools developed throughout the years are mainly based on library sizes, improving the chances of success in finding the desired properties in a biocatalyst. Some methods described include cultures on microtitre plates or microfluidic chips in smaller libraries and phage display, cell surface display or plasmid display in larger libraries (Dalby, 2007). Others imply analytical methods such as High Performance Liquid Chromatography (HPLC), Mass Spectroscopy (MS), capillary electrophoresis or assays based on fluorogenic and chromogenic substrates (Wahler et al., 2001). Some specific and recent examples of screening methods for biocatalysts are for instance applied in hydroxynitrile lyases, which are based on the detection of the hydrocyanic acid released when the catalyst is attached to the substrate (Rumbold et al., 2007). Some screening methods for lipases are described in Sandoval et al., 2007, who reports three different methods to measure synthetic activity of lipases in vitro and in vivo, directly from culture broth. In a more recent study, a specific fluorescent indicator is incorporated in reaction media, which will screen for hydrolases with high activity and enantioselectivity (Wang et al., 2009).

1.1.2 Biocatalysis: some trends and applications

Another interesting drift in pharmaceutical industry development is the so called “Design for the Environment” program, which implies considerations about the environment, health and safety during the development and design of several processes. These inherent considerations will fulfill the environmental regulations, which are a major step in industrial production nowadays. Some examples of “green practices” concern the minimization of waste and hazardous reagents and intermediates, in the case of the usage of organic solvents they should be totally recovered for reuse and expend the less amount of energy possible (US EPA, August 2002). Several studies have demonstrated that is possible to use wastes as starting materials or in the downstream processing, having high yields of conversion or recovery of the final product (Azevedo et al., 2009, Marques et al., 2010a). Another possibility is the use of green solvents as an alternative to organic solvents (in reactions with hydrophobic compounds). The most used green systems with green solvents are ionic liquids, supercritical fluids and cloud point and aqueous two phase systems. Recent publications have stated the relevance of these systems in catalysis, showing that in some cases an increase of the transformation yield was obtained (Pfruender et al., 2006, Wang et al., 2004). Catalysis itself is known to be a green system since

it achieves the goals to attain environmental and economic benefit, fulfilling the 12 principles of green chemistry (Anastas et al., 2003).

In Table 1, some characteristics of the use of biocatalytic systems are resumed. This information was adapted from Rozzell (1999) and Fernandes (2010a).

Studies concerning biocatalysis have been carried out using isolated enzymes with different purification grades or whole cells with given catalytic activities. In both cases and, in order to maximize the efficiency of the process, a combination of biocatalytic transformations, either separated in time and space or in one-pot or cascade is gaining scientific relevance. This raises new challenges in process development such as: combining the conditions for the all the enzymes in the system, verifying the possible interactions between them and the effect of each catalyst on the behavior and performance of the remaining catalysts. These parameters should be synchronized in order to emphasize the benefits of the multiple operation (Garcia-Junceda, 2008).

Table 1 – Characteristics of biocatalytic processes (Adapted from Rozzell, 1999 and Fernandes, 2010a)

Feature	Examples	Ref
Substrate Selectivity	Production of amino-acids by transaminase using L-glutamic acid or L-aspartic acid as amino group donor	Rozzell, 1985
Stereochemical specificity	Production of L-aspartic acid by aspartase adding amonnia to fumaric acid	Yukawa et al., 1982
Region selectivity	Production of 11-α-hydroxyprogesterone by *Aspergillus niger* using progesterone as substrate	Fouad et al., 2009
Functional Group	Chemo-selective hydrolysis of a nitrile by nitrilase in the presence of an ester or amide	Bengis-Garber et al., 1989
Mild conditions	Production of acrylamide by a nitrile hydratase with 100% conversion, at 10ºC	Watanabe, 1987
Environmental issues	Enzymes are biodegradable and generate few and less toxic wastes in comparison to chemical processes	Woodley, 2008 Rozzel, 1999
Efficiency	Immobilization of whole cells or of the isolated enzymes with high stabilization and activity, providing cost effectiveness processes like L-aspartic acid production by aspartate ammonia lyase.	Tosa et al., 1974

An example of the application of these processes is in redox biocatalysis, in which, with the proper combination of enzymes, a fast recovery of the co-factor is achieved and therefore it can be used continuously (Figure 2). Other valid example is in the steroid bioconversions and bioelectrocatalysis, where there is the coupling of oxidases with peroxidases. The oxidase-substrate reaction yields the oxidized substrate and the inactive reduced enzyme. The enzyme turns active by electron transfer, producing hydrogen peroxide, generating an electrochemical signal that can be detected by an electrode (if the aim is to act as a biosensor, for instance). The peroxidase will then dissociate the hydrogen peroxide in oxygen and water molecules. Enzymes such as cholesterol oxidase and glucose oxidase are being used in this kind of multienzyme–catalyzed procedure (Burton et al, 2008).

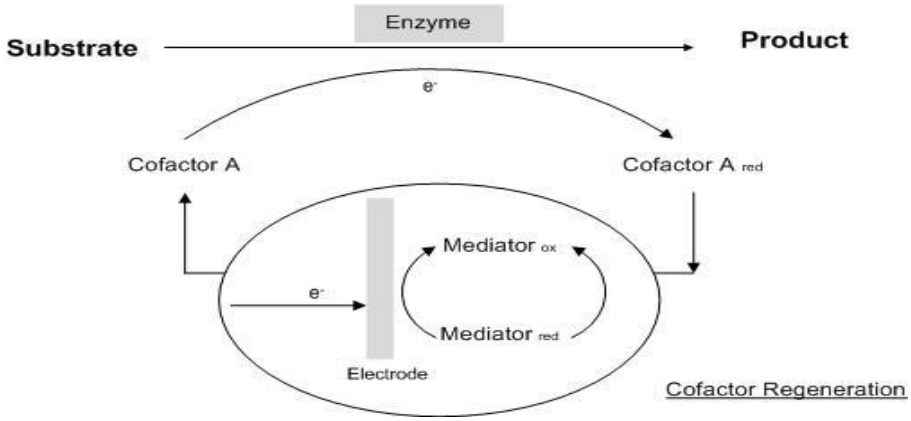


Figure 2 – Regeneration of the cofactor using an copolymerized mediator at an electrode surface as electron supply (Adapted from Burton et al., 2008)

In order to achieve a better description of the behavior of a given biocatalytic process, the representation of the reaction mechanism through mathematical models can be a key step in process development, since it gives the opportunity to evaluate the process feasibility and to explore the advantages and limitations of the overall process. In multienzymatic processes several considerations concerning the reaction itself and the process in general have to be carefully weighted (Table 2).

The support of mathematic modeling and computational tools is very helpful to process development and optimization since it leads to a better exploitation of the material and human resources. Such pattern further contributes to the possibility of replacing chemically catalyzed processes by enzyme catalyzed processes in a near future (Santacoloma et al., 2011)

Table 2 – Considerations to follow in the development of multienzymatic modeling (Adapted from Santacoloma et al., 2011)

Considerations		Description
Reaction	Involved compounds	Physical Properties of: Enzymes Substrates Cofactors Mediators
	Structure	Description of all possible routes to the desired product
	Interaction Matrix	Defining the relationship between each compound and the specific enzyme
	Evaluation	Analysis of the feasibility of the previous considerations and rearrangement of the model if needed
Process	Mode of operation	Batch Continuous Single-pot Multi-pot
	Reactor[1]	STR PFR PBR FBR MBR CSTR
	Compound Characteristics	Purity of the compounds Enzyme formulation (whole cell, immobilized, isolated)
	Control	Regulatory control (pH, temperature, Dissolved Oxygen) Supervisory control (concentrations of the compounds)
	Downstream	Defining the required purity of the product (e.g., enzymes activity and stability, yield)

[1]STR: Stirred-Tank Reactor; PFR: Plug-Flow Reactor; PBR: Packed-Bed Reactor; FBR: Fluidized-Bed Reactor; MBR: Membrane Bioreactor; CSTR: Continuous Stirred-Tank Reactor

1.2 Bioreactors

Bioreactors are vessels in which the bioconversion process takes place, designed to control biochemical and physical parameters, e.g. pH, temperature, mass transfer and reactant concentrations. These reactors can be tuned in configuration and modes of operation to maximize bioconversion yield in terms of product output while minimizing production costs. The main purpose of the use of bioreactors is to guarantee, maintain and control the appropriate environmental conditions during the reaction course.

Bioreactors may be classified concerning different characteristics. According to the mode of operation we can find batch, fed-batch (or semi-batch or semi-continuous) and continuous operation (Dunn, et al., 2003). From this base division, new sub-divisions have been suggested to a better illustration a determined operation mode, for instance stirred-tank reactors (STR), packed-bed reactors (PBR) and fluidized bed reactors (FBR). STR can operate in both batch and continuous mode, whilst PBR and FBR preferably are inserted in a continuous mode operation (Julien et al., 2007).

In stirred-tank batch reactors a perfect mixture of all the components in the media is often obtained. The associated simplicity of these reactors gives the advantage of a more controllable environment during the reaction course. However, when performing a batch operation, a pronounced batch-to-variation may occur, which hamper the scale-up processes. Other arising problem in STR may be the attrition resulting from the stirring. This problem may be overcome by the use of a PBR, in which the catalyst is immobilized in particles that are held inside the reactor and do not move with the flow, therefore, the flow velocities in the channels can be high in order to eliminate external mass transfer limitation. FBR are used when a fluid-like behavior is needed inside the reactor. The substrate is passed upward through the immobilized enzyme bed at high enough velocity to lift the particles from the fixed bed state (Coney, 1983, Eigenberger, 1992, Baron et al., 1996). In Figure 3, schematic examples of these three different reactors are shown and in Table 3 more characteristics of each bioreactor are given.

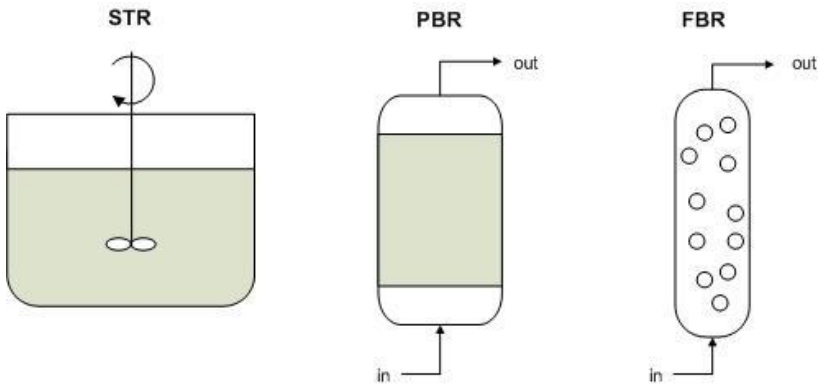


Figure 3 – Schematic representation of the three bioreactors**.** STR: Stirred-Tank Reactor; PBR: Packed-Bed Reactor; FBR: Fluidized-Bed Reactor.

Table 3 - Comparison of some characteristics of three different types of bioreactors (Adapted from Fernandes, 2010a, Santacoloma, et al., 2011)

Reactor / Characteristic	STR	PBR	FBR
Control	Easy	Difficult	Better than PBR
Multiphase	Yes	No	No
Immobilized enzymes	Yes	Yes	Yes
Enzyme Damage	Possible inactivation	Low	Possible inactivation
Volumetric Productivity	Low	High	High
Residence Time	-	High	Low
Shear Stress	Very High	Low	High
Conversion Rate	Low	High	Lower than PBR
Enzyme Recycle	Difficult	Yes	Yes
Flow	-	Low	High

Several studies using these bioreactors have been published concerning soluble enzymes, immobilized enzymes and whole cells as catalysts. The higher productivities obtained when the enzymes are in an immobilization support are a recurrent result. In Ates et al., 2006, a comparison study was made using tyrosinase in soluble and immobilized forms and a clear higher productivity was obtained when the latter was used. Also in this study, when comparing stirred-tank batch reactor and a PBR in continuous operation mode, the latter led to higher conversion rates. Similar results were obtained when using whole cells as biocatalysts (Kim et al., 2002, Gomez et al., 2003), once again emphasizing the importance of the immobilization process and the differences between batch and continuous operations. A fluidized-bed reactor was used and compared to stirred-tank reactor, both in continuous mode, for the degradation of phenol using *Pseudomonas putida* in solution (STR) and immobilized in calcium alginate beads (FBR). The results suggested that FBR displayed a better stability, a more accurate control and also the ability to work when low residence times must be taken into account (Gonzales et al., 2001). The degradation of phenol and hydrogen peroxide using isolated enzymes in the soluble form and immobilized in glass supports was also studied. The trials were performed in a continuous mode system and the enzymes were trapped inside a FBR. This report demonstrated the viability of the usage of FBR in continuous systems and also the importance of a model construction to predict the conversion results (Gomez et al., 2007).

In order to overcome the relatively slow implementation of biocatalysis on industrial scale, wide research efforts were made towards the development of a miniaturized scale of processing

techniques, with low volume reactors at the core, often termed microreactors (Fernandes, 2010a). The definition of microreactors is rather broad (Urban et al., 2006, Miyazaki et al., 2006, Yoon et al., 2005), but for simplification, in the present work the term microreactors will abridge vessels having a volume under 100 ml, thus providing a faster transfer of results (and high level of parallelization required to the several stages of the process), lower costs and lower manpower. Some examples used are microwell plates (useful for screening) and microfluidic devices, like microchannel reactors, minireactors (vessels with similar behavior to bench scale fermentation systems but with reduced volumes, e.g. 10 ml). Both miniaturized systems have obtained successful results in steroid biotransformation research (with whole cells or isolated enzymes) illustrating its potential and feasibility in the screening for the suitable organic solvent and in biotransformation itself (Marques et al., 2009, Marques et al., 2010a).

1.2.1 Reaction Media

A limitation that has also being overcome is the imperative use of an aqueous media when working with enzymes. Water, while playing a crucial role in the stabilization of the enzymes, is not the adequate environment to sparingly water-soluble substrates, which can limit the productivity and the downstream of the process. Non-aqueous or non-conventional reaction media such as organic solvents, ionic liquids and supercritical fluids are often used and studied, creating multiphase reaction systems (Krishna, 2002). In the majority of cases, a biphasic system is formed, composed by an aqueous phase (where the biocatalyst is incorporated) and an organic solvent as second phase. In Table 4 the advantages of using an aqueous/apolar phase in enzymatic catalysis is shown. However, as stated before, organic solvents do not correspond to the assumptions of green processes although in some systems this can be overcome by the use of a recycling system.

Table 4 – Advantages related to the use of non-conventional media in biocatalysis (Adapted from Léon et al., 1998, Purich, 2010)

Advantages
Enhanced solubility of apolar substrates and co-factors
Production of sparingly water-soluble compounds
Suppression of side-reactions
Non-dissociation of water-trapped solutes even when enzyme exhibits low affinity for its substrate
Lack of physicochemical meaning of pH in organic solvents (enzymes retain the pH of the last aqueous solution that they were exposed to)
Maintenance of a low concentration of toxic compounds in the aqueous phase
In situ product recovery

1.2.2 Immobilization

Another step forward in biocatalysis is the physical confinement of the enzyme (or of whole cells) while retaining catalytic activity, thus allowing its repeated or continuous use (Chibata, 1978) – immobilization. Several reasons are evoked that sustain immobilization, the most relevant are: allowance for high enzyme content and therefore high volumetric productivity, increased control of the reaction, easier recuperation of the used enzyme, reduced contamination of the final product, minimization of substrate inhibition, prevention from denaturation and possibility of reuse, which makes this procedure economically feasible. The major drawbacks are the mass transfer limitations imposed by the immobilization support and the possibility of activity loss during the immobilization (Purich, 2010, Fernandes, 2010b). Thus, enzyme immobilization enhances the stability and hence applicability of biomolecules as reusable and robust biocatalysts.

The techniques used to immobilize vary according enzyme characteristics and requirements. The major techniques include (Figure 4):

- Enzyme aggregation
- Entrapment/encapsulation in a support
- Binding to a carrier (ionic, hydrophobic and covalent)

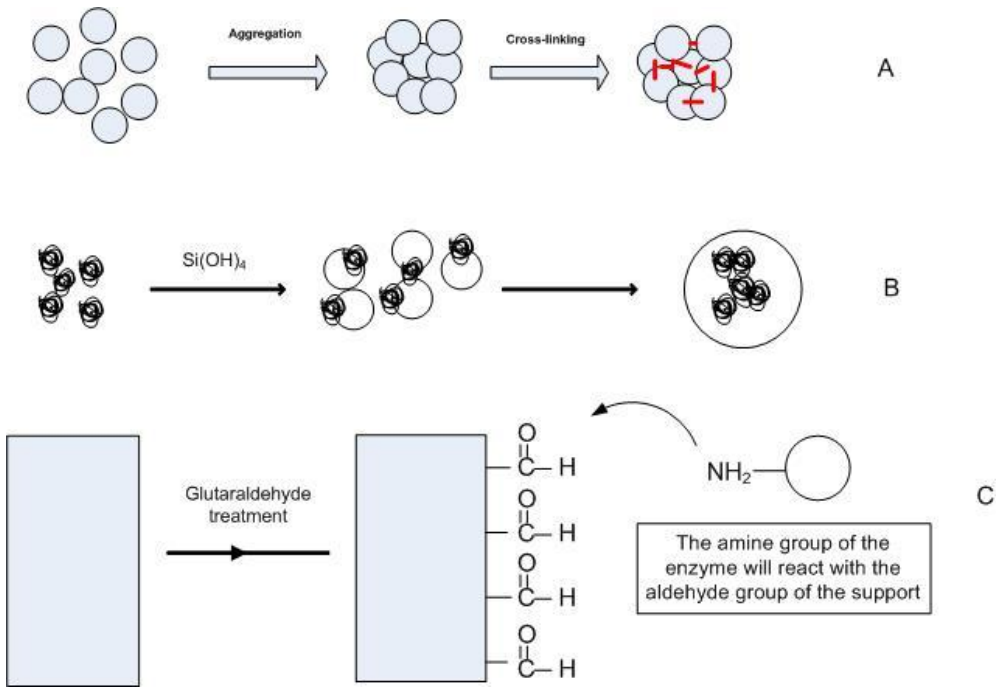


Figure 4 – Schematic exemplification of the different immobilization techniques. A: enzyme aggregation and cross-linking; B: entrapment in a silica support; C: glutaraldehyde treatment promoting linkage between the amine groups and the support (Adapted from Matijosyte et al., 2009, Betancor et al., 2008)

The induction of aggregates formation has been successfully explored as alternative to the use of an expensive support. Some examples of enzymes immobilized as CLEAs (cross-linked enzyme aggregates) are penicillin acylase, lipases and nitrilases. The use of this technique is restricted to enzymes that are able to resist to chemical cross-linking and the aggregates formed are size-limited, in order not to raise extended mass-transfer resistance (Matijosyte et al., 2009, Pchelintsev et al., 2008).

In the entrapment/encapsulation technique, the enzyme is contained within a structure, that can be composed by an organic polymer net (e.g. gelatin) or inorganic matrices (e.g. silica), a membrane (e.g. hollow fibers) or a microcapsule (e.g. reversed micelles). A widely used support is silica, which is a stable matrix, has a specific surface area and controllable pore size (Mei, et al., 2000). Immobilization in lipossomes is also considered, in this technique, dehydration and rehydration are performed and the resulting vesicule becomes larger and able to capture solute molecules (Betancor et al., 2008, Chaize et al., 2004).

Enzymes binds to a carrier through covalent, ionic or physical (van de Waals and hydrophobic) interactions. While physical interactions are the weakest, allowing a relatively easy leakage of the enzyme from the support, covalent interaction are the strongest, induced by chemical treatment with amide bond formation substances, alkylation or Ugi reactions. One example is the use of glutaraldehyde, which will promote the linkage between free amino groups of the support and the enzyme (Betancor et al., 2006, Fernandes, 2010b).

With a rational design of an immobilization process, a biocatalyst with an improved performance may be obtained, when compared to a free biocatalyst. The different immobilization formulations may theoretically be used in different reactor types, although with some limitations. Given the scope of the present work, the remaining bibliographic review will be focused in the immobilization processes used in microreactors described in literature. Some microreactors have been developed in which the immobilized particles fill the reactor or the enzyme is immobilized within the reactor itself. In the first case the beads or monolith layers are located inside the reaction chamber and then the product is recovered and the enzyme continuously reused. Some in examples of this method are, for instance, the co-immobilization of glucose oxidase and horseradish-peroxidase in a nonporous glass support (Bankar et al., 2011), package of glucose isomerase for high-fructose corn syrup production (Mosier et al., 2009) or immobilization of lipases for the interesterification of non-traditional oils (Usmani et al., 2010).

As examples of monolith reactors there are the ones prepared by molding a polymer monolith inside microchannels (Sakai-Kato et al., 2003) or by filling a silica monolith with an enzyme and entrapping it within the microchannel (Kawakami et al., 2005). A common method of immobilization uses biotinylated compounds which are physically attached to the reactor surface (Holden et al., 2004) while other methods involve the coating of the reactor surface with glutharaldehyde, aminopropylsilane/ methylsilane or similar compounds (Miyazaki et al., 2003, Kaneno et al., 2003).

As stated before, miniaturized devices are a powerful tool to speed up the rate of process development in a cost effective manner. While it mimics a biological environment (providing high surface-to-volume ratio and high heat and mass transfer) it is also easier to maintain in comparison with the conventional reactors. Further investigation on microfluidics could provide novel mechanisms and better understanding of fluidics in microchannels might enable new reaction pathways. One of the strongest advantages offered by microreactors has commercial purposes. Once optimized, a microsystem can be introduced into an industrial-scale plant, this occurs due to the high parallelization level of these devices. Parallel scale-out enables extension of reaction conditions of a single reactor, eliminates scale-up problems, provides high throughput operation of different reagents at a single operation and serves as an excellent tool for combinatorial processes. Even though, some problems may arise, for example in control and monitoring of the fluid and reaction conditions (Amador et al., 2003).

1.2.3 Scale-up

All scale-up procedures share a common pattern of development that encompasses the detailed characterization of the process and timely identification of process parameters that affect product yield, quality and consistency. Specifically to bioconversion processes, as the scale increases, more emphasis is required on chemical and process engineering considerations (Marques et al., 2010b).

Several issues arise when an economic analysis is performed to a bioconversion process. One factor is the difficult evaluation of the costs of the whole process (for instance the cost of the biocatalyst) and the costs of development of the process (Tufvesson et al., 2011), which may contribute to discard (in error) a process that could be economically viable. Other factor concerns the bottlenecks associated to the scale up procedure. Table 5 lists some items to considerate when the development and implementation of a large scale process is on sight, which can be helpful to make rational decisions (Tufvesson et al., 2010). All these features tend to lower the overall yield of the process and to difficult the success of the scale-up. Regardless of the relevance of the scale-up in biotechnological processes, there is no straightforward and uniform strategy to embark this issue and, consequently, the criteria are elaborated according to the individual characteristics of the processes, products and facilities. The growing availability of biocatalysts combined with new bioengineering tools is contributing to the development of industrial processes using enzymes, demanding further investigation in scale-up or scale-out strategies that can meet the industrial challenges, providing a continuous success of biocatalysis.

