Biotransformations

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1.- Introduction

Selective conversions of natural and/or synthetic substrates into useful products using whole cells or isolated enzymes have been gaining increase relevance among the methods at industrial scale. Enzymatic activity is often characterized due to its high chemo-, regio- and stereo-selectivity, which are very useful for synthesis of fine chemicals and intermediary molecular blocks. However, the success of a biotransformation at industrial scale depends upon a series of factors such as availability and low cost of the biocatalyst, efficient product recovery processes, competitive costs compared to conventional methods, environmental compatibility, and non-pathogenicity of the biological systems.

This chapter highlights general aspects of biocatalysis, recent findings and new strategies about modes of using enzymes and fluids in the biotransformation field.

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2.- Brief history of biotransformations

Biotransformations can be defined by the use of whole-cells and/or biocatalysts, to produce goods of social and/or economical values. Since ancient times, biotransformations had been intimately associated to fermentation processes of many foods and beverages in western as well as eastern cultures. However, the beginning of the biotransformation rational up today started in the nineteenth century by the pioneer work of several researchers. Among them, Kirchhoff who first described in 1814 the sugar conversion from starch by malted barley, followed by Payen and Persoz who extracted for the first time in 1833 the diastase from malted barley; Kühne who coined the term enzyme to described catalytic activity not linked to the cells in 1867, and Ostwald who established the definition of catalysis in 1894. Also, the classic studies performed by Pasteur during 1850 to 1870 period had provided three relevant methods for resolving racemic mixtures: crystallization, salt formation and microbial degradation of one enantiomer. Further experiments demonstrated the synthesis of methyl oleate performed by pancreatic lipase in methanolic medium, and also the reversibility of the reaction (Pottevin, 1906). All those experiments were crucial to establish the founding scientific basis of Microbiology, and Chemistry developed during the twentieth century, and had provided the background of present biotransformations status. However, it was necessary to wait until the 1950's in where the synthesis of steroids took off at industrial scale by modifying enzymatically natural products to give the shape of biotransformations at the

current level. The impact of furthers developments like the antiparasitic compound oxamniquine followed by the manufacture of semisynthetic penicillins and cephalosporins at large scale had provided the bases for the exponential growth of biotransformation field (Neidelman, 1990). The resolution of aminoacids by immobilized biocatalyst replacing the whole-cell process at large scale during the 70's became a definitive milestone for the use of enzymatic processes at industrial level (Turner, 1995).

3.- General considerations about enzymes

Enzymatic reactions are supplying the metabolic material sources of all living organisms. Enzymes can be defined as biocatalysts which accelerate biological reactions with no changing the thermodynamic equilibrium. Therefore, some reactions can be reversed by changing experimental parameters (e.g. solvent, ionic strength, pH, etc…). In general, biocatalysts can provide reaction rate accelerations than are exceeding 10^{12} times compared to non-enzymatic reactions, and at very low concentrations (10^{-3} to 10^{-5} % mole fractions compared to 10^{-1} to 1 % mole fraction in chemical catalysis counterpart). Also, since enzymes are biological materials can be degraded in nature without any impact in the environment.

Enzymes are selective catalysts generally used under mild experimental conditions contrary to chemical catalyzed synthesis in where high pressures and temperatures are generally required. As a result, enzymatic reactions do not require high energy costs, and intensive waste treatments. One of the main advantages to use enzymes is the ability to distinguish a single molecule in complex chemical and biological mixtures. In a substrate molecule, enzymes are capable to recognize a defined chemical group located in specific region of the molecule, and also differentiate between singular isomers. A typical example of biocatalysis stereo-selectivity is the synthesis of (*S*)-(+)-Naproxen by enzymatic resolution of racemate substrate. The relevance of the Naproxen synthesis specificity is based on the

biological activity of S-enantiomer which is 28 folds higher than the R-enantiomer. The synthesis of Naproxen, a non-steroidal anti-inflammatory optically active drug, is catalyzed by carboxyl esterase from the racemic substrate (R,S)- ethoxyethyl-[2-(6-methoxy-2-naphtyl)] propionate (Figure 1). After 90 minutes of reaction the conversion was 45 % with 84 % enantiomeric excess to (S)-(+)-Naproxen (Cretich *et al*., 2001).