Table 5 – Process considerations following a scale-up approach (Adapted from Tufvesson et al, 2010)

Considerations	
Immobilized enzymes	Agitation speed may damage particles Attrition may difficult particle recovery
Oxygen	Oxygen transfer rate decreases with scale enlargement, though the stirring speed and gas flow must be incremented Stirring and gas flow influence mass transfer rates
Two-liquid phase	The maintenance of the phases and interface demands good mixing In the regions of excessive mixing phase separation may occur and in regions of poor mixing the enzyme may be dysfunctional.
pH	Buffers are used to control pH, but effective agitation is required to avoid spots with high or low pH
Contamination	Size expansion increases contamination risk
Logistics	Reactions timings and biocatalyst storage may difficult operation decisions

1.3 Steroids

The present work is focused on the biotransformation of steroid molecules. Steroids are lipidic molecules that are characterized by the presence of a four-ring carbon atom core, called cyclopentanephenanthrene (Figure 5). All natural occurring steroids are originated from this basic structure or else bond scissions, ring expansions and contractions derived there from (Fahy et al., 2005, Fernandes et al., 2010).

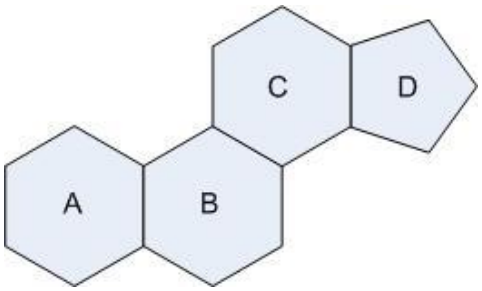
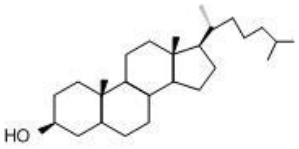
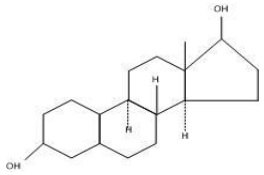
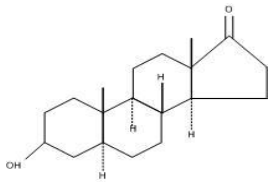
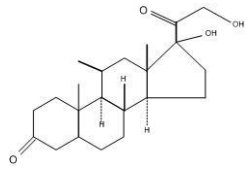
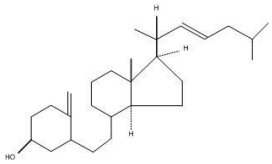
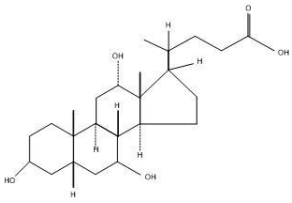


Figure 5 – Basic steroid structure (cyclopentanephenanthrene)

The different steroid molecules will vary with the different functional groups attached to these rings and also the number of carbons that constitute the molecule. These factors will determine the specificity of the biological response and also provide the subclasses division that is nowadays accepted (Fahy et al., 2005). The most commonly used raw materials in steroid industry are cholesterol and phytosterols and the process often requires multiple conversion steps before the final product is formed, generating numerous intermediates during the process (mostly due to the complexity of the molecules which present multiple chiral centers). Several efforts have been made aiming the improvement of steroids therapeutic properties and also to the increase the yield of the bioconversion steps required to reach the desired product and, as stated before, the miniaturized platforms have given precious help to pharmaceutical field, providing faster and cheaper results (Fernandes et al., 2010, Znidarsic-Plazl et al., 2010). In Table 6 the most relevant steroid subclasses are presented, providing its nomenclature, biological functions and structure schemes.

Table 6 – Relevant characteristics of steroids (Adapted from Fernandes et al., 2003, Fahy et al., 2005, Fernandes et al., 2007)

Class	Subclasses (e.g.)	Characteristics	Biological Functions	Structure
Sterols	Cholesterol Phytosterols	Methyl groups at C18 and C19 Iso-octyl side chain at C17	Components of membrane lipids	 Cholesterol
Steroids	Estrogens	18 carbon atoms	Hormones and signaling molecules	 β-estradiol
	Androgens	19 carbon atoms		 Androsterone
	Corticoids and Progestogens	21 carbon atoms Two carbon side chain at C17 position		 Cortisol
Secosteroids	Vitamin D	Characterized by a cleavage of the B ring		 Vitamin D_2
Bile acids	Cholic Acids			 Cholic Acid

Enzymes are currently among the well established products in biotechnology with an expected rise of demand of 6% per year and an expected €5 billion market in 2013 (Fernandes, 2010a). Nowadays the steroid industry has a well established position in the pharmaceutical market and biological conversions are widely used, both with whole cells and isolated enzymes, since some enzymes are able to catalyze several relevant reactions in commercially interesting steroids. Thus, the right enzymes may induce useful transformations in raw steroids, for instance hydroxylations, removal or introduction of double bonds, specific local cleavages, oxidation alcohol steroids and reduction, esterification or hydrolysis of esters steroids.

The chemical modification of steroid molecules often produces stereoisomers, therefore lowering the yield and purity of the final product, nevertheless it still competes with biocatalysis due to their low prices of manufacture (Fernandes et al., 2010). In Table 7, some relevant biotransformations concerning steroid compounds catalyzed by whole cells are described.

Table 7 – Microbial biotranformations of steroids with relevant industrial application (Adapted from Fernandes et al., 2010)

Reaction	Substrate	Main product	Microorganism/ Enzyme	References
11α-hydroxylation	Progesterone	Corticoids	*Rhizopus sp.*	Ulracher et al., 2006
11β-hydroxylation	Cortexolone	Cortisol	*Curvularia sp.*	Sonomoto et al., 1981
$\Delta^{1,2}$-dehydrogenation	Cortisol	Prednisolone	*Pseudomonas flourescens*	Naim et al., 2003
Side-chain cleavage	Phytosterols	Androstenedione Androstadienedione	*Mycobacterium sp.*	Gomes et al., 2008 Sripalakit et al., 2006

The present work concerns the oxidation of cholesterol using an enzyme, cholesterol oxidase. The oxidation of cholesterol to cholest-4-en-3-one (cholestenone) by cholesterol oxidase (EC 1.1.3.6) is the first step in the degradation of cholesterol. Therefore this reaction has been investigated as a model system for the bioconversion of sterols and steroids. In this particular case, the enzyme was used in an isolated form but several cholesterol oxidase-producing microorganisms have been described, for instance *Nocardia sp.*, *Rhodococcus sp.* and *A. simplex* mutants (Liu et al., 1996).

Cholesterol oxidases (3β-hydroxysterol oxidases) are flavin adenine dinucleotide (FAD) dependent enzymes that catalyse two reactions in one single active site: the initial oxidation of a Δ5-ene-3β-hydroxysterol to Δ5-ene-3β-ketosteroid with the reduction of molecular oxygen to hydrogen peroxide, followed by isomerization of Δ5 bond yielding a Δ4-ene-3β-ketosteroid as the final product (Aparício et al., 2008). A scheme of occurring reactions is show in Fig. 6.

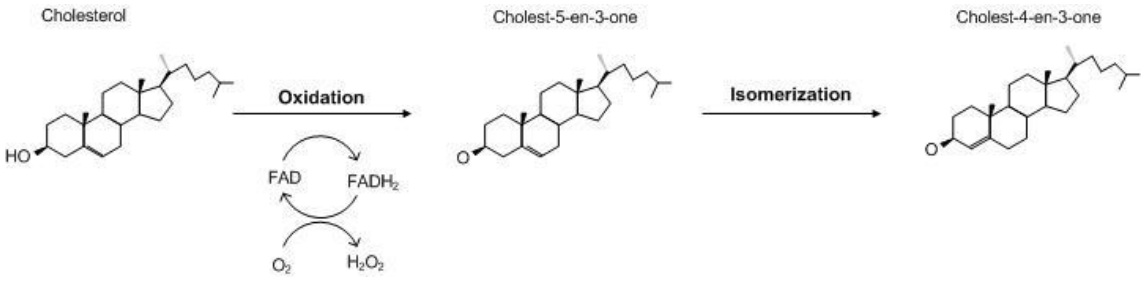


Figure 6 – Action of cholesterol oxidase over cholesterol molecule (Adapted from Aparício et al., 2008)

Due to its ability to form hydrogen peroxide, this enzyme has been used to determine the amount of serum cholesterol in biological samples, which has been useful in the assessment of some lipid disorders as arthrosclerosis and coronary heart diseases or in the determination of the risk of heart attack and thrombosis. In these procedures cholesterol samples are often esterified, which implies the use of cholesterol esterase before the treatment with cholesterol oxidase. Catalase will then catalyze the oxidative coupling of the hydrogen peroxide produced in the reaction with the aid of an aromatic dye (e.g. phenol and 4-aminoantipyrine), which is then detected by spectrophotometry (Figure 7) (Bordet et al., 2007, Mendes et al., 2007).

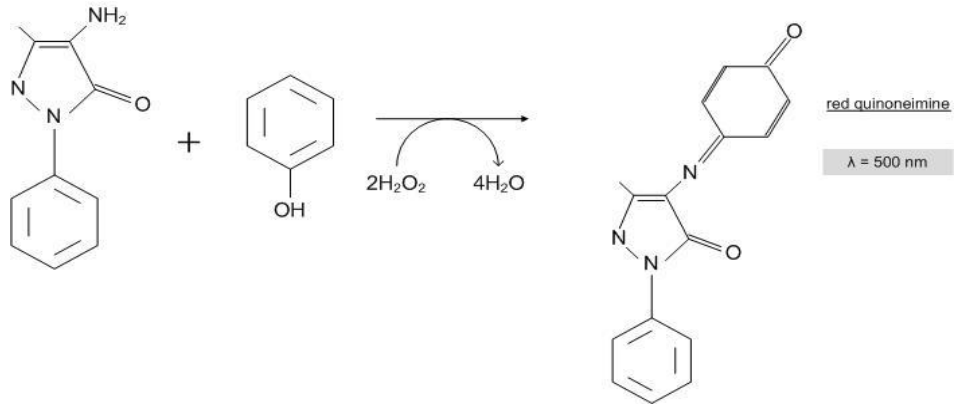


Figure 7 – The coupling of a phenol to 4-aminoantipyrine, catalyzed by a horseradish peroxidase originates a red quinineimine which can be detected by spectrophotometry at a wave length of 500 nm (Adapted from Aparício et al., 2008)

Other application of this enzyme concerns one's ability interact with constitution of lipid membranes, especially cholesterol and its interaction with phospholipids. Hence it is used for the search and study of lipid rafts, which are implicated in numerous biological processes such as membrane trafficking, signal transduction, cell adhesion mechanisms and viral and bacterial infections (London, 2002, Xu et al., 2001). Also, the production of cholestenone can alter the structural integrity of lipid membranes, for instance it can disrupt the midgut epithelial membrane of larvae of Coleoptera and Lepidoptera, acting as an effective insecticide (Aparício et al., 2008, Santos et al., 2002).

Other relevant applications are related to its capacity to use 3β-hydroxysterols as substrates, permitting its use for the determination of cholesterol and other sterols in foods (particularly in assays to detect phytosterols, given their cholesterol-lowering properties) and the relevancy of this enzyme in steroid-production industry. It is thought that this enzyme has broad substrate specificity, thus it can transform sterols (in principle it is able transform any 3β-hydroxysterols) into the corresponding ketones, thus it can be used as intermediate in the synthesis of other steroids with pharmaceutical or commercial relevance (Fernandes et al., 2010, Aparício et al., 2008, Fernandes et al., 2007).

1.3.1 Case-study

The aim of this work is to obtain a micro-industrial production platform using the oxidation of cholesterol by cholesterol oxidase yielding cholestenone, as a model reaction. This reaction produces hydrogen peroxide along the way and, although it can be useful in some clinical applications, it is also a constraint in the downstream process. Thus, the main objective is to incorporate two enzymes working properly together, using cholesterol oxidase to convert cholesterol while using catalase to dissociate the hydrogen peroxide into oxygen and water. Previous works had demonstrated that this kind of reaction system is feasible (Bankar et al., 2011, Crestini et al., 2011, Burton et al., 2008, Santacoloma et al., 2011, Marques et al., 2010c).

Besides the verification of the enzymes behavior by itself, immobilization techniques will be studied and activity profiles will be determined. The immobilization procedures comprehend the incorporation of both enzymes (separately and together) in e.g. PVA-PEG (poly(vinyl alchol)-polyethylene glycol) beads. Since the substrate is hydrophobic it will be incorporated in an organic phase, therefore a suitable screening system will be delivered to chose the suitable organic solvent to use as substrate and product pool. The reactors configuration to test will be operating in batch or continuous systems and, knowing in advance their advantages and drawbacks, several parameters will be adjusted in order to obtain higher substrate conversions as possible. The analytic methods include liquid chromatography to analyze the steroids and by spectroscopic methods.

This proposed base line strategy has an advantage of giving an extended and more realistic view over a scale-up protocol, since the removal of all by-products within the reaction process facilitates downstream, making it faster, easier and cheaper, inserting it in an high throughput approach and also enabling the reuse of both enzymes. These studies may be useful to describe continuous steroid biotransformations, making the implementation of this type of reactions feasible in industry. Moreover, it will provide effective inside view on the application of green practices.

Materials and Methods

2. Materials and Methods

2.1 Materials

Cholesterol oxidase (EC 1.1 .3.6) was purchased from BBI Enzymes Ltd. (Gwent, UK) and catalase (EC 1.11.1.6) from Sigma (St. Louis, MO, USA). 4-cholesten-3-one was purchased from Acros (Geel, Belgium), cholesterol and progesterone were obtained from Sigma (St. Louis, MO, USA) and hydrogen peroxide from Merck (Germany). The hydrated salts $NaH_2PO_4.H_2O$ and $K_2HPO_4.3H_2O$ were also purchased from Merck. To PVA (poly(vinyl alcohol))-based material (LentiKat® Liquid), was purchased from GeniaLab (Braunschweig, Germany). The needles used to form the PVA beads were obtained by Terumo (Tokyo, Japan) with an internal diameter of 0.8 mm and 50 mm of length. The syringes were also purchased from Terumo and had a 2 ml capacity. Polyethylene glycol (PEG) 600 was purchased from Fluka (Deisenhofen, Germany). Sodium bis(2-ethylhexyl) sulfosuccinate (AOT) was obtained from Sigma (St. Louis, USA). Magnetite (iron oxide 97.0%) was purchased from Merck (Germany) and glutaraldehyde from Sigma-Aldrich (St. Louis, USA). All other chemicals used during this work were of analytical or HPLC grade, purchased from assorted suppliers. For the solvent screening assay the following organic solvents (with the respective purity) were used:

Acetone (99.9%), acetonitrile (99.8%), toluene (99.8%), benzyl alcohol (99.0%), fufuryl alcohol (98.0%), furfural (98.0%), triethylamine (99.0%), methanol (99.9%), methyl cellosolve (99.0%), methyl ethyl ketone (99,7%), dimethyl formamide (99.0%), formamide (99.0%), ethanol (99.8%), ethyl acetate (99.7%), ethylene glycol (99.8%), diethyl ether (99.9%), diisopropyl ether (99.7%), hexanol (99.0%), hexane (95.0%), cyclohexane (99.0%), heptanol (99.9%), octanol (99.7%), nonanol (98.0%), decanol (99.0%), undecanol (99.0%), 2-methyl-1-butanol (98.0%), 2-methyl-1-propanol (99.8%), 2-methyl-2-butanol (99.5%), 2-propanol (99.0%), 2-butanol (99.8%), 2-pentanol (98.0%), 2-hexanol (99.0%), 2-heptanol (98.0%), 2-octanol (97.0%), 2-nonanol (99.0%), 2-decanol (98.0%), 2-undecanol (97.0%), 2-dodecanol (99.0%), 3-methyl-1-butanol (98.0%), 3-nonanol (95.0%), *n*-heptane (99.0%), *tert*-butanol (99.8%), dichloromethane (99.9%) and chloroform (99.9%), which were all obtained from Sigma-Aldrich (St. Louis, USA).

2.2 Methods

2.2.1 PVA-PEG

Catalase and cholesterol oxidase were entrapped (either separately or together in a mixture) in PVA beads according to the protocol established by Fernandes et al., (2009). Briefly, the LentiKat® liquid was heated in a water bath at 100ºC until complete melting . After cooling to approximately 30ºC, the enzyme solution (0.1 $g.l^{-1}$) was added in a 9:1 (v/v) proportion, that is 0.4 ml of enzyme solution to 8 ml 50 mM phosphate buffer pH 7,. The mixture was thoroughly mixed under magnetic stirring. The solution formed was extruded into PEG 600 where capsules were formed. The beads were left to rest in the PEG for one hour, harvested afterwards and washed with 50 mM phosphate buffer pH 7. The capsules were either immediately used or stored at in phosphate buffer at 4 ºC until use.

2.3.2 Sol-gel

In this procedure 6 eppendorfs, containing 100 µl of TMOS and 40 µl of HCl were prepared and submitted to 10 minutes of sonication (Transsonic T460 by Elma, Singen, Germany). After sonication, 160 µl of an enzyme solution of 0.1 $g.l^{-1}$ prepared in 50 mM phosphate buffer pH 7, was added to each of the previous eppendorfs and the mixture was mixed during 1 minute in a vortex (Reax 2000 by Heidolph Instruments in Schwabach, Germany). Afterwards, the resulting 300 µl were immediately added to 6 ml of AOT/isoctane 150 mM solution, previously prepared in centrifuge tubes, and submitted to 1 minute of vortex. The 6 tubes were centrifuged for 10 minutes at 4500 rpm (Sigma 201) and washed twice in 50 mM phosphate buffer pH 7 (with centrifugations between the washings). The resulting gel was transferred to eppendorfs containing 1 ml of 50 mM phosphate buffer pH 7 and left open for seven days inside a closed box containing a saturated NaCl solution (80 g NaCl and 200 ml H_2O), in order to create an water activity of 0,75 in the solution.

2.2.3 Magnetite

200 mg of magnetite (iron oxide 97%) obtained from Merck (Germany) was dissolved in 5 ml of PB and then 1 ml of glutaraldehyde 2.5 M 25% w/v was added. This solution was left for one hour at 90ºC under mild stirring and filtered with a magnet afterwards. The filtrated magnetite was then added to 20 ml of water and the mixture was left for one hour under mild agitation. This procedure was repeated three times to guarantee a proper washout of the glutaraldehyde. The 2 ml of an 0.1 $g.l^{-1}$ cholesterol oxidase and/or catalase prepared in 50 mM phosphate buffer pH 7 was added to the solid mixture and incubated at 30ºC for 1 hour. After a final wash with 50 mM phosphate buffer pH 7, the immobilized enzyme was filtered with a magnet and stored at 4ºC until use.

2.2.4 Kinetic parameters and activity profiles

Enzymatic kinetic parameters were determined in microreactors, with a 10 ml capacity, in 50 mM phosphate buffer pH 7 under a magnetic agitation of 600 rpm at 30ºC. The kinetic parameters (K_m and v_{max}) were determined by the initial-rate method both for free enzyme as well as immobilized enzymes. The trials were performed using 8 different initial substrate concentrations from 0.1 $g.l^{-1}$ to 1.0 $g.l^{-1}$. The catalase reaction occurred in an aqueous environment whilst cholesterol oxidase reaction occurred in biphasic (aqueous-organic) environment composed of ethanol in a 1:1 (v/v) proportion.

The activity profiles for cholesterol oxidase and catalase were determined in different conditions of pH and ionic strength of the phosphate buffer. This was accomplished by varying the pH of the phosphate buffer from 3 to 11 and by the variation of the molarity of the buffer, from 0 to 1M, respectively. The trials were performed using both enzymes in batch microreactors composed by a 50 mM phosphate buffer solution and ethanol in a 1:1 (v/v) proportion. The concentration the enzymes were 0.1 $g.l^{-1}$ and the substrates were 1.0 $g.l^{-1}$. The reactions occurred for 2 hours, at 30ºC under 600 rpm.

2.2.5 Solvent screening

Preliminary tests were performed to assess the effect of the organic solvent on a 96-well microtiter plate surface. For this purpose, pure organic solvents were added to the wells and the plate was covered with a breathable sealing tape (Sigma, USA) and left overnight at 30ºC without agitation. Afterwards, tests were performed to verify the interaction of the organic solvents in hydrogen peroxide reading. A volume of 100 µl of each organic solvent was deposited on the plate and 100 µl of phosphate buffer with a 0.64 M of hydrogen peroxide in solution was added. The interaction with the spectrophometric readure was verified by a comparison with a 200 µl phosphate buffer control solution containing the same hydrogen peroxide concentration (0.64 M) and the organic solvents in which a clear deviation from the control solution was verified, were eliminated from the screening process. Another assay was performed using magnetic agitated microreactors containing 2.5 ml of 100 mM phosphate buffer solution with 0.1 $g.l^{-1}$ of soluble cholesterol oxidase and 2.5 ml of each organic solvent containing 1.0 $g.l^{-1}$ of dissolved cholesterol in order to eliminate the organic solvents in with the bioconversion was null.

During the remaining assays, 2.5 ml of the organic solvents were placed in 10 ml screw caped vessels together with 2.5 ml of 100 mM phosphate buffer pH 7 containing 0.1 $g.l^{-1}$ of the cholesterol oxidase. Assays were conducted during 2 hours at 30ºC under magnetic agitation (600 rpm). Samples were collected and treated accordingly to the established protocol either for steroid analysis or hydrogen peroxide.

2.2.6 Bioconversion trials and continuous mode operation

2.2.6.1 Bioconversion trials

In all bioconversion trials either for stability evaluation, different aqueous-organic system ratio, co-immobilization trials, hydrogen peroxide toxicity and oxygen supply trials the defined conditions were temperature of 30ºC, using 10 ml capacity microreactors under 600 rpm magnetic stirring.

For the stability trials a concentration of 0.1 g.l-1 of the free was prepared (in 2.5 ml of buffer) and 2.5 ml of each organic solvent with the substrate incorporated (1.0 $g.l^{-1}$) was added. The aqueous phase, with the free enzyme, was kept in the reactor under the same conditions until a next reaction was performed. The reactions occurred with a time difference of 20 to 25 hours ending at 200 hours.

When evaluating different aqueous-organic ratios, different aqueous-organic ratios were tested, from 1:1 (aq/org) to 1:10 (aq/org) performing a total amount of 2 ml inside each microreactor. The organic phase used was composed by *n*-heptane and the reactions occurred for 2 hours at 30ºC under magnetic agitation. After the reaction period all the content was withdrawn (sacrifical well approach), extracted and analyzed to cholestenone content. This procedure was performed to both free and immobilized form of cholesterol oxidase.

Using the co-immobilization of both enzymes in the same support, the protocol used was the same referred in PVA-PEG section. These trials were performed during a total period of 5 hours, and samples were withdrawn each 60 minutes. Inside each batch reactordifferent combinations of the two enzymes were placed, namely catalase and cholesterol oxidase immobilized within the same support and catalase and cholesterol oxidase immobilized in different supports but acting together inside the same reactor; *n*-heptane was used as organic phase.

The hydrogen peroxide toxicity evaluation was performed in batch reactors using the enzyme in the free and in the immobilized form. The reactions occurred for 2 hours using concentrations from 0 to 10M of hydrogen peroxide dissolved in the aqueous phase and *n*-heptane as the organic phase.

To evaluate the behavior of the enzyme when in presence of different oxygen concentrations four assays were performed: a control reactor with no oxygen content (the oxygen was removed by the use of a nitrogen inflow), a reactor with the oxygen amount present in the room atmosphere (uncovered) and two reactors, submitted to 5 and 20 minute inlet of air, providing higher concentrations of oxygen. The bioconversion trials occurred during 2 hours using immobilized cholesterol oxidase inside a biphasic environment composed of 100 mM, pH 7 phosphate buffer and *n*-heptane.

2.2.6.2 Continuous mode operation

In continuous mode, both enzyme concentrations were 0.1 $g.l^{-1}$. Figure 8 and 9 represent the two different setups proposed and assembled in the continuous operation mode. Different temperatures were applied to the system: whilst the reactions occurred at 30ºC, the main reservoir was maintained at 4ºC by a refrigeration device (Thermomix), provided by Braun (Germany), to avoid solvent evaporation. An HPLC pump (L-6000 from Merck Hitachi) was used and also a peristaltic pump (LKB from Pharmacia) was incorporated into one of the continuous setups (Figure 9). Two different pumps were used since the organic solvent used interferes with the silicon tubes. For that reason the organic phase had to pass through tubes a different composition and the HPLC pump was chosen.

The packed-bed reactor used for cholesterol oxidase had a 2 ml capacity with dimensions of 1.5 cm length and 1 cm external diameter. The fluidized-bed reactor where catalase was incorporated had a 3 ml capacity with dimensions of 7.7 cm length and 0.7 cm external diameter. The main reservoir contained a two phase aqueous-organic system composed of 100 mM phosphate buffer solution and *n*-heptane, respectively. The main reservoir was covered with a PTFE lid and additionally with a parafilm layer. In these, six inlets/outlets were inserted: 1) to enable the withdrawal of the organic phase to begin the reactional circuit (out), 2) to enable the withdrawal of the aqueous phase continuous monitoring (out) , 3) air inlet, 4) to release the built-up pressure from the system, 5) to enable the organic phase to return to the main reservoir after completing the circuit (in) and 6) to enable the aqueous phase to return to the main reservoir (in) (Figure 10).

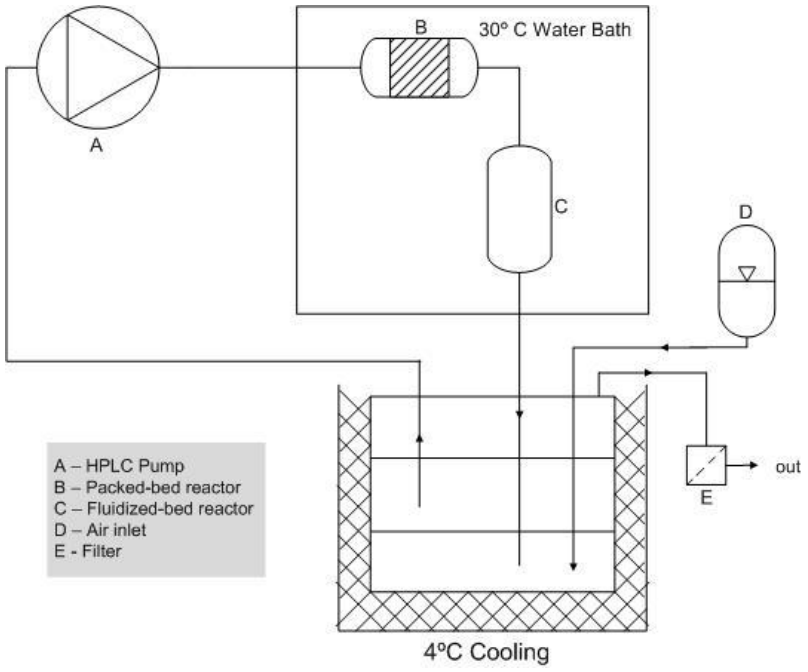


Figure 8 – Schematic representation of the continuous mode setup without the continuous hydrogen peroxide monitoring

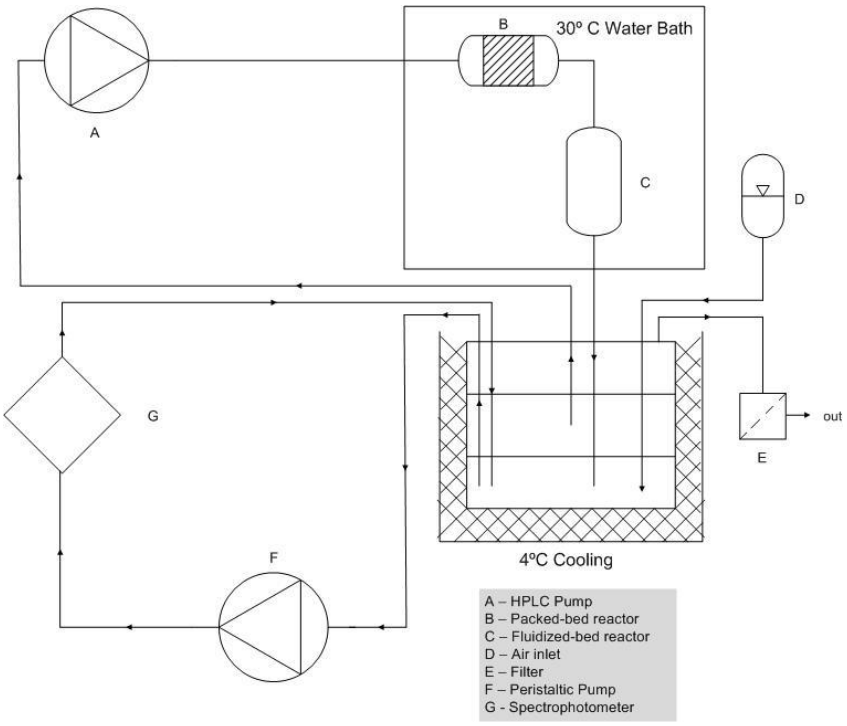


Figure 9 – Schematic representation of the continuous mode setup with the hydrogen peroxide monitoring incorporated

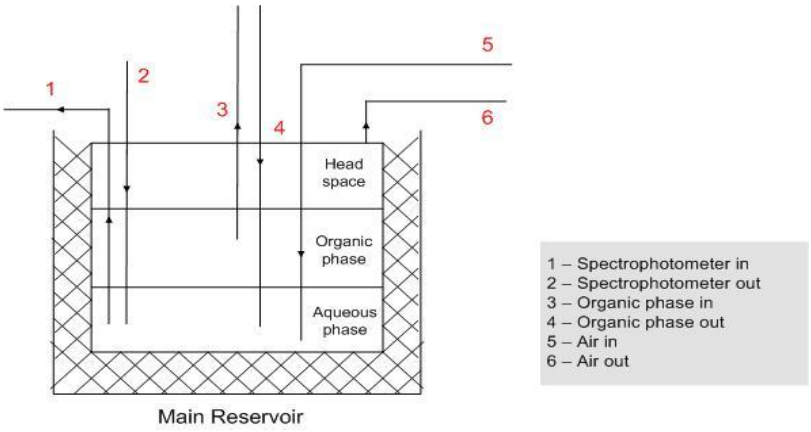


Figure 10 – Schematic representation of each inlet/outlet inserted in the main reservoir and the respective function

To evaluate the behavior of the enzyme over time using higher volumes of reactants, a total volume of 100 ml (1:1 aqueous/organic) was used (the same used in the main reservoir of the continuous mode setup) in a stirred-tank reactor (STR). Samples of 500 µl were withdrawn throughout 100 hours, during which time the reaction ran without stoppages. The STR had a magnetic stirring of 600 rpm, was thermostatisized at 30 ºC, contained immobilized cholesterol oxidase and *n*-heptane (carrying the substrate) was used as organic phase.

2.2.7 Statistical analysis

Data were analyzed using statistical analysis software (SPSS 14.0). The statistical analysis was obtained by one-way ANOVA, which was used to detect differences among variables. Statistical confidence was set at 95%. All trials were performed in triplicates.

2.2.6 Analytical methods

2.2.6.1 Hydrogen peroxide analysis

The hydrogen peroxide degradation was mononitored according to Li and Schellhorn (2007) in 96-well UV-transparent microtiter plates (Greiner, Bio-One GmbH, Germany) at 240 nm in a microtiter plate spectrophotometer (SpectraMax Plus 384, Molecular Devices, Inc., CA, USA). A volume of 20 µl was collected from the aqueous phase at the outlet of the packed-bed reactor and diluted in 280 µl of phosphate buffer prior to analysis. Analysis was performed in parallel in a spectrophotometer U-2000 Hitachi (Tokyo, Japan). In the continuous mode setup, an on-line monitoring protocol of the hydrogen peroxide was established. This setup was composed by an UV cuvette, connected to the aqueous phase of the main reservoir, which was placed inside the spectrophotometer. A peristaltic pump allowed the recirculation of the aqueous phase from the reservoir to the cuvette and back to the reservoir (Figure 9). This system allowed for a continuous readout of the overall buffer's absorbance and the direct evaluation of the hydrogen peroxide content and catalase activity.

2.2.6.2 Steroid analysis

Samples from the steroid biotransformation media were extracted with a solution of progesterone (0.12 $g.l^{-1}$) in *n*-heptane in a 1:1 proportion. This solution was used as an internal standard. After mixing on a vortex (Reax 2000 by Heidolph Instruments, Schwabach, Germany) for 1 minute the samples were centrifuged at 4500 rpm during 5 minutes (Sigma 201). The organic phase was collected and analyzed with HPLC. HPLC analysis was performed by using a Licrospher Si-60 column (5µm particle size, Merck, Germany) with 1 $ml.min^{-1}$ isocratic elution with UV detection at 254 nm to determine cholestenone concentration. To determine cholesterol the UV detection was performed at 215 nm). The mobile phase was composed of *n*-heptane and absolute ethanol (90:10 v/v).
Conversion yields were calculated on the basis of product formation since, besides 4-cholesten-3-one, no other product was formed, as assumed from the chromatograms, given the absence of unidentified peaks.

2.2.6.3 Protein content analysis

The quantification of protein was performed by the BCA method (Smith et al., 1985) using a commercial kit from Pierce Biotechnology (Rockford, IL, USA).

2.2.6.4 Dissolved oxygen analysis

To determine the dissolved oxygen concentration, Oxodish® 24-well microtiter plates (Presens, Germany) containing oxygen probes were used. The assays were carried out both under orbital agitation (200 rpm) and non-agitated conditions in two phase aqueous-organic systems composed of either ethanol and 100 mM phosphate buffer pH 7 solution or *n*-heptane and 100 mM phosphate buffer pH 7 solution.

Results and Discussion

3. Results and Discussion

3.1 Enzyme Immobilization

During the present work three different immobilization methods were performed. The immobilization of enzymes in and/or onto solid carriers, under optimized conditions, is often used in biocatalysis to improve enzyme operational stability and control as well as easiness of downstream when compared to the corresponding soluble enzymes process (Lyer et al., 2008). Nonetheless, some immobilization procedures may hamper a proper enzyme function due to mass transfer and partition limitations induced by the involving matrix (Houng et al., 1994). The three immobilization methods studied were the PVA-PEG, the sol-gel encapsulation and the immobilization on magnetite methods. These were chosen after a compromise was established between operational stability (taking into account the mode of operation of the microreactors available) and downstream processes (avoiding laborious protocols and enabling continuous product production and extraction).

Concerning the different methods, the PVA-PEG immobilization method was already been proved effective to relevant biotransformation processes (Fernandes et al., 2009, Marques et al., 2011) while sol-gel bioencapsulation procedures have been applied in a large number of biological materials (Catana et al., 2005, Gill et al., 2000). One example of the application of the latter is the entrapment of catalase. Besides the relevant increase of stability, a 50% activity loss had occurred (Jurgen-Lohmann et al., 2005).

To enhance simplicity in downstream (enzyme recovery) at the microreactor level, the immobilization of the enzyme onto magnetite was tentatively used. Horst et al. (2006) immobilized catalase onto this support and a higher maximum activity, when comparing to the free enzyme, was predicted.

In this study, the feasibility of the immobilization supports when incorporated inside the packed bed reactor (PBR) and the fluidized bed reactor (FBR) used in the continuous mode setups were also taken into account. Easy product recovery, large mass transfer specific area (in the case of PBR) and fluidity of the support (in FBR) were aimed at.

The activities of both enzymes (cholesterol oxidase and catalase) either isolated or combined, in the three different immobilization supports, were studied (Figure 11). As it can be observed, in sol-gel and magnetite immobilization, the enzymes presented no activity. For these activity trials, a substrate concentration of 1.0 $g.l^{-1}$ was used (as determined in Marques et al., 2010c). For the three immobilization methods and also for the free enzyme, a concentration of 0.1 $g.l^{-1}$ was prepared and to perform the assays 0.7 g (wet weigh) of each support was placed inside each microreactor.

All reactions, either using free or immobilized enzymes, occurred in immiscible aqueous-organic systems composed of ethanol and a phosphate buffer pH 7, 50 mM solution in a 1:1 proportion.

In this case, the solvent was used to deliver the substrate since cholesterol has low solubility in water (below 1 μM) (Marques et al., 2010c), and additionally to mimic the biphasic environment that will figure in the next trials (in the case of catalase).

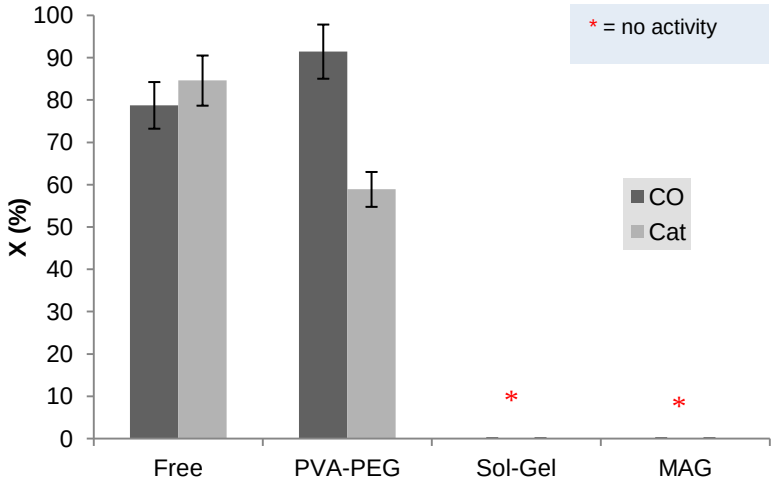


Figure 11 - Activity trials for both enzymes in the soluble form and in the three immobilization procedures (CO: cholesterol oxidase, Cat: catalase).

The activity loss related to sol-gel and magnetite immobilization in both enzymes had a strict correlation with the immobilization protocol. In the sol-gel immobilization, although it has been proven to work in other enzymes such as cholesterol esterase as horseradish peroxidase (Singh et al., 2007 and Kumar et al., 2000), the specific protocol used in this work was different. For instance, the latter authors' used tetraethyl-orthosilicate (TEOS) instead of tetramethyl-orthosilicate (TMOS) used in this work and TMOS may have inactivated the enzyme due to release of methanol during the immobilization process. Jurgen-Lohmann et al. (2005) reported that, after the immobilization procedures in several sol-gel matrixes, catalase presented structural integrity however the support itself had played a negative role in oxygen yielding and mass transfer. Additionally, the catalytic activity may also have been lost during the drying process.

In the case of magnetite immobilization protocol, Herzog et al. (1974) had demonstrated that glutaraldehyde had a negative effect on the catalytic activity of catalase when ethanol was used as hydrogen donor. According to this author, the loss of enzymatic activity was related to the reaction of glutaraldehyde with the lysine residues of the proteins and also to the active-site inhibition that resulted from the treatment with glutaraldehyde or other denaturing agents. The presence of this reagent may have equally disturbed cholesterol oxidase normal function. These immobilization procedures must be optimized and a dedicated work should be necessary, which was out of scope for the present.

Unlike the results obtained for cholesterol oxidase and catalase in the sol-gel and magnetite supports, when the PVA-PEG supports were used, promising conversion yields were obtained with both immobilized enzymes when compared to the free enzymes.

Although in catalase, the PVA-PEG support lowered the conversion yield when compared to the free enzyme, in the case of cholesterol oxidase that parameter was not affected by the immobilization procedure, since similar conversion yields were observed either in free or immobilized forms of the biocatalyst.

Both reactions occurred in a biphasic system and, although this reactional system favored cholesterol oxidase behavior, it has prejudiced the behavior of catalase. The immobilization support brought mass transfer limitations and protection from the surrounding environment to both enzymes. In the case of cholesterol oxidase, the mass transfer limitations tend to be higher than in the case of catalse, since the substrate for cholesterol oxidase will preferably stay in the organic phase and has to dislocate from this phase to the immobilization matrix, which will preferably be in the interface. In the case of catalase, the mass transfer limitations tend to be lower since both substrate and enzyme are in the aqueous phase. Despite this fact catalase lost more activity than cholesterol oxidase when immobilized, evidencing that other factors related to the immobilization protocol must have played a role besides the mass transfer limitations.

After the immobilization procedures all washing buffers were analyzed for protein content by SDS polyacrylamide gel electrophoresis, where no trace of protein was observed, demonstrating that the enzymes in the prepared solutions were all immobilized (data not shown).

3.2 Kinetic Parameters

In the determination of an enzyme kinetic parameters, in this case through the initial-rate method (Equation 1), it is implicit that the initial reaction rate (v) equals -d[S]/dt at t=0, assuming that the initial substrate concentration remains unaltered. It is assumed that the enzyme concentration is constant throughout the reaction, which determines how the initial velocity will depend on the substrate concentration ([S]) to evaluate the Michaelis constant (K_m) and the maximal velocity (v_{max}), from a plot of v versus [S], or 1/v versus 1/[S] (Michaelis and Menten, 1913).

$$v = \frac{v_{max} \times [S]}{K_m + [S]} \quad (1)$$

After running 8 different initial substrate concentrations (from 0.1 g.l^{-1} to 1.0 g.l^{-1}), the kinetic constants were determined according to the Lineweaver-Burk approach (Lineweaver and Burk, 1934). The determined values for K_m and v_{max} are shown in Table 8 for the free and immobilized enzymes in all the immobilization matrixes, knowing in advance that sol-gel and magnetite had loss total activity during the immobilization process.

Table 8 - Kinetic parameters of free and immobilized enzymes obtained using a media composed of water/ethanol (9:1)

	Catalase		Cholesterol oxidase	
	K_m (M)	v_{max} (M.s^{-1})	K_m (M)	v_{max} (M.s^{-1})
Free	2.85 x 10^{-4}	2.38 x 10^{-6}	1.57 x 10^{-6}	2.74 x 10^{-6}
PVA-PEG	8.72 x 10^{-3}	2.48 x 10^{-3}	6.54 x 10^{-7}	1.67 x 10^{-6}

When comparing the free and immobilized enzyme (Table 8), a higher K_m is observed for the immobilized enzyme. This reduction of the enzyme's affinity to its substrate has two reasons: i) a possible conformational change in the enzyme catalytic centre caused by the immobilization procedure or, ii) mass transfer limitations. In spite of these characteristics, which can be assumed to occur in catalase, in cholesterol oxidase, the immobilization procedure did not affect the enzyme's affinity towards the substrate. This can be explained by the protective characteristics that the immobilization support once again provided. Although all the above assumptions may be applied, in this case, the free enzyme was also prone to suffer from the environment surrounding the enzyme, such as the composition of the surrounding media (e.g. organic solvents). This explains why the immobilization in a PVA (and other similar polymers) support may be the answer in biphasic reactions. This kind of support, unlike others, is easy to perform, has a high enzyme concentration capacity, is relatively inexpensive and has good mechanical stability when compared to other hydrogels (Fernandes et al., 2009).

When comparing the enzymes used for this work (in both free and immobilized forms) with previous works, different values were observed. In Table 9 some examples are given concerning these kinetic parameters for the free enzyme and for different immobilization methods for both enzymes.

Table 9 – Kinetic parameters for free and immobilized cholesterol oxidase and catalase

	Source/ Immobilization Support	**Reactional system**	**K_m (M)**	**v_{max} ($M.s^{-1}$)**	**References**
Free cholesterol oxidase	Unknown	Aqueous-organic[2]	2.6×10^{-4} 7.6×10^{-2}	3.0×10^{-6} 1.15×10^{-3}	Çirpan et al., 2003 Akkaya et al., 2009
	Brevibacterium sp. *Streptomyces sp.*	Aqueous-organic (ethanol)	2.3×10^{-1} 2.17×10^{-4}	1.20×10^{-5} 1.58×10^{-5}	Srisawasdi et al., 2006 and Doukyu et al., 1998
	Pseudomonas fluorescens	Organic (hexane)	6.10×10^{-5}	1.10×10^{-5}	
Free catalase	Bovine liver	Aqueous	2.51×10^{-6}	2.4×10^{-3}	Çetinus et al., 2000
Immobilized cholesterol oxidase	Polymers	Aqueous-organic (isopropanol)	$2{,}72 \times 10^{-3}$	$9{,}8 \times 10^{-4}$	Akkaya et al., 2009
	Polymers	Aqueous-organic (PTSA)	8.2×10^{-5}	4.0×10^{-3}	Çirpan et al., 2003
	PVA-PEG	Aqueous-organic (*n*-heptane)	1.6×10^{-4}	4.76×10^{-5}	Marques et al., 2011
Immobilized catalase	Chitosan film	Aqueous	2.76×10^{-6}	1.02×10^{-4}	Çetinus et al., 2000

[2]In Cirpan et al., (2003), *p*-toluene sulphonic acid (PTSA) was used as organic phase and in Akkaya et al., (2009) isopropanol was used as the organic phase.

The differences obtained in the kinetic parameters shown in Table 8 and 9 must be related with the different enzyme sources, different media compositions and immobilization support used. Some similarities are found when comparing the free enzymes with the immobilized enzymes, since, also in these cases the enzyme affinity decreases when the enzyme is immobilized (due to the reasons already mentioned previously) and, generally, the v_{max} is also lower. Once again it is to notice that during these assays the media was composed by water and ethanol (in 9:1 proportion), which may have played an important role in the enzymes kinetic parameters, since ethanol is miscible in the aqueous media. Nevertheless, the values obtained for both enzymes, either in the free or immobilized form, are acceptable.

3.3 Activity profiles

The determination of the process variables of enzymes, both in free and immobilized forms, is essential to the progression of enzymatic studies, since it contributes to establish the optimal experimental conditions. By assessing BRENDA database (http://www.brenda-enzymes.org, assessed on 30 September, 2011) it was verified that both enzymes had an equal optimum temperature. Both cholesterol oxidase (derived from *Brevibacterium sp.*) and catalase (derived from bovine liver) are referenced with an optimum temperature of 30º C. Moreover, in MacLachlan et al. (2000), the same temperature was estimated to be the optimum. Knowing this parameter, the optimum pH and ionic strength of the buffer was determined.

These trials were performed using an enzyme concentration of 0.1 $g.l^{-1}$ and a 1.0 $g.l^{-1}$ substrate concentration for both enzymes, summing to 5 ml inside the batch reactor. The reaction lasted for 2 hours. In the case of the ionic strength of the buffer, the samples were prepared containing buffer molarities ranging from 0 to 1M. From Figure 12 and 13, is can be seen that the optimal range of buffer concentration goes from 50 mM to 150 mM, for both enzymes either in the free and immobilized from, as depicted from the higher relative activities obtained.