Another advantage of biocatalysis is the lack of specific group activation requirement in substrate molecule, like in chemical catalysis which additional work and time are required. Also, in some cases enzymatic reactions are the only way to perform a transformation of substrates (e.g. hydroxylation of terpenes and steroids by oxygenases) (Holland and Weber, 2000). These properties are relevant because of the lack of side reactions, and concomitantly the number of purification steps, operational costs, and time are substantially reduced. The main advantages and general properties of enzymes are summarized in Table 1:

Biocatalysts are sensitive to experimental settings, so in order to get high rates of substrate conversion adjustment to optimal conditions are required. The most influential external parameters for enzyme reactions are pH, temperature, ionic strength (in aqueous media) or salt content (in organic media), and internal parameters are enzyme source, protein structure, and molecular properties (molecular weight, isoelectric point, etc…), mixture composition (e.g. crude extract, pure crystalline enzyme,) and chemical composition (e.g. buffer properties, stabilizers). For each enzyme optimal operational parameters must be established under regular operational conditions. All types of natural sources are providing different kind of enzymes that can be used for special biotransformation purposes. Plant enzymes like the proteases papain, ficin, and bromelain, and more specialized enzymes such as pectinases, phenol oxidases and others plays relevant role in the enzyme world market. Animal enzymes are the common industrial sources of rennets, and lytic enzymes like proteases and lipases. However, the large scale supply of plant and animal biocatalysts

depends on many external factors like growth cycle, seasonal changes, plagues, and also political and economical policies. Moreover, the concerns about the presence of viruses in animal enzyme preparations is increasing the pressure over enzyme makers to produce certified batches with more detailed biological and chemical analyses. So, the preferred sources of enzymes for industrial purposes are microbes, which exceed 90 % of the total biocatalyst market. There are many advantages of using microbial enzymes, among them microbial diversity which allow to select a specific microorganism for a defined enzymatic process, and the ability to manipulate the microbial genome and protein engineering using molecular tools to enhance the production and/or modify enzyme properties. Furthermore, recent findings of extremophile microorganisms and new biocatalysts open the possibilities for the development of new biotransformation processes (Hough and Danson, 1999; Faber and Kroutil, 2005). Additionally, microbial enzymes are in general more robust compared to similar ones from other natural sources. Also, biocatalyst production by fermentation can be scaled up and down to a required level with minimal changes in the equipment, and also costs could be reduced by recycling agro feedstocks and/or industrial wastes.

The International Union of Biochemistry and Molecular Biology (IUBMB) had defined six major groups containing at least 4,000 biocatalysts (Table 2). However, the total number of enzymes was estimated in more than 25,000. The enzyme classification is based on reaction type they catalyze, and the classes are divided into six digits code until individual enzyme identification is determined. In a recent study on 134 biotransformations at large scale had revealed that 44 % of processes based on hydrolases, and 30% on redox reactions are used to synthesize 89 % of chiral compounds mostly required in fine chemical and pharmaceutical industries (Straathof *et al*., 2002). However, the search of new biocatalysts with improved or novel activities for the development of alternative bio-industrial processes is actually the main trend in chemical industries. Although, other biological molecules able to

catalyze reactions like Ribozymes (catalytic RNA), and Abzymes (catalytic antibodies) are under consideration for the development of alternative industrial processes.

From the historic point of view the industrial use of enzymes and biotransformation field has moved from commodity chemicals to more sophisticated molecules like chiral or enantiopure intermediary compounds. A typical example of commodity chemicals is the biosynthesis of acrylamide by nitrile hydratase from acrylonitrile developed by Mitsubishi Rayon Co (Figure 2). The biotransformation process was developed using immobilized *Rhodococcus rhodochrous* J1 cells on fixed bed reactors at 10 °C with a conversion yield higher than 99.9 % and final aqueous concentration of 50 % wt% acrylamide. Currently acrylamide production by biotransformation using this process is about 40,000 Tons per year. There are many advantages of the biotransformation process compared to the chemical one: high acrylamide purity and molecular weight, lack of heavy metals in the product, reduction of the amount of wastewater, no heat or high pressure required (Yamada & Kobayashi, 1996). Presently, bioconversions through single reaction or biocatalytic process and/or in a series of coupled enzymatic reactions, named biotransformations, are commonly described using eukaryotic and prokaryotic cells systems form yeast, plant, fungi and microbes, and also vegetative cells or spores (Larroche & Gross, 1997; Schulze & Wubbolts, 1999; Faber & Kroutil, 2005; Serra *et al*., 2005).

4.- The biotransformation market

According to a specialized report the market for basic, intermediate, fine chemicals and biopolymers made by biotransformation-biocatalysis was approximately U$ 34 billion per year (Anomymous, 2006). The impulse of the market is made possible because of the recent advances in physiology, genetics, biochemical engineering, and downstream processing allowing enzymes to be more available and efficient in the chemical industrial arena. In

addition, the social pressure to chemical manufacturing industries to bring clean production and long-term sustainability has been increased since the last decade and is know as “Green Chemistry” area in which enzymes play a key role for the development of a new chemical industry in the years ahead.

In 2000, the conversion of raw materials by the American chemical industry was estimated in U$ 27 billons to manufacture U$ 419 billon in products (Anonymous, 2001). However, the chemical industry is moving towards the biotransformation field, with an enzyme world market estimated by the Organization for Economic Cooperation and Development (OECD) in U$ 1.5 billion with a growing rate of 12 % per year (Bull *et al.*, 1998), with the largest technical enzyme market for detergent estimated in U$ 500 million (Schäfer *et al*., 2002). Since 1990 to 2002, the quantity of industrial biotransformations has been increased from about 60 to more than 130 (Straathof *et al*., 2002). Actually, the number of biotransformation processes at industrial scale over passed the number of 300 (Arnold & Glieder, 2003). More recently, a report by the OECD on 15 industrial bioprocesses had demonstrated the advantages of biotechnology processes compared to the chemical ones who had diminished the energy consumption and had increased efficiency of raw materials reducing the operating costs between 9 to 90 %. A successful example of the bulk biotransformations at large scale is the implementation of biopulping process in the paper industry with a market valued between U$ 32 to 62 billons, and with predicted growth rate of 15 % per year (Jaworski, 2001).

In agriculture, the present biocatalyst feed market is about U$ 150 millions, mostly based in phytases or carbohydrolases to improve the nutrient utilization, with an increasing trend of 25 % per year (Anonymous, 2002). Common uses of bulk enzymes are listed in Table 3 (Rolle, 1998; Schäfer *et al*., 2007).

The world market for flavors and fragrances was estimated on U$ 16 billion on 2003. The quantities of flavors and fragrances are more than thousands, but only few hundred are economically relevant and produced for more than one ton per year. The extensive use of the substances is based on the widespread industrial use on beverages cosmetics, detergents, foods, pharmaceuticals. Most of the molecules or complex mixtures are obtained by extracting from plants and animals, and/or traditional chemical synthesis (Serra *et al*., 2005). From the chemical point of view flavors and fragrances are a broad group molecules involving from aldehydes (e.g. vanillin or 4-hydroxy-3-methoxybenzaldehyde), terpenoids (the main constituents of essential oils, typically limonene (4-isopropenyl-1-methyl cyclohexene), and menthol (2-(2-Propyl)-5-methylcyclohexanol)), esters, γ- and δ-lactones, ketones and other molecular species (Cheetan, 1993; Serra *et al*., 2005). Recently, new legislation in the EU and US had established the definition of natural for all marketed flavors, prepared only by physical methods or by biotransformations. Since the prices of flavors and fragrances in the market are depending on their quality and purity, and particularly organoleptic properties which are relying on stereochemical structure of molecules and mixtures; biotransformations and biocatalysis are very well suited to become strategic niche because they can be selected to run specific stereo- and regio-selective molecular synthesis.

However, the most powerful sectors that fueled biotransformations are fine-chemicals (including intermediary chemical blocks) and pharmaceuticals (Jaegger *et al*., 2002). In 2005, the biopharmaceutical market was about U$ 48 billons for only 130 biomolecules produced in large scale, and it is expected to reach U$ 100 billons at 2010 with about 17 % of all pharmaceutical market. At the present, the major therapeutic biopharma areas include oncology, anti-infectives, vaccines, and blood disorders (Downes, 2005). So, pharmaceutical industrial conglomerates are moving to develop new generation of drugs based on the basic concept of mimicking nature by searching new molecules and by the development of

molecule-molecule recognition therapies. Targets are cell-surface receptors and/or enzymes, which are always chiral molecules. For that reason, it is clear that only one enantiomer of a chiral drug has the desired biological activity, but other isomers may have not or worse than that, an unwanted biological activity. One of the worse cases in pharmacology history was the drug (±)-2-(2,6-dioxo-3-piperidyl)isoindole-1,3-dione best known as thalidomide, a prescription drug in the late 50's and early 60's to treat sleeping disorders and morning sickness during pregnancy. The R-enantiomer posses the desirable biological activity, meanwhile S-thalidomide is causing serious birth defects when taken during pregnancy. The first thalidomide formulation was a racemic mixture, and had caused terrible social and health problems during the 60's. As the counterpart during the 80's, a very good example of succeed in the pharmacologically arena was the synthesis of optically active Naproxen using hydrolases (Figure 2).