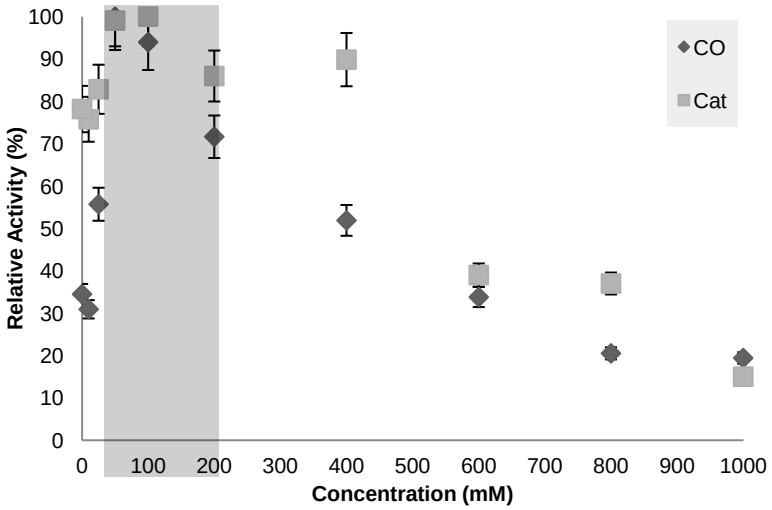


Figure 12 – Activity profile for free cholesterol oxidase (CO) and catalase (Cat) in different ionic strengths of the buffer. The grey area highlights the concentration range in which both enzymes obtained higher relative activities.

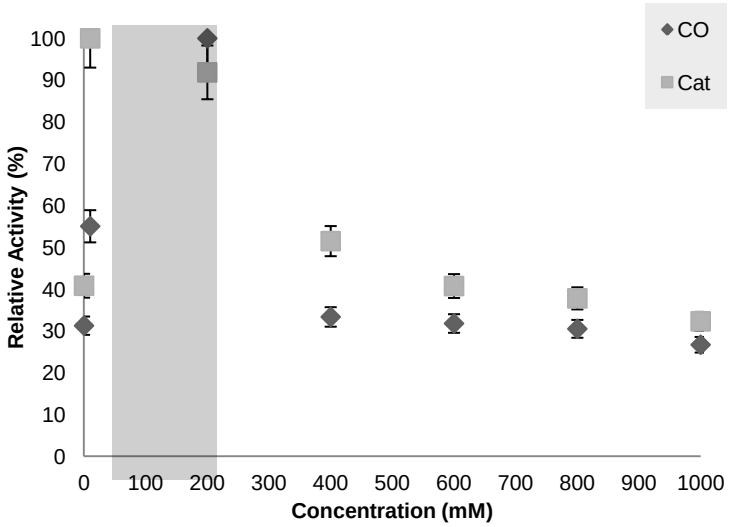


Figure 13 – Activity profiles for immobilized cholesterol oxidase (CO) and catalase (Cat) in PVA-PEG at different ionic strengths. The grey area highlights the concentration range in which both enzymes obtained higher relative activities.

These activity profiles correspond to what it was theoretically expected. Firstly, the obtained range of optimal activity was within the values reported (Lin et al., 2010, MacLachlan et al., 2000, Yazdi, et al., 2001) and also in the BRENDA database. Secondly, there is a slightly higher activity in the immobilized enzymes, even when they are out of the optimal range, which means that activity decay in the free form is more pronounced In free form, enzyme activity decay 80% (both enzymes) while in the immobilized form the activity decay reaches 70%. Once again this can be attributed to the protective effect of the immobilization support, in this case maintaining the enzyme catalytic activity in less favorable conditions.

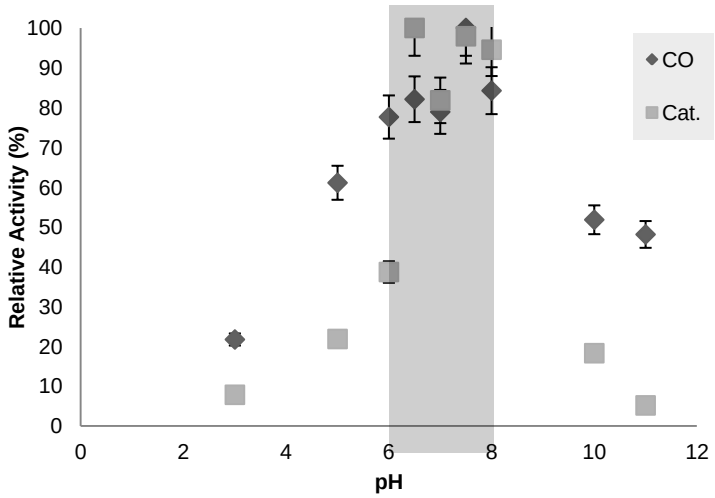


Figure 14 – Activity profile for free cholesterol oxidase (CO) and catalase (Cat) for different pH values. The grey area highlights the pH range in which both enzymes obtained higher relative activities

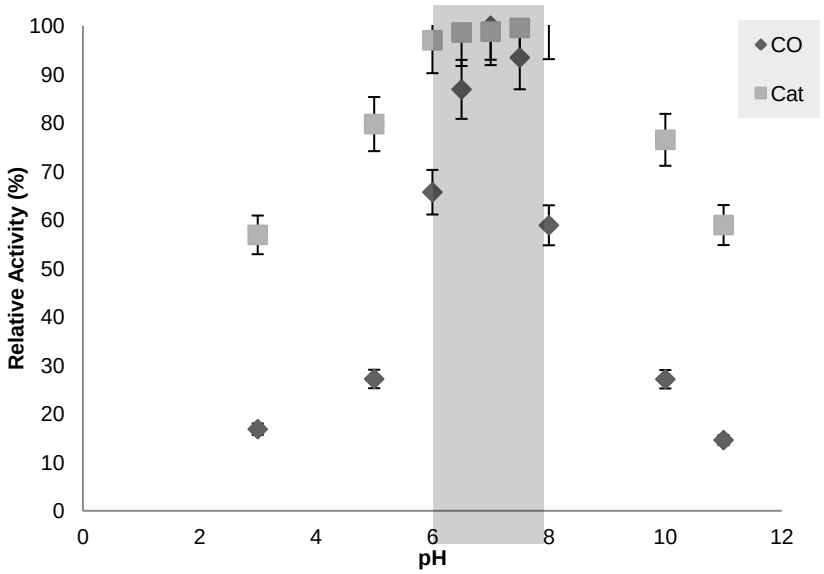


Figure 15 - Activity profile for immobilized cholesterol oxidase (CO) and catalase (Cat) in PVA-PEG at different pH values. The grey area highlights the pH range in which both enzymes obtained higher relative activities

A similar trend was obtained for pH activity profile, emphasizing the relevance of this parameter to the enzyme's proper usage. As it is know, the pH plays a role over the total net charge of the enzymes and the distribution of the charges on their exterior surfaces, affecting the reactivity of the catalytic active groups to the substrates. It may also affect the shape or charges in the substrate's surface thus, the determination of the optimum pH, is essential when performing enzymatic assays (Scopes, 2002). As it can be seen in Figure 14 and 15, the range in which both enzymes present higher relative activities goes from 6 to 8, achieving the highest activity at value of 7. Also for this parameter, immobilization plays an important protection role, especially in the case of catalase, enabling relative activities over 50% in all pH range tested. With this important step completed, it was established that both enzymes are able to work simultaneously in the same environment, under the same conditions, namely in phosphate buffer 100 mM pH 7.

3.4 Solvent screening

To perform the cholesterol oxidase enzymatic assays, the usage of a two-phase aqueous-organic system was evaluated. As already referred, due to the hydrophobic characteristics of cholesterol, an organic solvent is the most feasible response to increase the productivity (delivery of catalytic substrate) and facilitate the downstream process. During the preliminary assays, the organic phase was composed by ethanol. This solvent presents some disadvantages namely: it displays a relatively low solubility of cholesterol (molar fraction of 0.04, at 30 ºC, according to Chen et al., 2009) and does not form a distinct two-phase system since it is water-soluble. In this way, and to cope with the previous it was necessary to determine which organic solvent had the suitable characteristics to develop a continuous mode platform with the two enzymes working simultaneously. This screening assay was supported not only by experimental data, obtained by conversion reactions performed in 96-well microtiter plates and in magnetically stirred microreactors but also crossing these data with some physicochemical properties of the solvents, in order to obtain correlation patterns between them.

The first phase of this screening process was performed in three major steps: i) elimination of the organic solvents that interact with the microtiter plate's surface, to avoid misinterpretation of the chromatographic and spectrophotometric analysis, ii) elimination of the organic solvents that interact with the hydrogen peroxide reading and iii) elimination of the organic solvents in which the enzyme presented no catalytic activity.

For the first step, a set of 53 different organic solvents were tested (Table 10). A volume of 200 µl of each organic was deposited in each well. Those were left overnight in order to verify a possible interaction with the support. It was verified that some organic solvents have eroded, punctured or even left white deposits upon evaporation in the wells, namely, chloroform, diethyl ether, cyclohexane and hexane, therefore they were excluded from the remaining screening process.

The following step comprised a spectrophotometric assay in order to eliminate the organic solvents that provoked an effect on the hydrogen peroxide reading. A volume of 100 µl of each organic solvent was deposited on the plate and 100 µl of phosphate buffer with a 1 $g.l^{-1}$ of hydrogen peroxide in solution was added. The interference with the reading was verified by comparison with a control solution (200 µl of PB with 1 $g.l^{-1}$ H_2O_2). Organic solvents that had obtained an absorbance noticeably different from the control, were eliminated from the screening process. The organic solvents eliminated after this assay were: 2-octanol, benzyl alcohol, ethyl acetate, fufuryl alcohol, dimethyl formamide and toluene.

Table 10 – Set of organic solvents chosen to the initial screening process. The highlighted organic solvents represent the ones who have passed through the three first steps of the screening process and will continue to the following screening steps.

Organic Solvents		
Acetone	Diethyl ether	2-Pentanol
Acetonitrile	Diisopropyl ether	2-Hexanol
Toluene	Hexanol	2-Heptanol
Benzyl alcohol	Hexane	2-Octanol
Fufuryl alcohol	Cyclohexane	2-Nonanol
Furfural	Heptanol	2-Decanol
Triethylamine	Octanol	2-Undecanol
Methanol	Nonanol	2-Dodecanol
Methyl cellosolve	Decanol	3-Methyl-1-Butanol
Methyl ethyl ketone	Undecanol	3-Nonanol
Dimethyl formamide	2-Methyl-1-Butanol	*n*-Heptane
Formamide	2-Methyl-1-Propanol	*tert*-Butanol
Ethanol	2-Methyl-2-Butanol	Dichloromethane
Ethyl acetate	2-Propanol	Chloroform
Ethylene glycol	2-Butanol	

With the remaining organic solvents a bioconversion assay was performed. This step enabled the elimination of the organic solvents in which the conversion yield was 0 and the selection of the solvents in which the activity of the enzyme was higher. This final step lead to the elimination of: acetone, acetonitrile, furfural, triethylamine, methyl cellosolve, methyl ethyl ketone, formamide, ethylene glycol, diisopropyl ether and *tert*-butanol.

The choice of organic solvents for a screening process is still a trial and error process in the majority of cases. During this particular screening process the choice of the initial set of 53 solvents relied on the fact that they had been used to similar screening processes in both enzymes (Marques et al., 2009) and they presented characteristics from different classes of organic compounds. The aim was to infer which characteristics were the most important in steroid bioconversions. In Table 10, the whole initial set of organic solvents is presented and the 24 highlighted organic solvents represent the ones that have passed through the three first steps of the screening process and will continue to a final phase.

Figure 16 illustrates the substrate conversion obtained in each organic solvent for the cholesterol oxidase reaction, emphasizing the four organic solvents that have obtained better results and the respective conversion yields. As it can be seen, methanol, ethanol, 2-propanol and *n*-heptane presented acceptable conversion rates and the most suitable was chosen from this group. It is important to underline that these trials were made under non-optimized conditions.

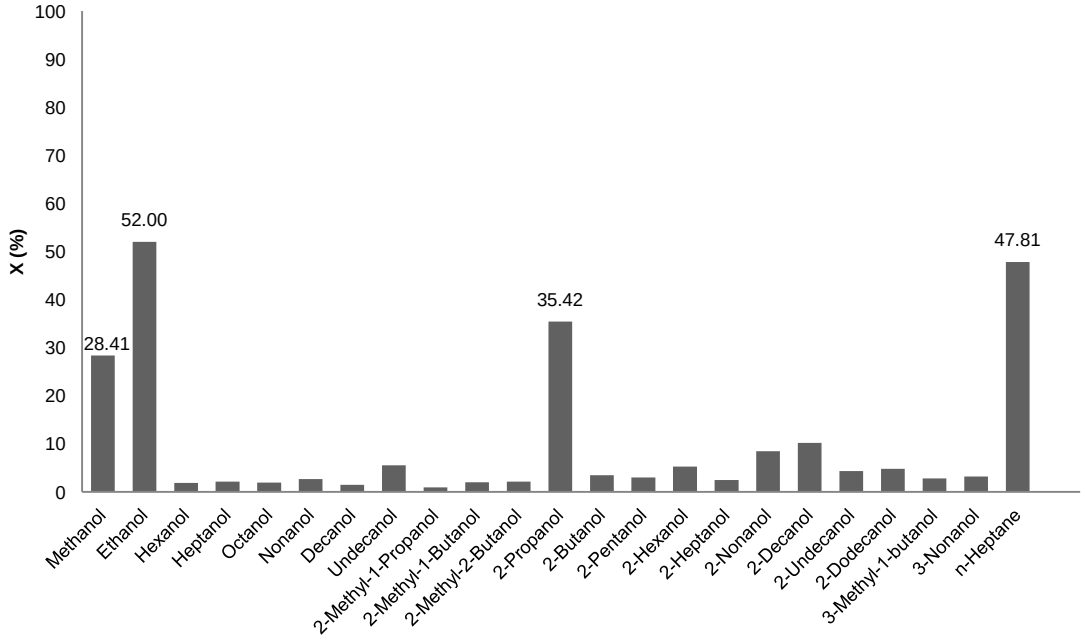


Figure 16 – Substrate conversions obtained with different organic solvents as substrate carrier for the cholesterol oxidase reaction using magnetically agitated microreactors containing free cholesterol oxidase (0.1 $g.l^{-1}$) in ph 7, 100 mM phosphate buffer and cholesterol dissolved into the organic phase (1 $g.l^{-1}$).

The second phase of the screening process comprehended three different steps: i) verification of a possible relation between the bioconversion yields obtained and some intrinsic properties of the solvents, ii) determination of the stability of the enzyme in the four referred organic solvents and iii) determination of the oxygen solubility in the organic solvents, since cholesterol oxidase reaction is dependent of the dissolved oxygen in the media.

In Table 11, the data for the first step is gathered. This information was collected from several sources and is assembled in the graphs below.

Table 11 - Physicochemical properties of the organic solvents used in the screening process

Solvents	MW ($g.mol^{-1}$)	Specific gravity ($g.cm^{-3}$)[3]	Vapor Pressure, 25ºC (torr)[3, 4]	LogP[4, 5]	Solubility (mM)[4, 5, 6]	Dielectric Constant, 30ºC[3, 5]	Henry's Law Constant, 25ºC ($atm.m^3.mol^{-1}$)[4, 5]
Methanol	32.0	0.79	127.00	-0.77	$2.45x10^{4}$	31.90	$4.40x10^{-6}$
Ethanol	46.1	0.79	59.00	-0.31	$1.70x10^{4}$	24.30	$5.20x10^{-6}$
Hexanol	102.2	0.81	0.75	2.03	69.00	12.10	$2,10x10^{-5}$
Heptanol	116.2	0.82	0.11	2.62	14.80	10.90	$1.89x10^{-5}$
Octanol	130.2	0,83	0.15	2.94	4.13	9.50	$2.50x10^{-5}$
Nonanol	144.3	0.83	ND	3.77	$9.70x10^{2}$	8.83	$3.09x10^{-5}$
Decanol	158.3	0.83	$7.50x10^{-3}$	3.90	0.25	7.20	$4.70x10^{-5}$
Undecanol	172.3	0.83	$5.50x10^{-3}$*	4.60	0.85	5.98	$4.51x10^{-5}$
2-Methyl-1-Propanol	74.1	0.81	9.00	0.79	$1.15x10^{3}$	17.93	$1.17x10^{-5}$
2-Methyl-1-Butanol	88.2	0.80	3.15	1.40	$3.40x10^{2}$	15.63	$1.18x10^{-5}$
2-Methyl-2-Butanol	88.2	0.81	19.20	1.22*	$1.3x10^{3}$	7.78	$1.33x10^{-5}$
2-Propanol	60.1	0.78	31.00	0.05	13.00	18.50	$1.10x10^{-5}$
2-Butanol	74.1	0.81	$1.13x10^{-5}$	0.61	$2.44x10^{3}$	15.20	$1.44x10^{-5}$
2-Pentanol	88.2	0.81	6.03	1.19	45.38	12.00	$1.51x10^{-5}$
2-Hexanol	102.2	0.81	26.30	1.76	$1.34x10^{2}$	11.10	$2.35x10^{-5}$
2-Heptanol	116.2	0.82	0.89	2.68	28.40	9.72	$8.22x10^{-5}$
2-Nonanol	144.3	0.83	0.11	3.40	0.46*	6.66	$1.82x10^{-5}$
2-Decanol	158.3	0.82	0.04	3.71	0.15*	5.50	$1.81x10^{-5}$*
2-Undecanol	173.3	0.83	0.01	4.41	$4.90x10^{-2}$*	ND	$7.26x10^{-5}$*
2-Dodecanol	186.3	0.83	$3.10x10^{-3}$	4.65*	0.11*	ND	$9.63x10^{-5}$*
3-Methyl-1-butanol	88.2	0.80	2.37	1.26*	$1.16x10^{-3}$*	1.92	$1.33x10^{-5}$*
3-Nonanol	144.3	0.82	0.10	3.22*	0.32*	4.49	$3.12x10^{-5}$*
***n*-Heptane**	100.2	0.68	45.70	4.66	$1.39x10^{-3}$	2.06	$2.06x10^{-4}$

[3]CRC handbook of Chemistry and Physiscs

[4]http://www.depreportingsvcs.state.pa.us/ReportServer/Pages/ReportViewer.aspx?%2fCPP%2fChemicals, assessed in August 26, 2011

[5]Marques et al., 2009

[6]Yaws et al., 1997

* Estimated using the US Environmental Protection Agency's EPISuite™

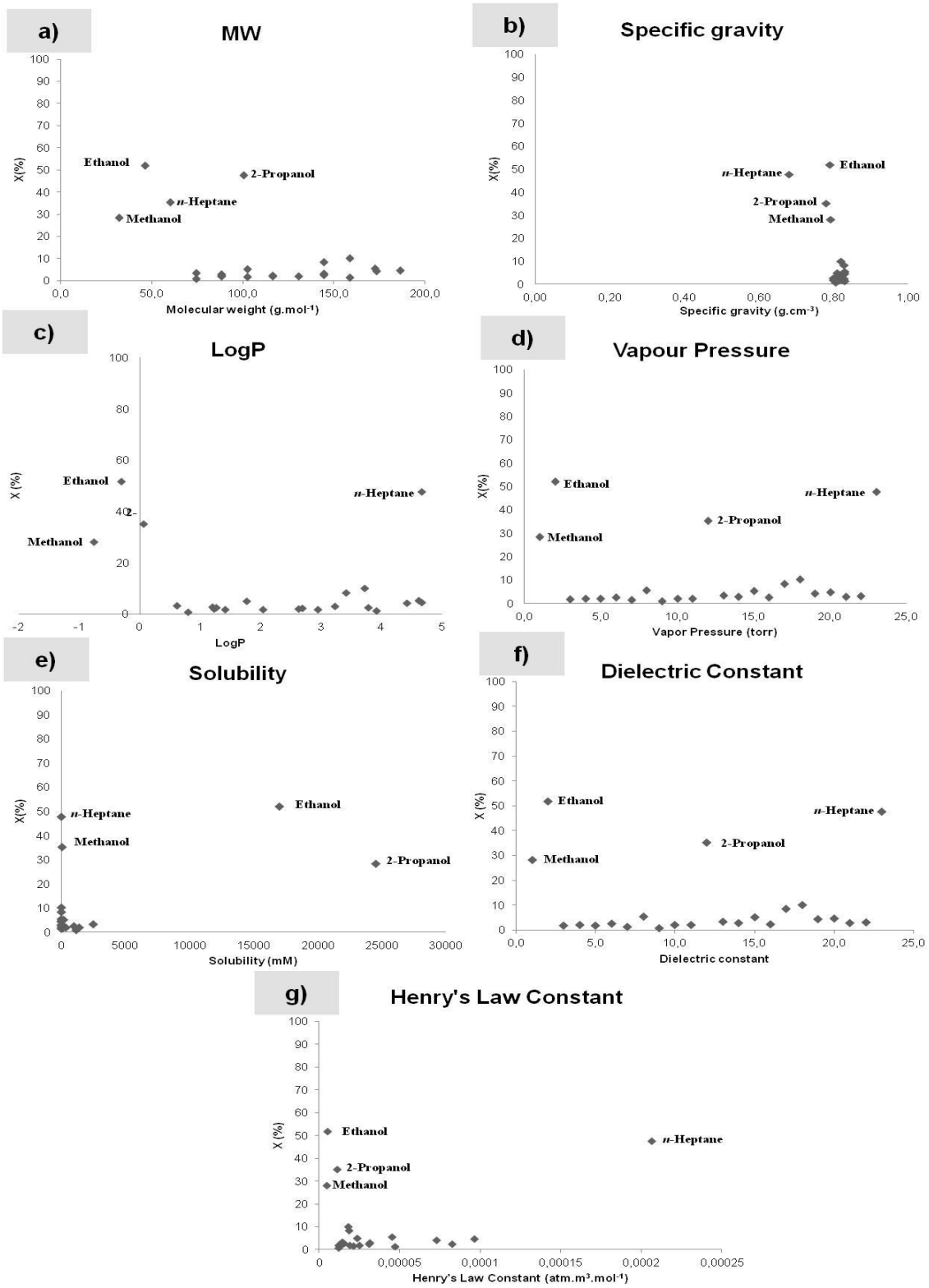


Figure 17 - Relationship between enzyme catalytic activity of cholesterol oxidase and solvent properties.

When looking to previous studies concerning the suitability of organic solvents in catalytic reactions, a pattern is extremely hard to find (Figure 17). Some author's claim a possible correlation between high conversions and logP (Cruz et al., 2004), others emphasize its simplicity, misleading the results obtained in experimental work. Filho et al., (2003) established that logP was not an adequate parameter to describe the performance of an alcohol dehydrogenase-catalyzed reaction and, moreover, that a single physicochemical parameter is incapable of satisfactory prediction of the enzymes behavior in biphasic systems. This suggests that a set of properties is required to the solvent choice, not only the hydrophobicity (logP) but also its functionality, in order to describe the interaction of the functional groups and the molecular structure of the solvents with the enzymes. Marques et al., (2009), verified that methanol and ethanol were the most suitable solvents when concerning the conversion of β-sitosterol using whole cells as biocatalysts. Also in this case a multi-parametric model was suggested (Equation 2).

$$AD = 0.644 \text{ Henry's constant} - 0.031 \text{ Dielectric constant} \quad (2)$$

In addition it emphasizes the importance of evaporation rate and reaction time as relevant parameters when screening for an organic solvent and not only the properties of the solvent itself. The addition of organic solvents to enzymatic bioconversions to promote the delivery of the substrate (and act in some cases as a pool for product) has been commonly used, thus, several works focused also on the impact that this addition may cause to the conversion rates. In Wolff et al. (1999), two models were constructed to quantify the impact of changes on the media, temperature and other parameters. The model reflected that the duration of the reaction had largely increased, therefore, the conversion ratios would probably decrease if the same time windows were applied, when comparing to solvent-free environment.

In the process of choosing an adequate organic solvent to implement biphasic systems in biocatalytic reactions generally the most relevant issues are affinity to the substrates and/or products (defined by the dissolution capacity), low price, non-biodegradable, non-toxic to humans and relatively low volatility. However the most important feature is the biocompatibility to the biocatalyst. The interlacement between the solvent properties and the conversions obtained will be taken into account during this discussion.