Typical examples of current biopharma targets for therapies are enzyme inhibitors, blocking cell surface receptors and/or metabolic pathways for the treatment of cancer, AIDS, diabetes, heart and immune diseases, microbial and parasitic infections like tuberculosis and filariosis. All of these molecules have in common high binding specificity, making pure enantiomers and chiral molecules the main candidates. It was estimated that the worldwide sales for single enantiomer molecule of pharmacological use exceeds U$ 100 billion, and more than half of new developed drugs reaching the market during the next decade will have optically pure active molecules (Jaeger *et al*., 2001). This tendency is so strong that some predictions had projected enantiopure pharmaceuticals will cover about 70 % of the total pharmaceutical market in the 21st century (Schulze and Wubbolts, 1999). Also, based on new US and EU legislations and regulatory constraints like patents issues, component purity associated with biological activities, biopharma industry will increase the development of processes based on biocatalysis and/or biotransformation for chiral molecules. Two different

strategies for production of chiral compounds were well established: resolution of racemic mixtures and asymmetric synthesis.

Bioremediation also can be considered as an special area of biotransformations, because is defined to bio-degrade or transform recalcitrant waste xenobiotics and toxic molecules in less harmful and/or reusable compounds. The list of pollutants human-made is vast, and covers a myriad of compounds ranging from explosives, aromatic hydrocarbons, pesticides, herbicides, and heavy metals to mention some. Application of bioremediation processes can be made from aquifers, to landfill leachates to industrial, city and military wastes. Microbial enzymes such as monoxygenases, dehalogenases, peroxidases were reported useful for many bioremediation process (Swanson, 1999; Anzenbacher & Anzenbacherová, 2001; Torres *et al*., 2003). However, bioremediation deserves a more detailed analysis, which is out the scope of the present chapter.

5.- Biocatalysts

The recognition of biocatalysts as key manufacturing tool for chemical and pharmaceutical synthesis has been increase since the advances of Biology and Biochemistry developed during the 70's. Biocatalysts can simplify, or in some instances even enable, the production process of complex chemicals and drug intermediates which are not able to be synthesized by standard chemical procedures.

5.1 Crude extracts

Biological crude extracts containing the biocatalyst are commonly obtained by cell cultivation, followed by a single and often simple separation step, generally centrifugation or extraction, to have cell-free extracts containing the desirable enzymatic activity. Study of enzyme activity and stability in crude extracts are key factors to take in account for the

development of any biotechnological process. So, catalytical properties such as substrate specificity, selectivity, and also operational parameters like thermostability, optimum pH, among others are relevant because they usually define the enzyme application range and type of process. As an example, crude lipolytic alcohol resistant extracts of *Aspergillus niger* MYA 135 produced in mineral medium with or without olive oil supplementation displayed different enzyme properties. Optimal conditions for olive-induced lipase were pH= 6.5 and 30-35°C, but had shifted to acid region (4.0-6.5) and 35-37°C when lipase extracts were produced under basal-media conditions (Pera *et al*., 2006). Another example is the response of lipolytic extracts from *Penicillium corylophilum* to hydrophobic solvents. The activity of lipolytic extracts were increase by 30 % when are incubated in presence of n-heptane with a reduced water activity of 0.95 (Baron *et al*., 2005). However, crude commercial lipases from *Candida rugosa* may be highly heterogeneous under similar culture conditions and can lead to irreproducible results, so crude extracts are not admissible for any process at industrial scale without proper activity controls. In order to normalize and discriminate enzymatic activities present in the batches, a simple methodology was proposed (Domínguez de María et al. 2006). In order to check the activity of *C. rugosa* lipase crude preparation, the catalytical performance parameter defined as % final yield x $(\text{mg catalyst})^{-1}$ in the transesterification of different alkanols with vinyl acetate was assayed. Synthesis of heptyl acetate was considered the parameter for quantification of lipases present in crude lyophilized powders. Furthermore, by changing the structure of the alkanols, it was even possible to evaluate in very ease way the relative percentage of the different isoenzymes of the crude catalyst.

5.2.- Whole cell biotransformation

The use of whole cell as catalytic agent has found widespread applications. Naturally cell-bound enzymes are potentially cost-effective because the biomass can be directly used. The whole-cell preparations are practical because are avoiding complex procedures of enzyme isolation, purification and immobilization which often results in loss of enzyme activity, also are time consuming and very expensive. Furthermore, the cell structure may act as natural matrix able to protect the enzymes from the possible negative action of external agents, providing an effect analogous to that exerted by common matrix used for enzyme immobilization.

5.2.1.- Naturally immobilized biocatalyst

Cell-bound membrane biocatalysts are typical examples of natural immobilized enzymes. Relevant examples are oxidative biotransformations with oxygenases, which are based on three main reasons (Li *et al*., 2002):

A) Their chemical counterparts either do not exist, or do not have the required regio- and stereo-selectivity.

B) They use oxygen as a cheap and environmentally friendly oxidant in contrast to toxic chemical oxidants, which also requires recovery and intensive waste treatment.

C) They can be used to prepare chiral building blocks and pharmaceutical intermediates, or to modify natural products with desirable biological activities.

Oxygenases-catalized reactions are also particularly fascinating since direct oxy-functionalization of unactived organic molecules is a challenge to synthetic chemist. Industrial-scale processes that use oxygenases are preferably based on whole-cell biotransformation. First, the *in-vitro* cofactor regeneration, though feasible, is relatively expensive. Second, oxygenases often consist of multiple components frequently located in the cell membrane. Fragrance compounds such as *cis*-jasmone ((*Z*)-3-methyl-2-(pent-2-enyl)-

cyclopent-2-enone), (R)-(+)-limonene (1-methyl-4-prop-1-en-2-yl-cyclohexene), (R)-(-)-carvone ((2-methyl-5-(prop-1-en-2-yl) cyclohex-2-enone), among others, were biotransformed by mycelium-bound oxygenases from *Trichosporum cutaneun* CCT 1903 (Pinheiro & Marsaioli, 2007). From the industrial point of view, the relevance of the mono-terpene biotransformations is based on the organoleptic properties of products: non-oxygenated terpenes are responsible for the fatty unpleasant notes in fragrant mixtures; meanwhile oxygenated terpenes are responsible for the floral and woody notes of essential oils.

Another typical example of naturally immobilized biocatalyst is *Carica papaya* lipase (CPL), which is being tightly bond to the water-insoluble fraction of crude papain. To date, several CPL applications have already been described, such as fat and oil modification, esterification and inter-esterification reactions using organic solvents, and more recently, asymmetric resolution of different non-steroidal anti-inflammatory drugs (Domínguez de María *et al*., 2006).

Thus, the use of whole cells presenting surface-bound enzymes may provide an alternative to the traditional way of performing enzymatic reactions.

5.2.2.- Morphology and biotransformation

The search of fungi for the production of commercially important products has increased rapidly over the past half century. Filamentous fungi are morphologically complex microorganisms, and exhibiting different structural forms throughout their life cycles. Fungal morphology can be associated with the expression of different biomarkers, among them some enzymes of industrial relevance. When fungi grown in submerged culture are exhibiting different morphological types, ranging from dispersed mycelial filaments to densely interwoven mycelial masses referred as pellets, which strongly affect the overall cell

performance. A particular fungal morphology is determined not only by genetic factors but also by environmental conditions such as spore ratio, medium composition, pH, temperature, reactor type and power input (Papagianni, 2004; Rodríguez Porcel *et al*., 2005). Furthermore, it is frequently reported that there is a direct relationship between morphology and some fungal product formation. Thus, pellet growth type is preferable for citric acid production by *Aspergillus niger* (Pera & Callieri, 1997; 1999), exo-biopolymer production by *Cordyceps militaris* (Park *et al*., 2002) and progesterone 11α-hydroxylation by *Rhyzopus nigricans* (Žnidaršič *et al*., 1998). While dispersed mycelia favors penicillin production by *Penicillium chrysogenum* (Smith *et al*., 1990), fumaric acid production by *Rhizopus arrhizus* (Byrne & Wars, 1989) and enzyme production by *A. niger* (El-Enshasy *et al*., 2006).

5.2.3 Cell surface engineering

The concept of using recombinant microorganisms for surface display of heterologous gene product has recently gained a lot of interest due to its promising application in Biotechnology. Functional proteins, such as enzymes, can be genetically immobilized on the cell surface via anchor proteins (Murai *et al*., 1997).