With the results obtained in this work, a similar pattern was observed. While the low hydrophobicity of the solvent benefited ethanol, methanol and 2-propanol, other solvents, with logP values below 1 have displayed a practically null substrate conversion (2-butanol, 2-Methyl-1-propanol), evidencing what was stated in Wolff et al., (1999) that, even with highly hydrophobic solvents, the reaction time must be increased or else some solvents may significantly reduce the conversion rates. Moreover, *n*-heptane exhibited the highest logP value of the whole set and yet a near 50% substrate conversion was achieved (Figure 17b). This suggests that solvents with a *log P* value below 2 are toxic, whereas those with a *log* P_{oct} value above 4 are biocompatible, leaving a transitional region within. Nonetheless, the model has led

to misleading predictions when compared to experimental data, namely when solvents with different functional groups are involved

Another interesting result was obtained when looking to the correlation between the Henry's law constant and enzyme activity. Henry's constant represents the partition of the substance between the atmosphere and the aqueous phase. Generically speaking it is a measure of the volatility of the organic compound. Equation 3 expresses the Henry's Law at constant temperatures, where p is the partial pressure of the substance, c is the concentration and k_H the Henry's law constant.

$$p = k_H c \quad (3)$$

It was expected that higher values of this constant were related to higher volatile species and, therefore, higher conversions were achieved due to a decrease in organic solvent quantity (since with the evaporation of the solvent the substrate tends to crystallize). However, in this case, the reaction only lasted 2 hours and the volatility of the compound may have not affected the conversion results. Nonetheless, this evaporation has a drawback that consists in the crystallization of the substrate which hinders biotransformation due to mass transfer limitations.

As it can be seen in Figure 17 - g, the high volatility of *n*-heptane had a positive effect over the performance of the enzyme. In the case of methanol, ethanol and 2-propanol a low volatility helped the catalytic process but the same cannot be said to compounds with equally low Henry's law constants, once again making it hard to find a pattern (Figure 17 - g).

In agreement with this correlation (Henry's law constant vs. conversion rates obtained for cholesterol oxidase reaction) it was also the correlation observed when crossing the vapor pressure data with the substrate conversions obtained (Figure 17 - d). Lower vapor pressure solvents tend to have slower evaporation rates. Therefore, since the evaporation of the organic solvent can lead to a higher availability of the substrate, the conversion yields could increase when the solvents present a higher vapor pressure. On the other hand, if the higher vapor pressure is observed, the crystallization of the substrate may occur, lowering the conversion yields. Thus, particular care should be given when designing experiments concerning organic solvents with a higher volatility, since the results may be misinterpreted due to alterations in certain environmental conditions. This phenomenon may be minimized using closed vessels, since it allows to reach a liquid-vapor equilibrium.

For the dielectric constant, which represents the polarity of the solvents, it was expected that less polar solvents obtained better conversion values (Marques et al., 2009). As in the referenced article, ethanol and methanol corresponded to that expectations but the same has not occurred in 2-propanol and *n*-heptane (Figure 17 - f).

In the remaining figures the trend was similar. It is also suggested that, in these correlation studies, a multi-parametric analysis is required. Moreover, it is again emphasized that logP can not be chosen as the main influencing parameter in solvent screening for biocatalysis. Also, some of the parameters studied may only be applicable with larger exposure times to the solvents. In that case, evaporation rates must be taken in to account and, probably, a different substrate concentration and larger organic phase volume were required, decreasing the screening throughput. One of the initial goals of this work was to build a multiparametric model, which was not achieved due to lack of consistence results and lack availability of the analytic equipment. Since the solvent screening is not a simple task, because it will always be a compromise between the different characteristics, a more dedicated study is needed to achieve this pre-defined goal.

3.4.1 Stability trials

At this point, the screening process had reduced the initial set of organic solvents to four. Nevertheless, the main objective was to choose only one of them to this specific work. The screening was refined with the aim of verifying which of the four was the most effective solvent under large experimental run times (in the scope of a continuous mode operation). These stability assays were performed both for immobilized and free cholesterol oxidase. The results obtained for the free enzyme are shown in Figure 18.

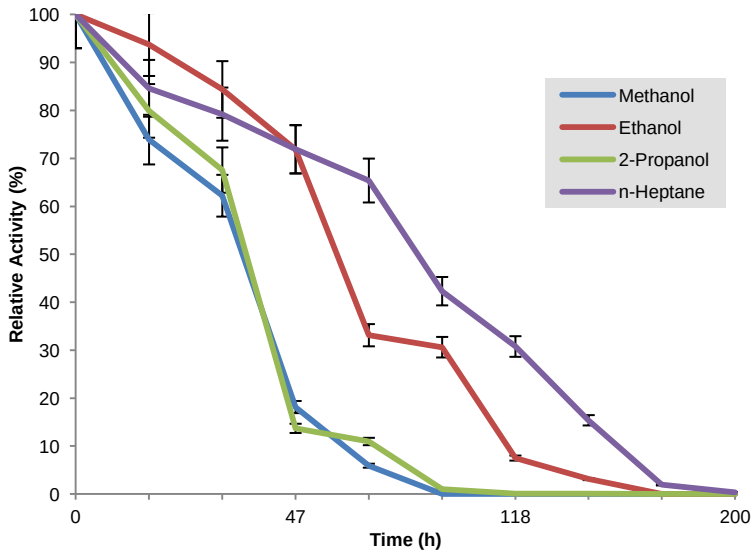


Figure 18 - Stability of free cholesterol oxidase in the presence organic solvents using magnetically agitated microreactors containing an aqueous phase of 100 mM, pH 7 phosphate buffer and cholesterol dissolved into the organic phase.

In this figure, the enzyme had a higher stability in ethanol and *n*-heptane when compared to 2-propanol and methanol. During the first 47 hours, the activity of the enzyme was high (>70%), especially in ethanol and *n*-heptane. From this stage on, the activity decay was sharper and, at 70 hours of reaction, the enzyme had already lost more than 50% of the initial activity in all four

solvents. While in methanol and 2-propanol the enzyme lost complete activity at about 100 hours of reaction, in ethanol and *n*-heptane the enzyme presented activity for almost 200 hours (in the case of *n*-heptane complete loss of catalytic activity was reached later than in ethanol). Although the activity loss percentage was similar in both ethanol and *n*-heptane, generally, the enzyme had higher activities when in contact with the latter. In figure 16, ethanol obtained the highest substrate conversion and, in figure 18, the conversion obtained when using ethanol as the organic phase were again the highest. After larger periods of time *n*-heptane was shown to provide higher relative activities than ethanol, demonstrating the stability of the free enzyme when in contact with this organic solvent. This elucidated the importance of the study of the enzyme activity along large periods of time, describing a more accurate evaluation of the enzyme's behavior. Determining the biocompatibility of organic solvents requires the characterization of both molecular and phase toxicity. Molecular toxicity is related to the logP coefficient and is due to the dissolution of the solvent molecules into the aqueous phase (Léon et al., 1998). Ethanol, since has a relatively high solubility in water may be included in this group when concerning a long-term biphasic reaction. Phase toxicity is caused by the presence of an aqueous/solvent interface (low solubility solvents are included in this group), which may lead to the partitioning of essential components from the aqueous phase, limiting their availability (those characteristics tend to have a more accentuate effect over whole-cell biocatalysts). Nonetheless *n*-heptane and other solvents with low octanol/water partitioning coefficients can present this kind of toxicity (Léon et al., 1998). This means that we are dealing with two different toxicity types and that may be the reason why different stability patterns were achieved.

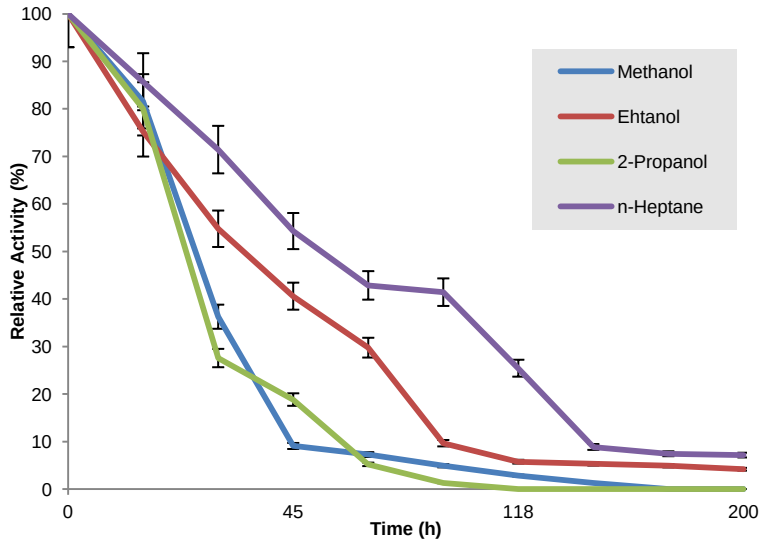


Figure 19 - Stability of immobilized cholesterol oxidase in the presence organic solvents using magnetically agitated microreactors containing an aqueous phase of 100 mM, pH 7 phosphate buffer and cholesterol dissolved into the organic phase.

With the immobilized enzyme, *n*-heptane provided once again a better stability to the enzyme. In this case, ethanol offered lower relative activities during the whole period of the trial, unlike in the free enzyme. It was also noticed that the activity of the enzyme was maintained for longer

periods of time than in the last assays, which can be once again attributed to the protective characteristics of the immobilization support. While in methanol and 2-propanol the enzyme exhibited a total loss of activity after 200 hours of the experiment, ethanol and *n*-heptane enable a residual activity of the enzyme after the same period of time. Nevertheless the enzyme was more stable than in the free form for the four organic solvents tested, due to the already mentioned protective character of the immobilization support.

These stability trials were not performed to catalase but this will be taken into account. The degradation of hydrogen peroxide by catalase is a secondary reaction while cholesterol oxidase plays the most relevant role. The secondary reaction may always be adjusted through, for instance, reactor engineering, concerning the possibility of a lower activity in the presence of a specific solvent.

3.4.2 Oxygen availability trials

After the stability trials, methanol and 2-propanol were excluded from the screening process and the two remaining organic solvents were submitted to an oxygen availability trial. Most industrial microbial processes are aerobic, in these processes, oxygen is an important nutrient that is used by the microorganisms for growth, maintenance and metabolite production, and scarcity of oxygen affects the process performance (Gracia-Ochoa et al., 2009). In the case of this specific reaction it is known that oxidases use molecular oxygen as electron acceptor to produce hydrogen peroxide. Therefore, the availability of oxygen in the reaction media is also an essential parameter when the reaction viability is being evaluated.

The intent of this trial was to estimate the oxygen concentration in a biphasic environment to assess experimental conditions in which there are no oxygen limitations. This is a crucial parameter since in small scale aeration is difficult to perform. To simulate this, two conditions were used to mimic efficient aeration and non-ideal aeration. This was achieved by conducting the experiments in microtiter plates with agitation (forced aeration) and without agitation (passive aeration).The reaction was performed for 2 hours, the enzyme (in free form) and substrate concentrations were 0.1 $g.l^{-1}$ and 1 $g.l^{-1}$, respectively. As referred in the *Materials and Methods* section, these assays were performed in a 24-well microtiter plates equipped with oxygen probes and connected to the proper software.

The first step was to calculate the oxygen concentration present in aqueous phase. Knowing the solubilities of oxygen in both solvents and the oxygen transfer coefficients it was possible to calculate the amount of oxygen available to be used in the reaction of cholesterol oxidation. The expression that follows those assumptions is:

$$\frac{dC}{dt} = k_L a(C - C_L) - q_{O_2} x \qquad (4)$$

Where $k_L a$ represents the oxygen transfer coefficient (s^{-1}), C the oxygen concentration in the media (mM), C_L the oxygen concentration in the liquid (mM), q_{O_2} the consumed oxygen during the reaction period (mM) and x the enzyme concentration (mM). In order to be enough oxygen in the media available to the reaction:

$$k_L a(C - C_L) > q_{O_2} x \qquad (5)$$

And in equilibrium:

$$k_L a \,.\, C > q_{O_2} . x \qquad (6)$$

The amount of oxygen consumed during the reaction was estimated using the enzyme kinetic parameter v_{max} previously determined (v_{max} = 2.74 x 10^{-6} M.s^{-1}). The oxygen consumed during the reaction was assumed to be the same in all the reactions since the enzyme and the reaction times did not vary between each trial ($q_{O_2} = 3.8 \times 10^{-7}$mM). The remaining values to perform further calculations are gathered in Table 12.

Table 12 – Oxygen transfer coefficients and oxygen concentrations

Considered Data		
k_La (w/ agitation)	0.077 s^{-1}	Marques et al., 2010c
k_La (w/o agitation)	0.015 s^{-1}	
$C_{ethanol}$[7]	0.24 mM	
$C_{n\text{-heptane}}$	12 mM	

[7]The values considered concerning ethanol calculations correspond to the ones for water, due to the solubility of this solvent in the aqueous phase, forming a monophasic media

Table 13 resumes the obtained values obtained by applying equation 4, showing that the oxygen availability was not a limiting step to this reaction.

Table 13 - Evaluation of the molecular oxygen availability to the catalytic reaction

		k_La .C	$q_{O_2.x}$
Ethanol	w/ agit	0.018	1.27x10^{-9}
	w/o agit	0.004	1.27x10^{-9}
n-heptane	w/ agit	0.924	1.27x10^{-9}
	w/o agit	0.180	1.27x10^{-9}

One important phenomenon that plays an important role in the oxygen availability during a reaction is the oxygen transfer rate (OTR). Due to the importance of gas exchange in reactions where the lack of oxygen is a limiting factor, particular care is given to favor oxygen transfer into the liquid phase. A favorable OTR was achieved when agitation was imposed to the system, thus, when the plate was under agitation, the oxygen concentration read in the probe was

higher than when the plate was not submitted to agitation. This happened due to the renovation of the air that the agitation provided, enabling the different portions of the media to be read in the probe, particularly when *n*-heptane was used as substrate pool (Figure 21). As it can be seen by the figures 20 and 21, the concentrations read on the plates during the reaction period reached a steady state. This happened due to the measurement range of the plate itself. According to the specifications of the plate the maximum range of reading is 250% of air saturation (OxoDish® by PreSens), which probably was below the values obtained during the trial performed. It was also noticed that *n*-heptane obtained higher concentration of oxygen. This happened due to the higher oxygen concentrations present in this solvent (Table 12), which provided a higher oxygen availability to be used during the reaction. In ethanol, the concentration is lower since it is soluble in water, thus it forms a monophasic media. The oxygen concentration is assumed to be the same in water and in ethanol.

The oxygen solubility values in water were obtained from Lewis (2006). It is known that the concentration of molecular oxygen in water varies with the increase of the temperature. Although the temperature was set to 30ºC it was registered by the probe that the initial temperature of the reaction was 25ºC and after 1 hour it raised to 30ºC, thus the reference oxygen solubilities varied along the reaction time. The values of oxygen solubility at different temperatures were considered since the probe incorporated in the microtiter plate is calibrated to read the oxygen values in water and not in the solvent, therefore referenced solubility values were incorporated in figures 20 and 12.

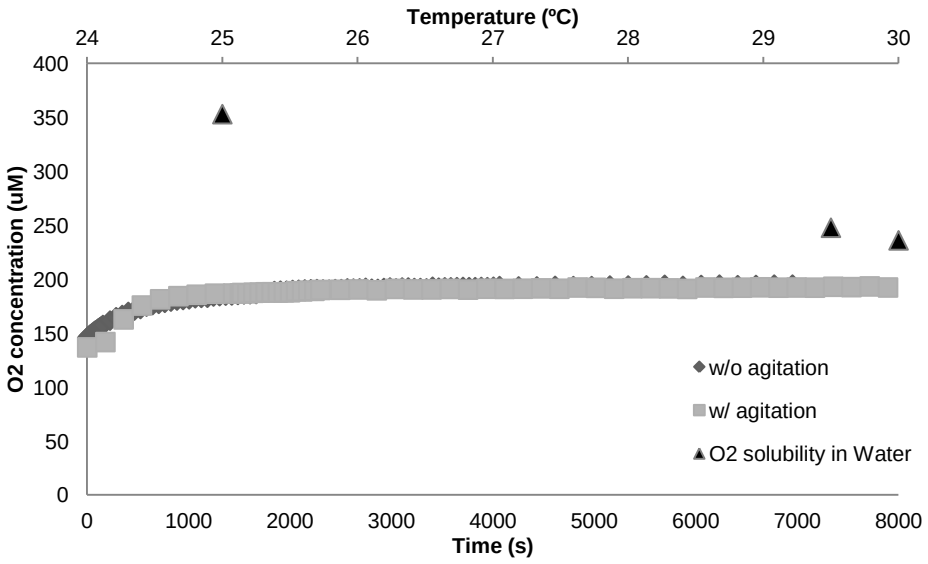


Figure 20 - On-line measurement of the oxygen solubility when ethanol was used as organic phase in 24-well microtiter plates incorporating an oxygen probe. The plates were even static or agitated at 200 rpm.

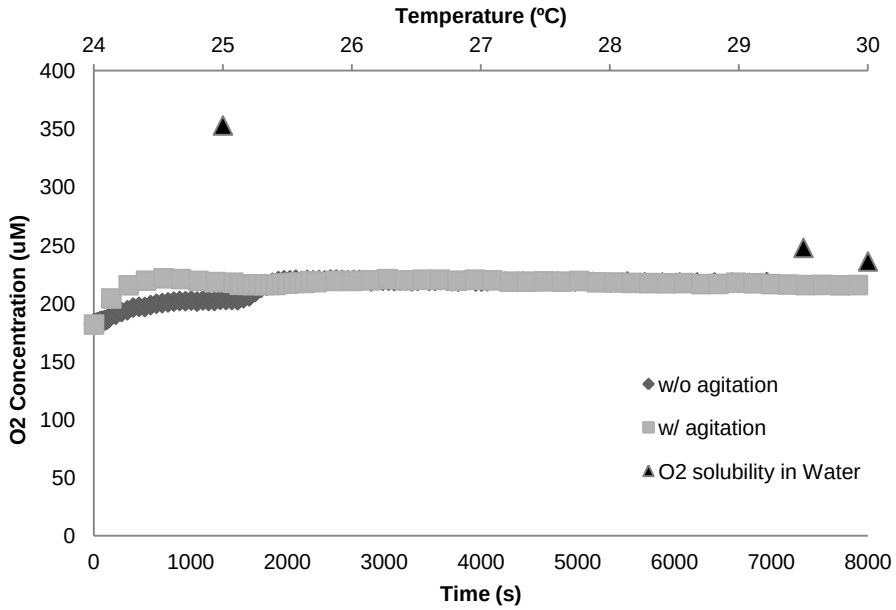


Figure 21 - On-line measurement of the oxygen solubility when *n*-heptane was used as organic phase in 24-well microtiter plates incorporating an oxygen probe. The plates were even static or agitated at 200 rpm.

Altogether, these results have shown that both solvents were suitable to use as organic phase in the proposed bioconversion trials. However, *n*-heptane had demonstrated not only a longer stability but also higher oxygen availability, providing the most balanced set of results between the two organic solvents. Moreover, it can eventually be used in on-line measurement device, since the eluent used in HPLC is mostly composed by this solvent. The *n*-heptane insolubility in water provided an easier withdraw of the samples along the reactions, which made the analytical process faster and simpler and also the capacity to carry larger amounts of cholesterol in comparison with ethanol.

3.5 Bioconversion Trials

The bioconversion trials executed envisaged an optimization process towards a continuous mode operation. It had a major objective of incorporating cholesterol oxidase and catalase within the same reactional system, as a multi-step enzymatic process using micro sized devices. The adoption of green chemistry guidelines in processes with industrial potential has been widely explored. Examples of such practices involve the use of recoverable reagents, mild temperatures and the in-process elimination of the by-products produced (Marques et al., 2010a). This system eliminates the by-products formed during the reaction which, besides the green approach, it provides enzyme industrial feasibility due to the aid in downstream process.

The trials provided a more detailed adjustment of some system variables in order to proceed to the construction of the continuous system. The stability trials were performed once again but in this case concerning the bienzymatic reaction and not only each enzyme per se. The utilization of *n*-heptane as organic phase was compared once again with the utilization of ethanol and predictive models were constructed to evaluate the stability of the enzymes. Although *n*-heptane is not inserted into the green solvents role, during the continuous mode operation the organic solvent will be reused, therefore, despite the lack of green properties in *n*-heptane, the overall process follows a green approach. In the utilization of this setup, immobilized enzymes were a preferable choice but soluble enzymes were also tested as a comparison with catalase and the several methods of using cholesterol oxidase and catalase, such as cholesterol oxidase and catalase immobilized within the same support and cholesterol oxidase and catalase in different immobilization supports but acting together inside the same reactor.

In order to analyze these results additional information was gathered with the usage of two different activity models, bi-exponential adjustment model and exponential deactivation model, presented in Equations 7 and 8, respectively (Aymard et al., 2000).

$$Act = Ae^{\beta t} + (1 - A)e^{\alpha t} \qquad (7)$$

$$Act = e^{-kt} \qquad (8)$$

In the bi-exponential model Act stands for the enzymatic activity, A, β and α are the three variable parameters and t is the reaction time. In the deactivation model Act is the enzymatic activity, t the reaction time and k the variable parameter.

Following the observation of the results obtained, the most accurate model was chosen to predict the behavior of the enzymes. As an example, Figure 22 shows the behavior of the two distinct models and a clear approximation of the bi-exponential adjustment model to the experimental results. The remaining data relative to the exponential deactivation model is not shown in this thesis for ease of understanding.

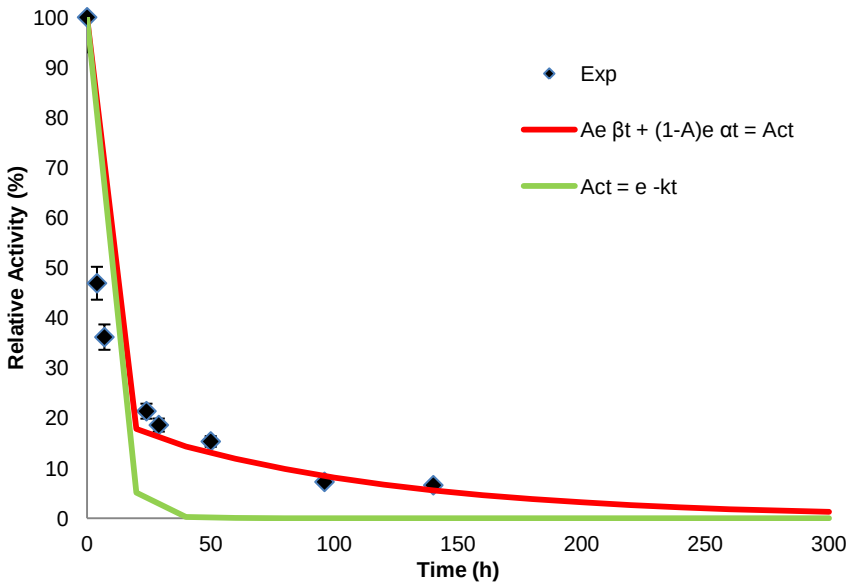


Figure 22 – Results obtained using two different predictive models demonstrating an approximation of the model represented by the red line to the experimental results. Data corresponds to immobilized catalase in PVA-PEG within *n*-heptane (with residual water content) using a 10 ml, magnetically stirred microreactor.