In-vivo immobilization of foreign proteins on the surfaces of Gram-negative bacteria is usually accomplished through fusion of the target protein with endogenous outer membrane proteins such as LambB, PhoE, OmpA, Lpp-OmpA, OprF, invasin, and ice-nucleation protein (INP) (Chen & Georgiou, 2002; Wernerus & Stahl, 2004). The target proteins are either fused to the N-terminal or C-terminal or inserted into a permissive region. A successful example is the immobilization of thermostable lipase from *Pseudomonas fluorescens* on cell surface of a solvent-resistant *Pseudomonas putida* GM 730 using INP anchoring motif from *Pseudomonas syrinage*. The surface-immobilized lipase retained full enzyme activity in two-phase water-isooctane reaction system after incubation at 37°C for 12

hours, while the activity of free form enzyme decreased to 65 % of its initial value in the same experimental conditions. This biocatalyst was efficiently used for olive oil hydrolysis, triglyceride synthesis and chiral resolutions (Jung *et al*., 2006).

Another relevant example was reported in Gram-positive bacteria, which are coding for one or more enzymes termed sortases, catalyzing the covalent anchoring of substrate proteins on their cell wall. They recognize an amino acid sequence, designated sorting motif, present close to the C-terminal end of the substrate proteins, cleave within this motif and catalyze anchoring of the polypeptide chain to the peptide cross-bridge linking the peptidoglycan strands in a transpeptidation reaction. It have been reported that *Bacillus subtilis* codes for two different sortases (Pallen *et al*., 2001), but the sorting sequences recognized by them are yet unknown. In order to immobilize proteins on the *B. subtilis* cell surface, the *srtA* gene coding for sortase A of *Listeria monocytogenes* with the known sorting motif (LPXTG) was introduced into *B. subtilis* (Nguyen & Shumann, 2006). This sortase recognizes a peptidoglycan cross-bridge that is identical to that present in *B. subtilis* cell wall (Navarre & Schneewind, 1999). To anchor the normally secreted *B. amyloliquefaciens* α-amylase on *B. subtilis* cell surface, its C-terminus was fused to the C-terminal region of *Staphylococcus aureus* fibronectin binding protein B containing the sorting motif.

Yeast cell surface engineering also has been used in many biotechnological fields (Ueda, 2004). Novel whole cell biocatalyst such as glucoamylase, α-amylase, CM-cellulase, β-glucosidase, and lipase were constructed. In this case, proteins are immobilized by using the C-terminal half of α-agglutinin (Kondo & Ueda 2004). More recently, *Rhizopus oryzae* lipase was displayed on cell surface of *Saccharomyces cerevisiae* by genetically immobilization procedure (Shigara *et al*., 2005). Hydrolytic and esterification activities of this new immobilized enzyme were higher than its commercial preparation, probably also because of lipases generally are interfacial biocatalysts.

5.3.- Immobilized biocatalyst

Immobilization systems are applied to enzymes, cellular organelles, microbial, animal and plant cells. These systems are intended to enclose the biocatalyst into a confined space in order to retain their activities and be reutilized several times over a long period of time. Several different methods have been used for biocatalyst immobilization, including adsorption onto insoluble materials, entrapment in polymeric gels, encapsulation in membranes, cross-linking with bi-functional or multifunctional reagents and linking to insoluble carriers.

5.3.1.- Adsorbed biocatalyst

Adsorption methods involve the binding of the biocatalyst to a water insoluble support by using ionic or covalent chemical links, bio-specific coupling, or junctions. Natural polymers such as polysaccharides (e.g. cellulose, dextran, and agarose derivatives), proteins (e.g. gelatin, albumin), synthetic polymers (e.g. polystyrene derivatives, polyurethane) and inorganic material (e.g. sand, clay, ceramics, magnetite) are commonly used for this purpose.

Recently, it was reported a procedure for immobilization of lipase from *Mucor miehei* by adsorption on micro-porous, asymmetric hollow fiber membrane reactors (Shamel *et al*., 2007). The biocatalyst was used to hydrolyze palm and olive oils at 40°C, average transmembrane pressure of 115 mm Hg, oil and aqueous flow rates of 2.5 and 3.0 ml/min, respectively. It was also experimentally proven that lipase adsorption increase with temperature and was higher on hydrophobic membrane than hydrophilic ones.

Hydrophilic and hydrophobic silica gels, synthesized by polyethyleneimine coating and silanization, respectively, were used as a carrier for a lipase from *Bacillus*

stearothermophilus L1. Covalently bound lipase was more stable under an extreme condition (pH 10 and 50°C) than lipase immobilized by physical adsorption (Hwang *et al*., 2004).

Catana *et al*. (2007) had evaluated a bioconversion system using both free and Amberlite-immobilized commercial inulinase (2,1-beta-D-fructan fructanohydrolase) from *Aspergillus niger*. The apparent activation energy of inulin hydrolysis decrease when immobilized biocatalyst was used. Furthermore, immobilized inulinase was successfully reused in repeated biotransformation runs with no significant decrease in product yield. This procedure is making a promising system for the development of a highly effective production of high fructose syrups from fructans.

Immobilization methods based on affinity principles utilizes specific binding of a biomolecule to a ligand, which is typically immobilized onto a surface. The enzyme to be immobilized is linked to a biomolecule either through chemical or genetic conjugation strategies. Bioaffinity-based immobilization methods are superior to physical or chemical ones due to they are providing reversible and oriented enzyme immobilization, which are rendering higher catalytic activity (Saleemuddin, 1999). There are a variety of affinity tags based on different recognition principles, including affinity ligand pairs and metal chelation (Huang *et al*., 2007; Yeliseev *et al*., 2007). Recently, calmodulin protein (CaM) was used as an affinity tag for reversible enzyme immobilization on surfaces (Daunert *et al*. 2007). This strategy takes the advantage of both the reversible calcium-mediate binding of CaM to its ligand phenothiazine and the ability to produce fusion proteins between CaM and a variety of enzymes to be reversible immobilized in an oriented fashion to many surfaces. Two different enzymes were selected, organophosphorous hydrolase (OPH) and β-lactamase, and tow different solid supports, a silica surface and cellulose membrane modified by covalently attaching a phenothiazine ligand. The immobilized CaM-OPH retained more than 80% of the free enzyme activity while the immobilized CaM-β-lactamase showed the feasibility of using

a phenothiazine surface in several consecutive loading and regeneration cycles. In presence of Ca^{+2}, CaM adopts a conformation that favors interaction between hydrophobic pockets in CaM and phenothiazine, while in the presence of a Ca^{+2}-chelating agent such as EGTA, the interaction between CaM and phenothiazine is disrupted, thus allowing for removal of the CaM-fusion protein from the surface under mild conditions. CaM also acts as a spacer molecule, orienting the enzyme away from the surface and toward the solution, which minimizes enzyme interactions with the immobilization surface. An additional and interesting advantage lies in that the support can be regenerated by passing through EGTA, and then reused for immobilization of the same or a different enzyme.

In last decades, microbial cell immobilization was attracting more interest for study different bioconversions. Since the adsorption phenomenon is based on electrostatic interactions between the charged support and the microbial cell, the actual zeta potential on both surfaces plays a significant role in cell-support interactions. As recent example, biodegradation of phenol by using covalent immobilized *Trichosporon cutaneum* onto polyamide and polyacrylonitrile membranes were described. In addition, immobilizations on polymer membranes are very suitable and could be used at industrial scale (Godjevargova *et al*., 2006).

In order to reduce costs, chrysotile, a plentiful and low-cost inorganic material, was evaluated as an immobilization matrix for *Mycobacterium* sp. cells. This biocatalyst system was used to efficiently transform β-sitosterol to 4-androstane-3,17-dione in an organic-aqueous two liquid phase system (Wendhausen *et al*., 2005). Sitosterol side chain cleavage is a key step for therapeutic steroids production, with an estimated world market above a thousand tons/year.

Many attempts have been made to immobilize enzymes using various nanostructures such as mesoporous media, nanoparticles, nanofibers, and nanocomposites. Especially, the