3.5.1 Stability assays

The following figures (23 to 28) represent the stability of both enzymes in different conditions and the prediction of the enzymes behavior up to 300 hours runs, following a bi-exponential adjustment model. All experimental assays were performed at least for 100 hours. The experimental procedure was similar to the one explained for previous stability assays and proper construction of figures 18 and 19. The aim was to choose suitable conditions to perform a continuous mode operation using both enzymes, specifically to decide if both enzymes should work separately or together in the same reactor system and if they should be co-immobilized or use catalase either in a free from or immobilized.

Catalase behavior showed a relative activity with high values within this time window. In both immobilized and free forms the enzyme had slower activity decay, when compared to cholesterol oxidase performance. As expected, when *n*-heptane was present, the activity had a higher activity loss despite the protection character of the immobilization support, which can indicate that this organic solvent interacts with catalase activity. Figure 23 - b shows a predicted relative activity over 60% until 300 hours of use, which can be an indicator that it could be the proper choice to use in the subsequent runs, towards the production platform. Is noteworthy that these results correspond to the usage of catalase alone, without the presence of cholesterol oxidase or its substrates and products. This can be seen as control results for catalase behavior.

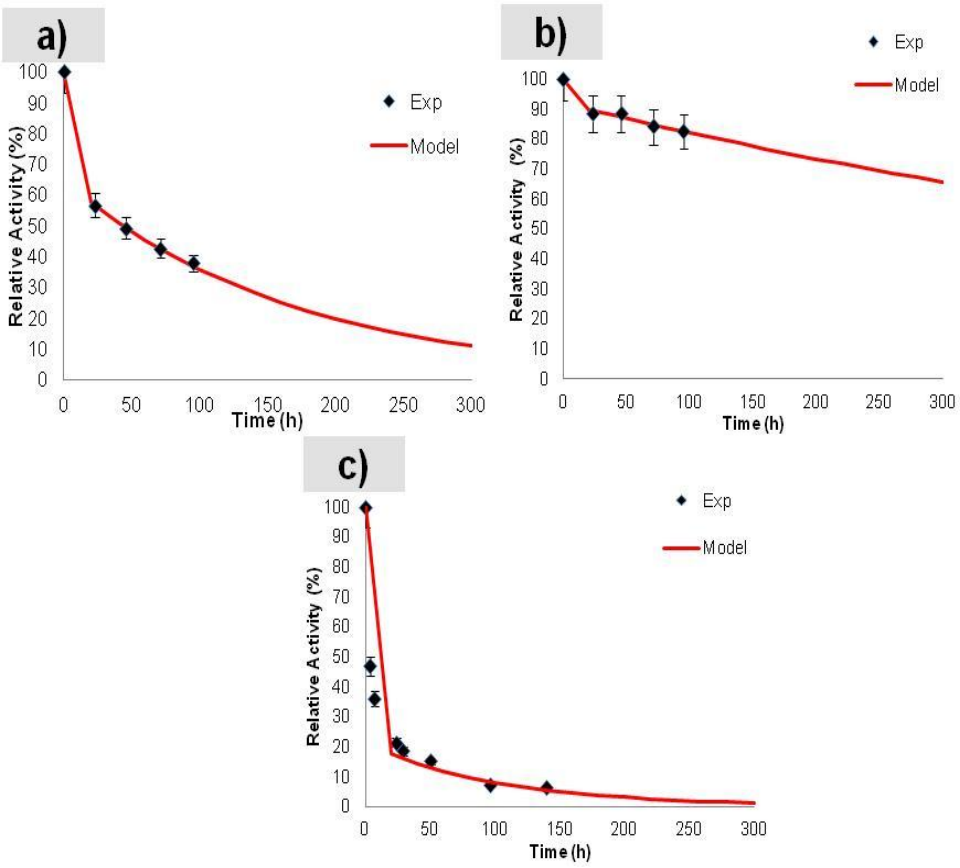


Figure 23 - Catalase stability along 300 hours run using 10 ml magnetic stirred microreactors in different batch conditions. a: free catalase within phosphate buffer; b: immobilized catalase in PVA-PEG within phosphate buffer; c: immobilized catalase in PVA-PEG within *n*-heptane with 1% water content.

The figure below reveals once again some relevant points already given during this work. Not only a preferable usage of the immobilized enzyme but also a higher stability of *n*-heptane in a long term perspective (Figure 24). Beyond these facts new data is revealed related to the usage of an organic monophasic system to perform this reaction. Figures 24 - e and 24 - f are here represented as a control data and when comparing the model obtained in 24 - f with 24 - d a similar activity is predicted (e.g. at 300 hours). This fact opens a possibility to discard the usage of an aqueous phase or alter the 1:1 proportion of the two phase aqueous-organic system.

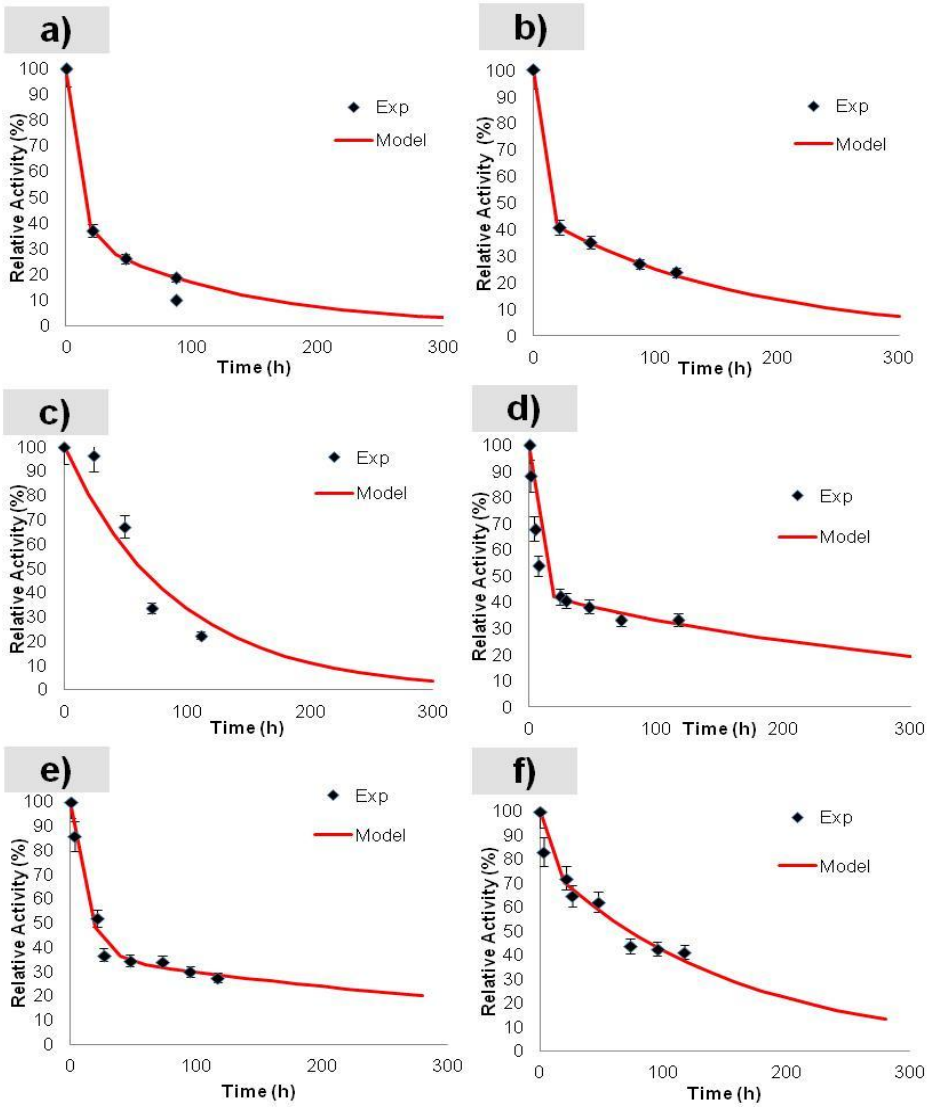


Figure 24 - Cholesterol oxidase stability along 300 hours runs using 10 ml magnetic stirred microreactors in different batch conditions. a: free cholesterol oxidase within ethanol; b: immobilized cholesterol oxidase in PVA-PEG within ethanol; c: free c.o within *n*-heptane; d: immobilized cholesterol oxidase in PVA-PEG within *n*-heptane; e: immobilized cholesterol oxidase in PVA-PEG in a biphasic system composed by ethanol and phosphate buffer; f: immobilized cholesterol oxidase in a biphasic system composed by *n*-heptane and phosphate buffer.

The following results comprehend the use of cholesterol oxidase and catalase simultaneously, within the same immobilization support. This has the advantage of allowing the use only one reactor in the continuous mode system. This procedure followed the same protocol as it was described before and it was tested in different batch conditions. The stability curves obtained show lower relative activities when using the enzymes by itself within this support. It can be explained by a possible interaction of each enzyme conformation with the other, hindering a proper forthcoming of the substrate to the active center.

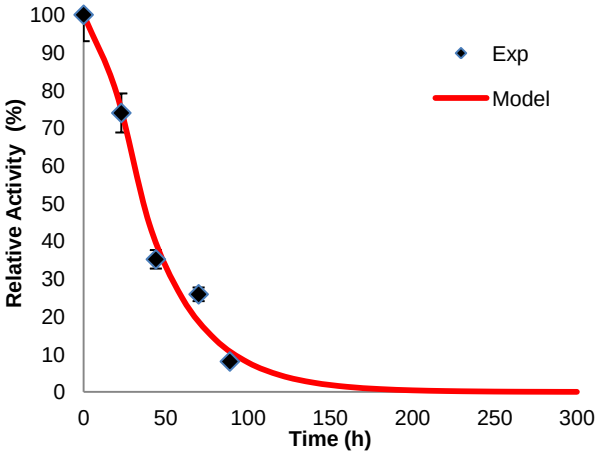


Figure 25 - Catalase stability along 300 hours runs using 10 ml magnetic stirred microreactors when co-immobilized with cholesterol oxidase in a PVA-PEG support and in phosphate buffer

Figure 25 relies on the behavior of catalase when inserted in the conjunct support and a clear diminish of the enzyme activity is noticed when compared with the curve obtained in figure 23b.

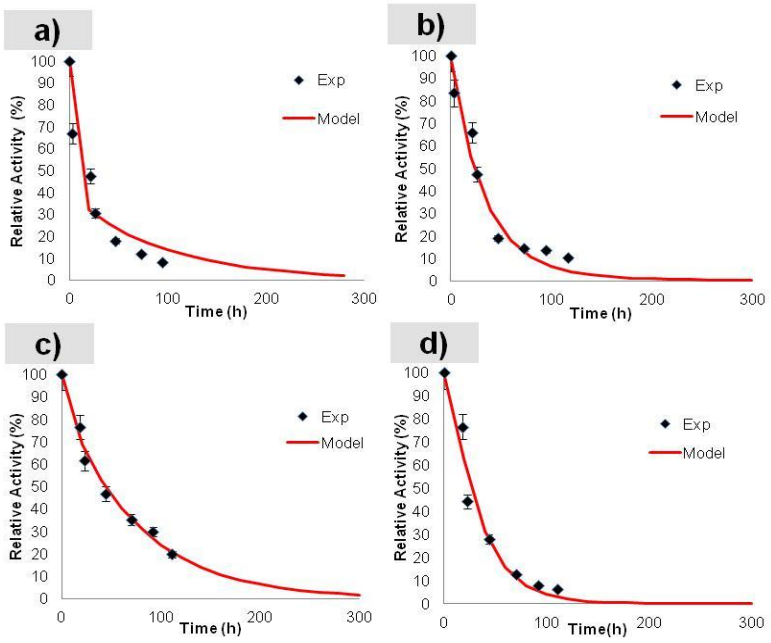


Figure 26 - Catalase stability when co-immobilized with cholesterol oxidase in PVA-PEG support and in different batch conditions. a: within ethanol; b: within *n*-heptane; c: in a biphasic system composed by ethanol and phosphate buffer; d: in a biphasic system composed by *n*-heptane and phosphate buffer.

Once again the higher stability of the enzymes when inserted in an organic system, composed of *n*-heptane, could be observed. Also in this case, the behavior of the enzymes in a non-aqueous system was tested, suggesting a possible route to follow during the continuous mode operation optimization.

3.5.2 Different aqueous-organic system ratios

Following the stability assays, the priority was to work with both enzymes simultaneously in the same system. This could be achieved by: i) the incorporation of both enzymes within the same immobilization matrix or, ii) by using both enzymes in different supports or iii) by using both free enzymes inside the same reactor, which is less preferable. The later was not chosen due to the constraints that the use of the free enzyme brings to the downstream processes. It may be viable if an adequate cut-off membrane was used, enabling the reuse of the free enzymes but this will significantly increase the complexity of the process. When using an immobilized enzyme one of the benefits is the possibility of their reuse without the need of a specific filtration system. The reuse makes a process more economically viable, thus recycling an expensive component provides to cost reduction of the overall process. There was also the choice between using one or two reactors in the continuous design which was also taken into account in this context and discussed in more detail in *Continuous-Mode Operation* section.

The first trial was performed to determinate the effect of the reduction of the water content in the two-phase aqueous-organic system. This procedure was performed to both free and immobilized form of cholesterol oxidase and the results are shown in the following figure.

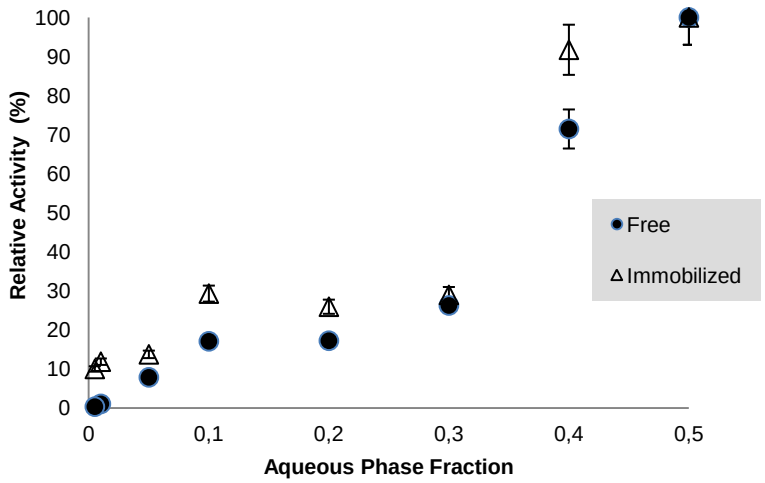


Figure 27 - Effect of the aqueous phase fraction (v/v) over cholesterol oxidase activity using 10 ml, magnetically stirred, biphasic microreactors.

These results revealed that, even when the enzyme was immobilized, it was not advisable to remove the aqueous phase from the reactor. The decrease of the aqueous phase fraction significantly lowered the conversion yield, which only achieved promising values for an aqueous phase fraction in excess of 30%. Water is essential to enzymatic activity, thus it provides the physiological pH for the enzymes to work (and organic solvents do not present physiological pH). The differences observed may have occurred due to alterations in the interfacial area arising from the variation of the volume of both phases, as reported in (Cruz et al., 2001)

The process used in the present work lacks optimization and if the aim was the reduction of the aqueous phase, some alterations of the immobilization process can be the solution. The water content may be added to the support or the use of other supports may be more suitable. In this case, the aim was to verify the intensity of the effect that the reduction of water had over cholesterol oxidase performance, since it has already been shown that this parameter influenced the stability of the enzyme (Figures 23, 24 and 26).

3.5.3 Co-immobilization assays

The next trial was mainly to evaluate the differences between the usage of both enzymes simultaneously in the same support or separately (either in the free form or immobilized). Inside each batch reactor different combinations of the enzymes were placed and the results obtained are showed in the next two figures. The results gathered in Figure 28 represent the activity obtained after one hour of reaction.

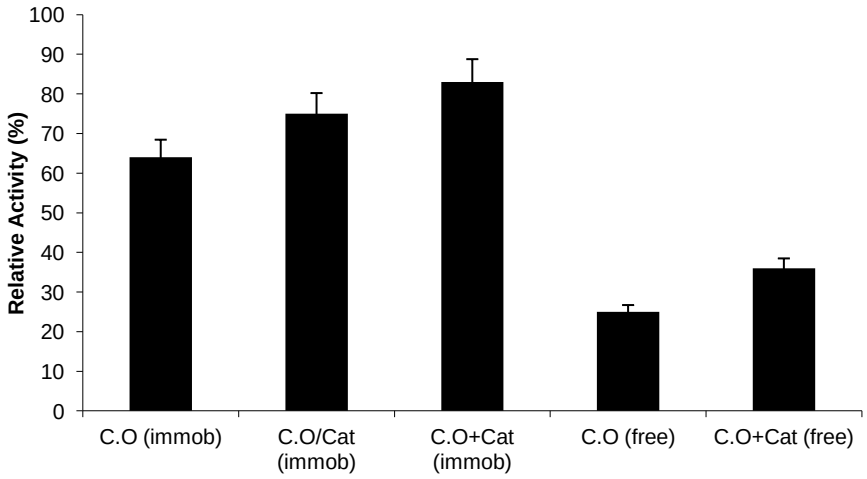


Figure 28 – Cholesterol oxidase (CO) and catalase (Cat) activity after 1 hour of reaction in different combinations. *C.O/Cat*: both enzymes within the same support; *C.O+Cat*: both enzymes in the same reactor but immobilized separately.

The highest activity was observed when cholesterol oxidase and catalase were separately immobilized when compared to the remaining enzyme formulations considered (Fig. 28). This can be due to a hydrogen peroxide free environment given by the action of catalase over the reaction media or simply to sampling errors. The results concerning the behavior of the enzymes after 5 hours of reaction are shown in Figure 29 and reveal the same results that are disclosed by the figure above.

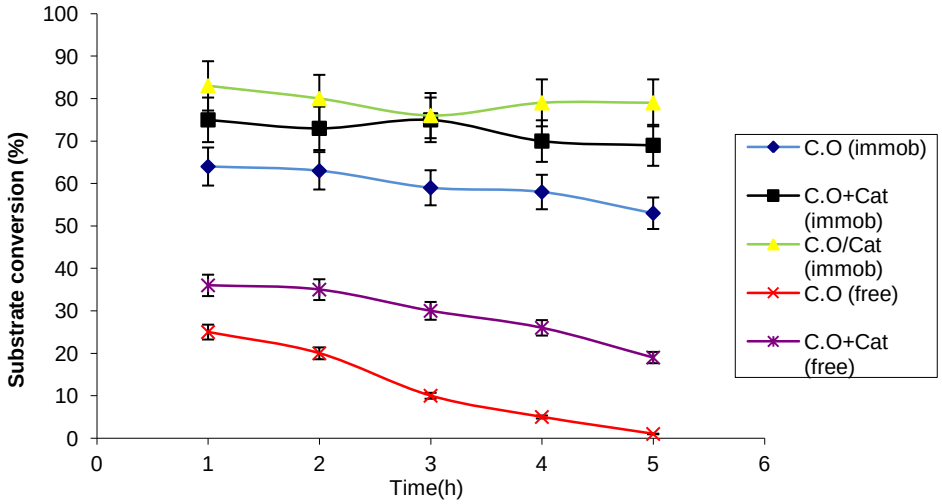


Figure 29 - Cholesterol oxidase (CO) and catalase (Cat) activity curves after 5 hours of reaction. *C.O/Cat*: both enzymes within the same support; *C.O+Cat:* both enzymes in the same reactor but in different supports.

As expected, the enzymes in the free form had obtained lower activity profiles than the ones immobilized. It was as well expected that cholesterol oxidase and catalase immobilized within the same support did not perform as well as cholesterol oxidase alone, due to a possible interaction of the enzymes with each other or with the support, as seen in figure 26. The results have showed that further optimization was needed to effectively use a co-immobilization procedure and, therefore, the enzymes will be placed in separate immobilization supports, using the PVA-PEG protocol.

Enzymatic co-immobilization has been widely used and satisfactory results have been obtained. Not only it may provide cost reductions due to the use of a unique immobilization support but it may reduce the time of diffusion of the substrate to the second enzyme (Ge et al., 1999). Conventional immobilization enables multi-step reactions or co-factor regeneration, which may be applied in several industrial processes (Erhardt et al., 2008), such as in biofuels enzymatic cells fabrication area (Shim et al., 2011).

3.5.4 Oxygen supply assays and solvent evaporation

The oxygen supply trial consisted on the evaluation of the enzyme behavior when different concentrations of oxygen were present inside the reactor. This trial aimed to establish whether or not an oxygen supply device should be integrated in the continuous mode setup. This is an important parameter since it is already known that a certain amount of oxygen is necessary to perform the reaction but it was already shown that the oxygen content reaches a steady state in the reaction media, providing enough oxygen to the reaction progress. The question was to evaluate if a higher amount of oxygen supply would inhibit the bioconversion, as it had been reported by Marques et al. (2010).

Four assays were performed: a control reactor with no oxygen content (the oxygen was removed by the use of a nitrogen inflow), a reactor opened to atmosphere with no forced aeration; two reactors, submitted to 5 and 20 minutes of forced aeration, providing higher concentrations of oxygen

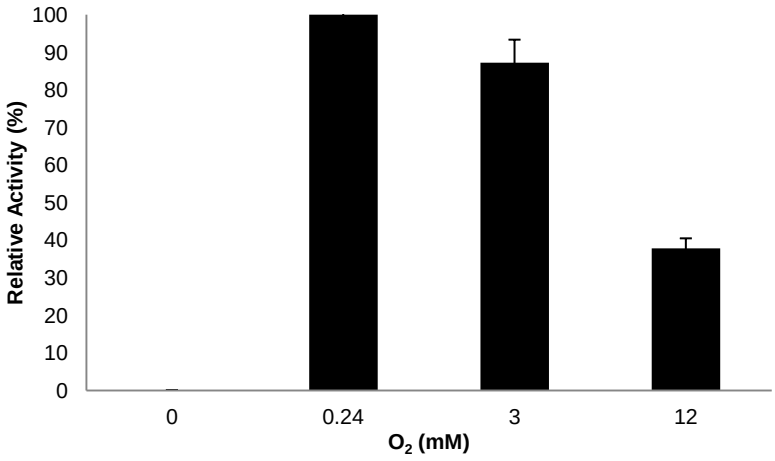


Figure 30 - Activity of cholesterol oxidase in the presence of different oxygen concentrations using 10 ml, magnetically stirred, biphasic microreactors.

All assays were performed with immobilized cholesterol oxidase and in a biphasic environment with *n*-heptane composing the organic phase. The reactions occurred for 2 hours and the cholestenone production results are shown in Figure 30 in terms of the variation of the relative enzymatic activity with the oxygen concentration.

The oxygen concentrations were determined through a calibration curve (oxygen concentration vs. time), which was measured with an oxygen probe, as stated in Marques et al., (2010c). The probe was calibrated to measure the oxygen concentrations not only in aqueous media but also in organic solvents (data not shown). The results revealed that within a media without oxygen the reaction did not occur, demonstrating that oxygen is an essential component to the enzyme function. Also, that higher conversion rates were obtained with an open reactor and lower concentration of oxygen. Moreover with an oxygen concentration increase over 10 fold, the

conversion loss was not in the same proportion, providing a satisfactory enzyme activity. The same did not happen with a 12 mM concentration of oxygen, where the enzyme activity was low and inhibition may have occurred, which was in agreement with the results obtained in Marques et al., 2010c.

The difference between supplying additional oxygen and use an open reactor relies on the fact that, with the open reactor, the evaporation of the organic solvent will occur, especially in a long term operation. There was the possibility of operating in a closed circuit with the reactor remaining closed, as it was shown to be possible in Figure 21 to *n*-heptane. Nonetheless, in the long term this might not be the right solution since the reaction is oxygen-dependent and the incorporation of an oxygen supply device is imperative.

3.6 Continuous-mode operation

3.6.1Toxicity of hydrogen peroxide

Other important aspect that was left to test was to inquire if hydrogen peroxide alters the behavior of cholesterol oxidase. Although it is known that this component it often a drawback in downstream processes and might have an inhibitor role in biocatalysis (Masouka et al., 1996, Woodley, 2008), the quantification of the toxicity is an important parameter to discuss in a optimization process.

These trials were performed in a batch reactor, at 30ºC with magnetic stirring and the enzyme was used in the immobilized form and. The reactions occurred for 2 hours. The tested concentrations revealed, as expected, a reduction in the enzyme activity as the hydrogen peroxide concentration increased. The maximum concentration tested was 10M and when this amount was present in the reaction media the enzyme had lost over half the activity, comparing with free-hydrogen peroxide media.

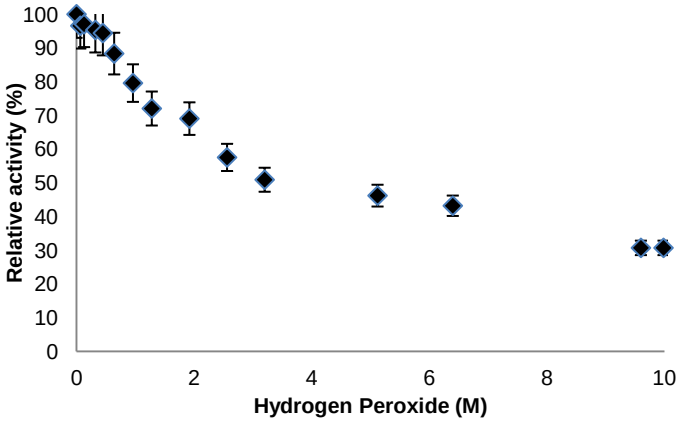


Figure 31 - Activity of cholesterol oxidase in the presence of different concentrations of hydrogen peroxide using 10 ml, magnetically stirred, biphasic microreactors.

Figure 31 illustrates the inhibition power of H_2O_2 and the urge to a proper elimination system, showing that besides the need to purify the final product there is also a toxic character towards cholesterol oxidase if no catalase is used.

3.6.2 Continuous mode setups

The used continuous mode system was totally assembled in the BERG laboratories, limited to the available devices and space. The setups are schemed in Figure 8 and 9 in the *Materials and Methods* section.

Two setups were assembled during this work. Both were constructed following the results obtained and discussed previously. The variable parameters inherent to this bi-enzymatic process were chosen according to a continuous mode operation point of view and are resumed in Table 14.

Table 14 – Resume of the determined conditions defined during the previous assays

Reactional Temperature = 30ºC
Aqueous media composed of a 100 mM, pH 7 Phosphate Buffer
Enzymes concentration = 0.1 $g.l^{-1}$
Cholesterol concentration = 1 $g.l^{-1}$
Immobilization of the enzymes is essential (PVA-PEG method)
n-heptane is the suitable organic solvent to use as substrate pool
Hydrogen peroxide has an inhibition effect over cholesterol oxidase function
Bioconversion efficiency is favored if the two enzymes are immobilized separately
The system has to be oxygenated (preferably at a concentration of 3 mM)
The system must be closed to avoid evaporation

In Marques et al., (2011), a continuous setup concerning the usage of cholesterol oxidase and catalase within the same system was also assembled. In this case a microchannel reactor was used to perform the steroid bioconversion. At the output of the microchannel, a packed-bed reactor (PBR) containing immobilized catalase was coupled. This system, as the one assembled in this work, was operating at 30ºC, with cholesterol concentration of 1 $g.l^{-1}$, with an oxygen inlet and the enzymes were also immobilized in a PVA-PEG support. The results revealed that, in the steroid bioconversion step, the enzyme could keep 30% of its initial activity after 300 hours of reaction. The activity of catalase inside the PBR was enough to achieve a 100% reduction of hydrogen peroxide. To improve this system the authors refer the increase of the residence times (higher than the tested 14 $ul.min^{-1}$).

In the present setup the guiding lines gathered in Table 14 were all incorporated: both enzymes were immobilized in PVA-PEG support; both reactors were at 30ºC and the phosphate buffer had a concentration of 0.1M phosphate buffer pH 7; the organic phase was composed by *n*-heptane; the hydrogen peroxide decomposition was achieved by the use of catalase; the main

reactor was closed, at low temperatures temperature and had an appropriated oxygenation system.

The enzymes were placed in separate reactors. This choice was made by the results obtained in the bioconversion trials concerning the possibility of a co-immobilization of the enzymes (Figures 28 and 29). The reactor conformation and disposition had to do with the fact that the enzymes had to be placed in reactors with two different operation modes. As it is observed in the schemes one reactor is identified as being a plug-flow reactor (PFR) and the other is a fluidized-bed reactor (FBR). The PFR carried the immobilized cholesterol oxidase since it is advantageous to use a reactor in which the enzyme is occupying all the possible spaces, increasing the residence time inside the reactor, the specific area of mass transfer and consequently the conversion yield. The same reactor will not be advantageous to use in catalase reaction because the formed hydrogen peroxide is a gas and, reaching a reactor with few spaces to diffuse may cause problems like clogging, increasing back-pressure of the system and rupture of the vessels. The fluidized-bed reactor was placed vertically and the liquid entrance was made from the bottom to the top, to favor the diffusion of the gases and the beads, which were able to move inside the reactor due to the flow movement. An amount of 4.4g of immobilized cholesterol oxidase was placed inside the PBR and an amount of 3 g was placed inside the FBR.

The setup schemed in figure 9 presents the incorporation of a spectophotometric analysis into the system. This additional section provided an online monitoring of the hydrogen peroxide amount present in the aqueous phase of the reservoir. This provided the control of the absorbance of the buffer and, therefore, the inference of catalase activity during the operation.

The trials to test both setups were performed using a flow rate of 0.5 $ml.min^{-1}$. Also in this work flow rates of 1.5 $ml.min^{-1}$ and 1.0 $ml.min^{-1}$ were tested (data not shown). No conversion was obtained during these trials, suggesting that higher residence times were needed to improve the yields. The samples were withdrawn, extracted and analyzed with a 30 minute interval, from the organic phase contained in the main reservoir.

The results obtained using the continuous mode setups using a 0.5 $ml.min^{-1}$ flow rate were not satisfactory, compared to the ones obtained using isolated batch stirred-tank reactors and with Marques et al., (2011), where microchannel reactors were used with a flow rate of 0.14 $ml.min^{-1}$.

The obtained results during these experiments were inferior to the ones obtained in Marques et al., (2011). The observed differences may be related to the use of different reactors, and the lower residence time. Whilst in this work a 100 ml STR was coupled to the continuous system, as a reservoir for the organic and aqueous phase, a microchannel was used to perform the cholesterol oxidation, which led to higher substrate conversions. This fact in combination with the higher residence times had played a preponderant role in the yields observed. If the inflow

was diminished to 0.14 ml.min-1, the residence time will increase 3.5 fold inside the PBR and the FBR used in this continuous system.

A stirred-tank reactor (STR) comprising a two-phase aqueous-organic system was assembled using higher volumes compared to previous trials in order to evaluate a scale-up approach. The STR had a total volume of 100 ml (1:1 aqueous/organic), the same used in the main reservoir of the continuous mode setup. Samples were withdrawn throughout 100 hours, during which time the reaction ran without stoppages.

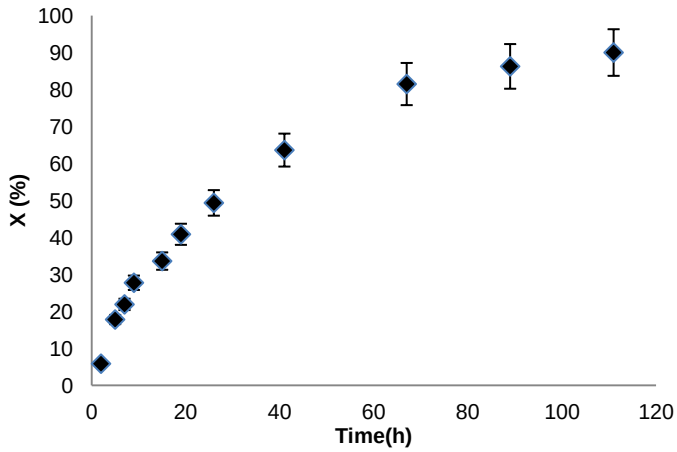


Figure 32 - Substrate conversion achieved in a STR of 100 ml for a reaction period of 100 hours

The results obtained in batch-mode show a near complete substrate conversion after the 100 hours in which the reaction occurred. It must be taken into account that direct comparison between both systems can not be performed directly, since they deal with different hydrodynamics. Whilst using a stirred-tank batch reactor has the vantage of its simplicity, higher reaction control, larger residence times and even lower operation costs, a continuous stirred-tank reactor provides a lower volume requirement, lower energy expense and, most importantly, higher reactants concentration capacity, due to the ability to operate in a longer term operation.

Nevertheless, when looking to the results obtained for the continuous system (Figure 33), there is an apparent evolution for a stationary after 40 hours of operation and, between 70 and 100 hours of operation, the conversions obtained were approximately constant (though at a value far from the desired). The solution may pass through a reduction of the residence time, expecting that meanwhile this does not creates significant external mass transfer resistance.

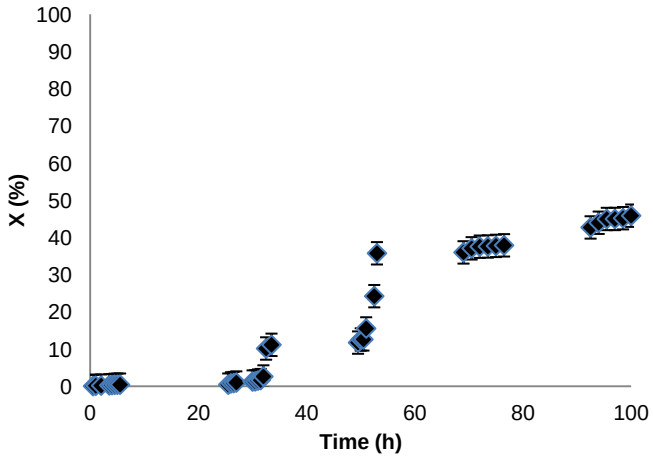


Figure 33 - Substrate conversion achieved using the continuous mode system for a reaction period of 100 hours with an inflow of 0.5 ml.min^{-1} and residence times of 4 minutes in the PBR and of 6 minutes in the FBR.

In this figure, during 100 hours of continuous reaction, the substrate conversion did not reached 50%. Despite the increment of conversion after 50 hours of operation, a much lower enzyme activity was achieved using this system. One reason for this poor conversion may be connected to the losses that occur in the vessels that provide the linkage between the components of the setup. Another reason is the fact that it was the organic phase that traveled through the vessels, therefore, the reaction occurred without an aqueous phase present in the reactors and in the vessels and the enzymes were in contact with *n*-heptane during the whole reaction period. Looking to figure 27 it could be observed that, without a water fraction, the enzyme only presents a 10% relative activity (if immobilized and in a 2 hour reaction) so, it is reasonable to think that the delay in proper enzyme function may be also related to this condition imposed by the setup characteristics.

It may also be related to factors that may be the weakness of the system: the precipitation of the product due to the temperature fluctuation that may have occurred in the main reactor at 4ºC and the length of the pipelines that may have contributed to large dead volumes and, consequently product losses.

Besides the decrease of activity, the temperature variations may also had provoked the precipitation of the substrate since, in the end of the reaction period, a large quantity of white residues were present in the interface of the water bath and covering the PBR surface (data not shown). These white residues were confirmed to be cholestenone that had never reached the main reservoir (from where the product was withdrawn) and, therefore, it was not accounted as bioconversion product. This fact may also indicate that the assembled system was too long and that the product withdrawn was performed too far from the reactor, which increased the system losses.

The refrigeration system is a main component of the setup since the main reservoir lid, as shown in *Materials and Methods* section, was not totally sealed to allow the withdrawn of samples and the insertion of the probes. Although, even if the main reservoir could be efficiently sealed, the evaporation of the organic solvent will eventually occur due to the air inlet installed. Additionally, in a completely sealed setup, without refrigeration, solvent evaporation may occur. This means that the refrigeration system, although bringing constraints, is an imperative component when assembling this long-term, continuous-mode bioconversion. During this work, several assays were performed without the refrigeration system. In all of them, the volume of the solvent had decreased significantly and the enzyme activity was near to zero (results not shown). Due to the air inlet and the 30º heating, *n*-heptane easily evaporated of the main reservoir. This reduction of the solvent may also be related to losses in the system vessels that could not have been totally sealed. The substrate precipitation when using the refrigeration system could not be quantified but certainly had influenced the substrate that reached the enzyme inside the reactor and the product that was formed, thus explaining the curve shown in figure 33.

Despite the unsatisfactory results obtained to cholesterol oxidase activity, catalase had demonstrated to be effective in the hydrogen peroxide removal using this system.

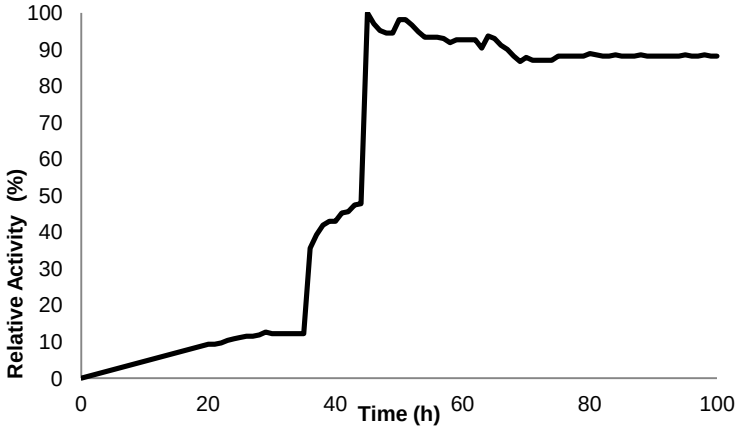


Figure 34 - On-line measurement of the hydrogen peroxide content in the continuous mode system using a spectrophotometer U-2000 from Hitachi (Japan)

Although working without an aqueous phase present inside the reactor and knowing that, due to the low substrate conversion, the hydrogen peroxide formed was not higher as it would have been in other conditions, the enzyme had lowered this toxic compound from the aqueous phase of the reservoir. The results analyzed in the spectrophotometer are shown in figure 34 where it is noticed an initial increase, a slightly decline and finally the stabilization of the catalase activity, which apparently had removed all the hydrogen peroxide inside the reservoir. This result suggests that catalase can be integrated with cholesterol oxidase for cholesterol oxidation in a continuous operation. The use of catalase enables the removal of hydrogen peroxide of the system that may inhibit cholesterol oxidase proper function.

Conclusions and Future Work

4. Conclusions and Future Work

The main objective of this work was to characterize an efficient system for the oxidation of cholesterol, anchored in the use of microreactors. A continuous mode setup was obtained that using the reaction of oxidation of cholesterol by cholesterol oxidase. This steroid biotransformation also yields hydrogen peroxide thus the incorporation of catalase into the system to degrade the hydrogen peroxide was also envisaged.

The immobilization of both enzymes was viable using a PVA-PEG method, described in Fernandes et al., (2009). The immobilization in sol-gel and magnetite matrixes was not successful and the enzymes ost their activity during the immobilization process.

The kinetic parameters of each enzyme, both in free and immobilized forms, were determined and compared to several reports on the use of cholesterol oxidase and catalase form different microbial sources. The values obtained for both enzymes were within the range of previous works and, as expected, the immobilized enzymes kinetic parameters had revealed mass transfer limitations.

The activity profiles of both enzymes corresponded to previous data gathered in which was stated that besides a 30ºC optimum temperature for both reactions a 100 mM phosphate buffer pH 7 provided the most suitable conditions to work through with both enzymes. The immobilization of both enzymes played an important protective role, enabling higher conversions within less favorable conditions, when comparing to the free enzyme.

The solvent screening process revealed that *n*-heptane was the most suitable organic solvent to use as the organic phase. The selection of the proper organic phase is an important step in process design since, when using hydrophobic substrates or products, the use of an organic phase as substrate and/or product pool increases the conversion yield. This organic solvent was chosen after several elimination steps (from an initial set of 54 organic solvents) that included: the elimination of the solvents that interacted with the MTP surface, that interacted with hydrogen peroxide reading or that presented no catalytic activity. Also, some physicochemical properties of the solvents were related with the conversions obtained when they were acting as organic phase and the discloser of a correlation pattern was attempted. Methanol, ethanol, 2-propanol and *n*-heptane reached the final step of the screening process and the stability of the enzyme when in contact with this four solvents was evaluated.

N-heptane provided not only a longer stability to the enzymes but also higher oxygen availability to the reaction medium, therefore it was stated as the most suitable substrate and product carrier. To avoid the evaporation of the organic phase, a proper isolation or refrigeration system in the overall production system must be taken into account. Additionally, this organic solvent offers the possibility to integrate an on-line product quantification coupled to a continuous system, e.g. HPLC, since the mobile phase in the HPLC analysis followed in this work is mainly composed by *n*-heptane.

When testing the behavior of the two enzymes (catalase and cholesterol oxidase) in the same reaction system once again, the use of *n*-heptane was feasible, compared to ethanol. It was demonstrated that the reduction of the aqueous ration hindered enzyme activity, even when those were immobilized. A ratio below 30% of aqueous phase was not advisable to this reaction.

The co-immobilization of cholesterol oxidase and catalase within the same support was assayed but better results were obtained when both enzymes were immobilized in separated supports. This fact must be related to mass transfer limitations that were more accentuated when both enzymes were in the same support. The quantification of hydrogen peroxide toxicity was also performed. It was shown that with 10M of hydrogen peroxide in the media the enzyme loses 60% of its activity, evidencing once again the importance of the incorporation of an reduction step in this system. Further optimization is needed if the co-immobilization of these two enzymes is envisaged.

Although it was already stated that there was enough oxygen available in the media to provide to the oxidation reaction, in a long term operation, oxygen inlet is a possibility. It was demonstrated that cholesterol oxidase had obtained satisfactory conversion rates at a oxygen concentration of 0.24 mM and 3 mM and that the enzyme function was compromised when 12 mM of oxygen were supplied to the media, evidencing a possible inhibitory value for the enzyme proper function.

In the design of the continuous mode setup seven parameters had been defined:

- the immobilization of the enzymes is essential to the process, thus it provides stability and reuse of the enzymes
- both enzymes are able to work within the same buffer solution (100 mM phosphate buffer pH 7)
- *n*-heptane is the suitable substrate and product pool for this reaction thus it provides higher stability and higher oxygen concentrations
- hydrogen peroxide has a negative effect over cholesterol oxidase activity
- co-immobilization of the enzymes in the same support has to be further optimized
- oxygenation of the system increases the cholesterol oxidase activity patterns
- the evaporation of the solvent has to be taken into account

The continuous mode setup was composed by a packed-bed reactor (PBR) and a fluidized-bed reactor (FBR). The PBR was chosen to cholesterol oxidase since it increases the residence time and the mass transfer area but the same could not be used for catalase since the formed hydrogen peroxide is a gas and to flow properly inside the reactor needs spaces to diffuse otherwise it may bring problems as clogging or vessel burst. The reactors had a 2 ml (PBR) and 3 ml (FBR) capacity and the residence time were 4 and 6 minutes, respectively.

During the assays performed with the continuous setup low bioconversion values were achieved. This must be due to:

- The refrigeration system incorporated in the system that may have lowered the temperature inside the reaction and the enzymes could be working out of its optimum range
- The crystallization of the product due to the low temperatures imposed to the main reservoir
- Eventual losses of the solvent through the system linkages
- Residence times inside the reactors were low
- Lack of an aqueous phase inside the reactor (and possible toxicity of long-term exposure to *n*-heptane)

To overcome these constraints several modifications could be applied to the setups. Although the incorporation of a refrigeration system is imperative in a long-term operation (due to solvent evaporation and oxygen inlet), the temperatures applied to the system may be carefully calibrated. The balance between the flow injection to the system and the size of the overall system must also be optimized to provide an adequate residence time inside the reactors and avoid solvent losses.

Despite the low conversion yields obtained, the proposed system works. Catalase had efficiently degraded the hydrogen peroxide present in the aqueous phase of the main reservoir and the monitoring system assembled was effective, providing an on-line catalytic activity monitoring of catalase during the whole reaction period.

Altogether these results demonstrated that further modifications have to be made to achieve suitable bioconversions using both enzymes in the same system. As underlined, these setups lack an optimization process, which could not been completed during the limited period of time available within the framework of the MSc thesis but it may be continued with the optimization of this system in future works. It was established that cholesterol oxidation coupled to hydrogen peroxide removal can be performed with cholesterol oxidase and catalase. Still, further optimization is needed if an industrial application is envisaged, providing enzyme reuse and by-product elimination, leading to cost reductions and industrial viability.

References

5. References

Akkaya, B., Sahin, F., Demirel, G., Tumturk, H., Functional polymeric supports for immobilization of cholesterol oxidase. *Biochemical Engineering Journal*, 2009; 43: 333-337.

Alcade, M., Ferrer, M., Plou, F., Ballesteros, A., Environmental biocatalysis: from remediation with enzymes to novel green processes. *Trends in Biotechnology*, 2006; 24: 281-287.

Amador, C., Gavriilidis, A., Angeli, P., Flow distribution in different microreactor scale-out geometries and the effect of manufacturing tolerances and channel blockage. *Chemical Engeneering Journal*, 2004; 101: 379-390.

Aparício, J., Martín, J., Microbial cholesterol oxidases: bioconversion enzymes or signal proteins? *Molecular BioSystems*, 2008; 4: 804-809.

Ates, S., Cortenlioglu, E., Bayraktar, E., Mehmetoglu, U., Production of $_{L}$-DOPA using Cu-alginate gel immobilized tyrosinase in a batch and packed bed reactor. *Enzyme and Microbial Technology*, 2007; 40: 683-687.

Aymard, C., Belarbi, A., Kinetics of thermal deactivation of enzymes: a simple three parameters phenomenological model can describe the decay of enzyme activity, irrespectively of the mechanism. *Enzyme and Microbial Technology*, 2000; 27: 612-618.

Azevedo, A., Rosa, P., Ferreira, F., Aires-Barros, R., Chromatography-free recovery of biopharmaceuticals through aqueous two-phase processing. *Trends in Biotechnology*, 2009; 27(4): 240-247.

Bairoch, A., The ENZYME data bank. *Nucleic Acids Research*, 1994; 22(17): 3626-3627.

Bankar, S., Bule, M., Singhal, R., Ananthanarayan, Laxmi A., Co-immobilization of glucose oxidase and catalase. *International Journal of Food Engineering*, 2011; 7(2): 1-13.

Baron, V., Willaert, G., De Backer, L., *Immobilised cell reactors*. In Immobilised Living Cell Systems: Modelling and Experimental Methods. Ed. by R.G. Wilaert, G.V. Baron and L. De Backer. John Wiley & Sons, 1996; Chapter 4.

Bershtein, S., Tawfik, D., Advances in laboratory evolution of enzymes, 2008. *Current Opinion in Chemical Biology*; 12: 151-158.

Betancor L., López-Gallego F., Hidalgo, A., Alonso-Morales, N., Dellamora-Ortiz, G., Mateo, C., Fernandez-Lafuente, R., Guisán, J., Different mechanisms of protein immobilization on glutaraldehyde activated supports: Effect of support activation and immobilization conditions. *Enzyme and Microbial Technology*, 2006; 39: 877-882.

Betancor, L., Luckarift, R., Bioinspired enzyme encapsulation for biocatalysis. *Trends in Biotecnology*, 2008; 26(10): 566-572.

Bordet, T., Buisson, B., Michaud, M., Drouot, C., Galéa, P., Delaage, P., Akentieva, N., Evers, A., Covey, D., Ostuni, M., Lacapère, J., Massaad, C., Schumacher, M., Mauxe, E., Delaage, M., Hendersion, C., Pruss, R., Identification and characterization of cholest-4-en-3-one, Oxime (TRO19622), a novel drug candidate for amyotrophic lateral sclerosis. *The Journal of Pharmacology and Experimental Therapeutics*, 2007; 322(2): 709-720.

BRENDA database, Available at http://www.brenda-enzymes.org, assessed on 30 September, 2011

Bruggink, A., Roos, E., Vroom, E., Penicillin acylase in the industrial production of a β-lactam antibiotics. *Organic Process Research & Development*, 1998; 2: 128-133.

Buchholz, K., Kasche, V., Bornscheuer, U., *Biocatalysts and enzyme technology*. John Wiley & Sons, 2005, pp 1-26.

Burton, S., Roes-Hill, M., *Oxidizing enzymes in multi-step biotransformation processes* In Multi-step enzyme catalysis: biotransformations and chemoenzymatic synthesis Ed. by Garcia-Junceda, E., Jonh Wiley & Sons, Inc., 2008.

Catana, R., Ferreira, B., Cabral, J., Fernandes, P., Immobilization of inulinase for sucrose hydrolosis. *Food Chemistry*, 2005; 91(3): 517-520.

Çetinus, S., Oztop, H., Immobilization of catalase on chitosan film. *Enzyme and Microbial Technology*, 2000; 26: 497-501.

Chaize, B., Fournier, D., Sorting out molecules reaction with acetylcholinesterase by enzyme encapsulation in liposome. *Biosensors and Bioelectronics*, 2004; 20: 628-632.

Cherry, J., Fidantsef, A., Directed evolution of industrial enzymes: an update. *Current Opinion in Biotechnology*, 2003. 14: 438-443.

Chen, W., Su, B., Xing, H., Yang, Y., Ren, Q., Solubilities of cholesterol and desmosterol in binary solvent mixtures of n-hexane + ethanol. *Fluid Phase Equilibria*, 2009; 287: 1-6.

Chibata, I., Industrial application of immobilization enzyme systems. *Pure & Applied Chemistry, 1978*; 50: 667-675.

Çirpan, A., Alkan, S., Toppare, L., Cianga, I., Yagci, Y., Immobilization of cholesterol oxidase in a conducting copolymer of thiophene-3-yl acetic acid cholesteryl ester with pyrrole. *Designed Monomers and Polymers*, 2003; 6(3): 237-243.

Comyns, A., Markets and Business In Focus on Catalysis. *Chemical Weekly*, 2010.

Comyns, A., Markets and Business In Focus on Catalysis. *Chemical Weekly*, 2011.

Crestini, C., Melone, F., Saladino, R., Novel multienzyme oxidative biocatalyst for lignin bioprocessing. *Bioorganic & Medical Chemistry*, 2011; 19: 5071-5078.

Cruz, A., Fernandes, P., Cabral, J., Pinheiro, H., Whole-cell bioconversion of β-sitosterol in aqueous-organic two-phase systems. *Journal of Molecular Catalysis B: Enzymatic*, 2001; 11: 579-585.

Cruz, A., Fernandes, P., Cabral, J., Pinheiro, H., Solvent partitioning and whole-cell sitosterol bioconversion activity in aqueous-organic two-phase system. *Enzyme and Microbial Technology*, 2004; 34: 342-353.

Dalby, P., Engeneering enzymes for biocatalysis. *Recent Patents on Biotechnology*, 2007; 1(1): 1-9.

Damborsky J., Brezovsky, J., Computational tool for designing and engineering biocatalyst. *Current Opinion in Chemical Biology*, 2009; 13: 26-34.

Doukyu, N., Aono, R., Purification of extracellular cholesterol oxidase with high activity in the presence of organic solvents from *Pseudomonas* sp. strain ST-200. *Applied and Environmental Microbiology*, 1998; 65(5): 1929-1932.

Eigenberger, G., *Fixed-bed reactors* In Ullmann's Encyclopedia of Industrial Chemistry, 1992, Vol. 4. 199-238.

ENZYME database, Available at: http://www.expasy.org/enzyme, accessed on 30 September 2011

EPA, *Design for the Environment Projects*. United States Environmental Protection Agency, 2002.

Erhardt, F., Kugler, J., Chakravarthula, R., Jordening, H., Co-immobilization of dextransucrase and dextranase for the facilitated synthesis of isomalto-oligosaccharides: preparation, characterization and modeling. *Biotechnology and Bioengeneering*, 2008; 100(4): 673-683.

Fahy E., Subramaniam, S., Brown, H., Glass, C., Merrill, A., Murphy, R., Raetz, C., Russell, D., Seyama, Y., Shaw, W., Shimizu, T., Spener, F., van Meer, G., VanNieuwenhze, M., White, S., Witztum, J., Dennis, E., A comprehensive classification system for lipids. *Journal of Lipid Research*, 2005; 46: 839-861.

Fernandes, P., Cruz, A., Angelova, B., Pinheiro, H., Cabral, J., Microbial conversion of steroid compounds: recent developments. *Enzyme and Microbial Technology*, 2003; 32: 688-705.

Fernandes, P., Cabral, J., Phytosterols: applications and recovery methods. *Bioresource Technology*, 2007; 98: 2335-2350.

Fernandes, P., Marques, M., Carvalho, F., Cabral, J., A simple method for biocatalyst immobilization using PVA-based hydrogel particles. *Journal of Chemical Technology and Biotechnology*, 2009; 84: 561-564.

Fernandes, P., Miniaturization in biocatalysis. *International Journal of Molecular Science*, 2010; 11: 858-879.

Fernandes, P., Enzymes in food processing: a condensed overview on strategies for better biocatalysts. *Enzyme Research*, 2010.

Fernandes, P., Cabral, J*., Steroid Bioconversion* In Encyclopedia of Industrial Biotechnology: Bioseparation and Cell Technology, Ed. by Flickinger, M., John Wiley & Sons, Inc., 2010.

Filho, M., Stilger, T., Muller, M., Liese, A., Wandrey, C., Is logP a convenient criterion to guide the choice of solvents for biphasic enzymatic reactions? *Angewandte Chemie International Editions*, 2003; 115:3101-3104

Fischer, A., Enkler, N., Neudert, G., Bocola, M., Sterner, R., Merkl, R., TransCent: computational enzyme design by transferring active sites and considering constraints relevant for catalysis. *BMC Bioinformatics*, 2009; 10: 54-70.

Foley, S., Colo, L., Oriel, P., Epstein, C., *Isomeration of glucose to fructose using glucose isomerase from Ampullariella*. 1981, United States Patent 4,308,349.

Fouad, W., Abbas, H., Elwan, M., Swellum, A., El-Dougdoug, A., Biotransformation of progesterone by microbial steroids. *Journal of Apllied Sciences Research*, 2009; 5(1): 137-143.

Garcia-Junceda, E., *Multi-step enzyme catalysis: biotransformations and chemoenzymatic synthesis*. Jonh Wiley & Sons, Inc., 2008.

Ge, Y., Wang, Y., Zhou, H., Wang, S., Tong, Y., Li, W., Coimmobilization of glucoamylase and glucose isomerase by molecular deposition technique for one-step conversion of dextrin to fructose. *Journal of Biotechnology*, 1999; 67: 33-40.

Gill, I., Ballesteros, A., Bioencapsulation within synthetic polymers(Part 1): sol-gel encapsulated biologicals. *Trends in Biotechnology*, 2000; 18: 282-296.

Golynskiy, M., Seelig, B., *De novo* enzymes: from computational design to mRNA display. *Trends in Biotechnology*, 2010; 28(7): 340-345.

Gomes, J., Malaviya, A., Androstenedione production by biotransformation of phytosterols. *Bioresource Technology*, 2008; 99: 6725-6737.

Gomez, J., Cantero, D., Kinetic study of biological ferrous sulphate oxidation by iron-oxidising bacteria in continous stirred tank and packed bed bioreactors. *Process Biochemistry*, 2003; 38: 867-875.

Gomez, J., Bodalo, A., Gomez, E., Hidalgo, A., Gomez, M., Murcia, M., Experimental behavior and design model of a fluidized bed reactor with immobilized peroxidase for phenol removal. *Chemical Engineering Journal*, 2007; 127: 47-57.

Gonzales, G., Herrera, M., Garcia, M., Peña, M., Biodegradation of phenol in a continuous processes: comparative study of stirred tank and fluidized-bed bioreactors. *Bioresource Technology*, 2001; 76: 245-251.

Herzog, V., Fahimi, D., The effect of glutaraldehyde on catalase: Biochemical and cytochemical studies with beef liver catalase and rat liver peroxisomes. *The Journal of Cell Biology*, 1974; 60: 303-311.

Holden, M., Jung, S., Cremer, P., Patterning enzymes inside microfluidic channels via photoattachment chemistry. *Analytical Chemistry*, 2004; 76(7): 1838-1843.

Horst, F., Rueda, E., Ferreira, M., Activity of magnetite-immobilized catalase in hydrogen peroxide decomposition. *Enzyme and Microbial Technology*, 2006; 38: 1005-1012.

Illanes, A., *Enzyme biocatalysis: principles and applications*. Springer Science, 2008, pp 1-50.

Iyer, P., Ananthanarayan, L., Enzyme stability and stabilization – aqueous and non-aqueous environment. *Process Biochemistry*, 2008; 43: 1019-1032.

Jurgen-Lohmann, D., Legge, R., Immobilization of bovine catalase in sol-gels. *Enzyme and Microbial Technology*, 2006; 39: 626-633.

Julien, C., Whitford, W., Bioreactor monitoring, modeling and simulation. *BioProcess International*, 2007; 5(1): 10-17.

Kaneno, J., Kohoama, R., Miyazaki, M., Ueharam M., Kanno, K., Fujii, M, Shimizu, H., Maeda, H., A simple method for surface modification of microchannels. *New Journal of Chemistry*, 2003; 27: 1765-1768.

Kawakami, K., Sera, Y., Sakai, S., Ono, T., Ijima, H., Development and characterization of a silica monolith immobilized enzyme micro-bioreactor. *Industrial & Engineering Chemistry research*, 2005; 44: 236-240.

Kelly, R., Dijkhuizen, L., Leemhuis, H., Starch and α-glucan acting enzymes, modulating their properties by directed evolution. *Journal of Biotechnology*, 2009; 140:184-193.

Kim, J., Oh, K., Lee, S., Kim, S., Hong, S., Biodegradation of phenol and chlorophenols with defined mixed culture in shake-flasks and a packed bed reactor. *Process Biochemistry*, 2002; 37: 1367-1373.

Kirk, O., Borchert, T., Fuglsang, C., Industrial enzyme applications. *Current Opinion in Biotechnology*, 2002; 13: 345-351.

Krishna, S., Developments and trends in enzyme catalysis in nonconventional media. *Biotechnology Advances*, 2002; 20:239-267.

Kumar, A., Malhotra, R., Malhotra, B., Grover, S., Co-immobilization of cholesterol oxidase and horseradish peroxidase in a sol-gel film. *Analytica Chimica Acta*, 2000; 414: 43-50.

Léon R., Fernandes, P., Pinheiro, H., Cabral, J., Whole-cell biocatalysis in organic media. *Enzyme and Microbial Technology*, 1998; 23: 483-500.

Lewis, M., Dissolved Oxygen In U.S Geological Survey Texas Water Resources Institute, 2006; Version 2.1 Book 9.

Li, Y., Schellhorn, H., Rapid kinetic microassay for catalase activity. *Journal of Biomolecular Technology*, 2007; 18:185-187.

Liese, A., Seelbach, K., Wandrey, C., *Industrial biotransformations*. Jonh Wiley & Sons, 2006.

Lin, Y., Fu, J., Song, X., Purification of an extracellular cholesterol oxidase from a *Bordatella* species. *Process Biochemistry*, 2010; 45: 1563-1569.

Lineweaver, H., Burk, D., The determination of enzyme dissociation constants, *Journal of the American Chemical Society*, 1934. 56 (3): 658-666.

Liu, W. Horng, W., Tsai, M., Bioconversion of cholesterol to cholest-4-en-3-one in aqueous/organic solvent two-phase reactors. *Enzyme and Microbial Technology*, 1996; 18: 184-189.

London, E., Insights into lipid raft structure and formation from experiments in model membranes. *Current opinion in Structural Biology*, 2002; 12: 480-486.

MacLachlan, J., Wotherspoon, A., Ansell, R., Brooks, C., Cholesterol oxidase: sources, physical properties and analytical applications. *Journal of Steroid Biochemistry & Molecular Biology*, 2000; 72: 169-195.

Marques, M., Carvalho, F., Magalhães, S., Cabral, J., Fernandes, P., Screening for suitable solvents as substrate carriers for the microbial side-chain cleavage of sitosterol using microtitre plates. *Process Biochemistry*, 2009; 44, 556-561.

Marques, M., Carvalho, F., Carvalho, C., Cabral, J., Fernandes, P., Steroid bioconversion: towards green processes. *Food and Bioproducts Processing*, 2010; 88: 12-20.

Marques, M., Cabral, J., Fernandes, P., Bioprocess scale-up: quest for the parameters to be used as criterion to move from microreactors to lab-scale. *Journal of Chemical Technology and Biotechnology*, 2010; 85: 1184-1198.

Marques, M., Fernandes, P., Cabral, J., Znidarsic-Plazl P., Plazl, I., On the feasibility of in situ steroid biotransformation and product recovery in microreactors. *Chemical Engineering Journal*, 2010; 160: 708-714.

Marques, M., Fernandes, P., Cabral, J., Znidarsic-Plazl P., Plazl, I., Continuous steroid biotransformation in microchannel reactors. *New Biotechnology*, 2011; doi:10.1016/j.nbt.2011.10.001

Masouka, N., Wakimoto, M., Ubuka, T., Nakano, T., Spectrophotometric determination of hydrogen peroxide: catalase activity and rates of hydrogen peroxide removal by erithrocytes. *Clinica Chimica Acta*, 1996; 254: 101-112.

Matijosyte, I., Arends, I., Vries, S., Sheldon, R., Preparation and use of cross-linked enzymes aggregates (CLEAs) of laccases. *Journal of Molecular Catalysis*, 2010; 62: 142-148.

Mei, L., Trevisan, H., Zanin, G., Preparation of silica with controlled pore sizes for enzyme immobilization. *Brazilian Journal of Chemical Engineering*, 2000; 17(1): 71-77.

Mendes, M., Recio, E., Anto, N., Guerra, S., Santos-Aberturas, J., Martín, J., Aparício, J., Cholesterol oxidases act as signaling proteins for the biosynthesis of the polyene macrolide pimaricin. *Chemistry & Biology Journal*, 2007; 14: 279-290.

Michaelis, L., Menton, M., Die Kinetik der invertinwirkung, *Biochemische Zeitschrift* , 1913, 49: 333-369.

Miyazaki, M., Kaneno, J., Uehara, M., Fujii, M., Shimizu, H., Maeda, H., Simple method for preparation of nanostructure on microchannel surface and its usage enzyme-immobilization. *Chemical Communications*, 2003: 648-649.

Miyazaki, M., Maeda, H., Microchannel enzyme reactors and their application for processing. *Trends in Biotechnology*, 2006; 24(10): 463-470.

Mosier, N., Ladisch, M., *Biocatalysis and the growth of industrial enzymes* In *Modern* biotechnology: connecting innovations in microbiology and biochemistry to engineering fundamentals. Wiley & Sons, 2009, pp 49-58.

Naim, N., Adham, N., El-Rehim, H., El-Hady, A., Prednisolone production using *Pseudomonas fluorescens* cells immobilized with polymer carrier produced by radiation polymerization. *Process Biochemistry*, 2003; 38: 1083-1089.

Northrop, J., Crystalline pepsin: Isolation and tests of purity. *The Journal of General Physiology*, 1930, 739-766.

Pchelintsev, N., Youshko, M., Svedas, V., Quantitative characteristic of the catalytic properties and microstructure of cross-linked enzyme aggregates of penicillin acylase. *Journal of Molecular Catalysis*, 2009; 56: 202-207.

Pfruender, H., Jones, R., Weuster-Botz, D., Water immiscible ionic liquid as solvents for whole cell biocatalysis. *Journal of Biotechnology*, 2006; 124: 182-190.

Purich, D., *Enzyme kinetics catalysis and control: a reference of theory and best practice methods.* Elsevier Inc., 2010.

Quax, W., Voorschoten, V., Luiten, R., Leiden, K., Schuurhuizen, P., Delft, N., *Glucose isomerase enzymes and their use*. 1994, United States Patent 5,376,536.

Rozzel, J., Commercial scale biocatalysis: myths and realities. *Bioorganic & Medicinal Chemistry*, 1999; 7: 2253-2261.

Rumbold, K., Krammer, B., Tschemmernegg, M., Pochlauer, P., Schwab, H., A novel screening assay for hydroxynitrile lyases suitable for high-throughput screening. *Journal of Biotechnology*, 2007; 129: 151-161.

Sakai-Kato, K., Kato, M., Ishihara, K., Toyo'ok T., An enzyme-immobilization method for integration biofunctions on a microchip using a water-soluble amphiphilic phospholipid polymer having a reaction group. *Lab Chip*, 2004; 4: 4-6.

Sandoval, G., Marty, A., Screening methods for synthetic activity of lipases. *Enzyme and Microbial Technology*, 2007; 40: 390-393.

Santacoloma, P.,Sin, G., Gemaey, K., Woodley, J., Multienzyme-catalyzed processes: next-generation biocatalysis. *Organic Process Research & Development*, 2011; 15: 203-212.

Santos, R., Monnerat, R., Grossi de Sá, M., Cordeiro, C., Gomes, A., Gander, E., Cholesterol oxidase interference on the emergence and viability of cotton boll weevil larvae. *Pesquisa Agropescuaria Brasileira*, 2002; 37(11): 1525-1530.

Scopes, R., *Enzyme activity and assays* In Encyclopedia of Life Sciences, Ed. by Macmillan Publishers Ltd, 2002.

Shim, J., Kim, G., Moon, S., Covalent co-immobilization of glucose oxidase and ferrocenedicarboxylic acid for an enzymatic biofuel cell. *Journal of Electroanalytical Chemistry*, 2011; 653: 14-20.

Singh, S., Singhal, R., Malhotra, B., Immobilization of cholesterol esterase and cholesterol oxidase onto sol-gel films for application to cholesterol biosensor. *Analytical Chimical Acta*, 2007; 582: 335-343.

Smith, P., Krohn, R., Hermanson, G., Mallia, A., Gartner, F., Provenzano, M., Fujimoto, E., Goeke, N., Olson, B., Klenk, D., Measurement of protein using bicinchonic acid. *Analytical. Biochemistry*, 1985; 150: 76.

Sripalakit P., Wichai, U., Saraphanchotiwitthaya, A., Biotransformation of various sterols to androstenones by *Mycobacterium* sp. and some steroid-converting microbial strains. *Journal of Molecular Catalysis: Enzymatic*, 2006; 41: 49-54.

Srisawasdi, P., Jearanaikoon, P., Wetprasit, N., Sriwanthana, B., Kroll, M., Lolekha, P., Application of *Streptomyces* and *Brevibacterium* cholesterol oxidase for total serum cholesterol assay by the enzymatic kinetic method. *Clinica Chimica Acta*, 2006; 372: 103-111.

Takamine, J., *Process of converting starchy material into sugar*, 1894. United States Patent 562,103.

Thomas, S., DiCosimo, R., Nagarajan, V., Biocatalysis: applications and potentials for the chemical industry. *Trends in Biotechnology*, 2002; 20(6): 238-242.

Tosa, T., Sato, T., Mori, T., Chibata, I., Basic studies for continuous production of L-Aspartic acid by immobilized *Escherichia coli* cells. *Applied Microbiology,* 1974; 27(5): 886-889.

Tucker, G., *Fundamentals of enzyme activity* In Enzymes in food processing. Ed. by Chapman & Hall, 1995.

Tufvesson, P., Fu, W., Jensen, J., Woodley, J., Process considerations for the scale-up and implementation of biocatalysis. *Food and Bioproducts Processing*, 2010; 88: 3-11.

Tufvesson, P., Lima-Ramos, J., Nordblad, M., Woodley, J., Guidelines and cost analysis for catalyst production in biocatalytic processes. *Organic Processes Research & Development*, 2011; 15: 266-274.

Urban, P., Goodall, D., Bruce, N., Enzymatic microreactors in chemical analysis and kinetic studies. *Biotechnology Advances*, 2006; 24: 42-57.

Usmani, G., Patil, H., Lipase catalysed interesterification for the production of oleochemicals from non traditional oils. *Rasayan Journal of Chemistry*, 2010; 3(2): 354-358.

Wahler, D., Reymond, J., Novel methods for biocatalyst screening. *Current Opinion in Chemical Biology*, 2001; 5: 152-158.

Wang, Z., Zhao, F., Chen, D., Li, D., Cloud point system as a tool to improve the efficiency of biotransformation. *Enzyme and Microbial Technology*, 2005; 36: 589-594.

Wang, B., Tang, X., Ren, G., Liu, J., Yu, H., A new high-throughput screening method for determining active and enantioselective hydrolases. *Biochemical Engineering Journal*, 2009; 46: 345-349.

Wedge, D., Rowe, W., Kell, D., Knowles, J., In silico modeling of directed evolution: implications for experimental design and stepwise evolution. *Journal of Theoretical Biology*, 2009; 257: 131-141.

Wohlgemuth, R., Biocatalysis – key to sustainable industrial chemistry. *Current Opinion in Biotechnology*, 2010; 21: 713-724.

Wolff, A., Zhu, L., Wong, Y., Straathof, A., Jongejan, J., Heijnen, J., Understanding the influence of temperature change and cosolvent addition on conversion rate of enzymatic suspension reaction based on regime analysis. *Biotechnology and Bioengineering*, 1999; 62(2): 125-134.

Woodley, J., New opportunities for biocatalysis: making pharmaceutical processes greener. *Trends in Biotechnology*, 2008, 26(6): 321-327.

Xu, X., Bittman, R., Duportai, G., Heissler, D., Vilcheze, C., London, E., Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). *The Journal of Biological Chemistry*, 2001; 276(36): 33540-33546.

Yaws, C., Hopper, J., Sheth, S., Han, M., Pike, R., Solubility and Henry's Law constant for alcohols in water. *Waste Management*, 1997; 17(8): 541-547.

Yazdi, M., Zahraei, M., Aghaepour, K., Kamranpour, N., Purification and partial characterization of a cholesterol oxidase from Streptomyces fradiae. Enzyme and Microbial Technology, 2001; 28: 410-414.

Yoon, S., Choban, E., Kane, C., Tzedakis, T., Kenis, P., Laminar flow-based electrochemical microreactor for efficient regeneration of nicotinamide cofactors for biocatalysis. Journal of *American Chemical Society*, 2005; 127: 10466-10467.

Znidarsic-Plazl, P., Plazl, I., Development of a continuous steroid biotransformation process and product extraction within microchannel system. *Catalysis Today*, 2010; 157: 315-320.