large surface area afforded by these nanostructures usually leads to high enzyme loading. Enzyme stabilization in nanostructures has also been described in many papers (Kim *et al*. 2006a). Sometimes, the apparent enzyme activity could be improved in nanostructures because of the relieved mass transfer limitation when are compared to macro-scale matrices using conventional enzyme immobilization techniques (Kim *et al*., 2006b). Among nanostructures, electrospun nanofibers have attracted a great attention as enzyme carrier. Electrospinning is the simplest, cheapest, and the most straightforward way to produce nanofibers by forcing a polymer solution or melt through a spinnerette with an electric field (He *et al*., 2007). Recently, Kim *et al*. (2005) successfully developed an active and stable enzyme system coating the surface of electrospun polymer nanofibers. This methodology uses covalent attachment of seed enzymes onto nanofibres consisting of polystyrene and poly(styrene-co-maleic anhydride) mixture, followed by a glutaraldehyde treatment that cross-linked additional enzyme molecules and aggregated from the solution onto the covalently attached seed enzymes molecules. On the other hand, polysulfone composite nanofibrous membranes containing poly(N-vinyl-2-pyrrolidono) or poly(ethylene glycol) were used to immobilize a lipase from *Candida rugosa*. This new biocatalyst system yielding enhanced thermal stability compared with free preparations (Wang *et al*., 2006).

5.3.2.- Entrapped biocatalyst

Another approach for enzyme immobilization is to entrap the biocatalyst into polymeric matrices, which usually retain the enzyme better than surface adsorption. Gelation of polyanionic or polycationic polymers by addition of multi-valence counter-ion is a simple and common method of enzyme entrapment. Alginates are one of the most frequently polymers used due to their mild gelling properties and lack of toxicity. Enzymes are entrapped by drop-wise addition of an aqueous solution of both sodium alginate and

biocatalyst to a hardening divalent cation solution salts (generally calcium or zinc). In order to avoid enzyme diffusion out of the gel matrix, the most important parameters of bioconversion efficiency are the degree of alginate cross-linking, and the molecular weight of the protein. However, the main issue of alginate gels is the poor mechanical resistance which decreased the half-life of the immobilized biocatalyst. In order to overcome this problem, an alternative strategy was coating alginate gel microspheres with other polymers. The coating procedure increased the strength of alginate gel matrix and had showed higher re-usability than non-coated ones, but on the other side imposes higher diffusional limitations of substrates and products going out or through the matrix.

Flavor compounds biosynthesized are considered natural under new legislation in US and EU. In this context, *Candida rugosa* lipase was entrapped in polyurethane foams and successfully used to produce ethyl butyrate in n-hexane (Pires-Cabral *et al*., 2007).

Whole cell immobilization by entrapment is also a useful alternative for biotransformation processes. *Bacillus subtilis* cells immobilized in calcium alginate were used for a semi-continuous production of α-amylase. The entrapment procedure led to an approximately 2.5-fold increase in the α-amylase yield in comparison to free cell culture (Konsoula & Liakopoulou-Kyriakides, 2006). Also, sodium silicate-based sol-gel entrapment method has been used to immobilize methanotrophic whole cells of *Methylomonas* sp. Immobilized cells almost kept the initial propylene epoxidation activity after 25 reaction batches. This stability behavior is relevant considering that free cells almost lost its activity after three reaction batches (Chen *et al*., 2004).

Another biotransformation approach by cell entrapment is the use of selective matrix in order to produce specific compound (Carballeira *et al.*, 2004). Free cell cultures of *Geotrichum candidum* NCYC49 are able to produce a mixture of cyclohexanol and ε-caprolactone (reduction and monooxygenation reactions) from cyclohexanone. However,

immobilized *Pterocladia* cells either in polyacryamide or in agar allows to have full control of the cyclohexanone biotransformation. Cells immobilized in 2.5% polyacryamide constitute a specific biocatalyst for the Baeyer-Villiger reaction, and specifically produce ε-caprolactone. On the other hand, when the *Pterocladia* cell immobilization is performed in agar the biocatalyst can acts both as selective for the reduction to cyclohexanol, if the reaction is performed at low shaking speed (100 rpm), or as selective for the Baeyer-Villiger reaction, if the reaction is performed at high shaking speed (250 rpm).

Another way to immobilize biocatalyst is to entrap it within manopores (Wang, 2006). Enzymes entrapped into mesoporous glass, either physically or through covalent binding, were greatly stabilized. Particularly, covalently bound α-chymotrypsin in nanoporous silica gel glass showed a half-life 1000 times higher than that of native one in organic solvents (Wang *et al.*, 2001).

5.4.- Protein engineering

One challenge of modern Biotechnology is the improvement of bioprocess by changing the biomolecules properties. Enzyme design with special capabilities involves DNA manipulation to construct hybrid biocatalyst. There are two complementary strategies currently available for generation hybrid enzymes: rational redesign and directed evolution. In both cases, the enzyme gene encoding, a suitable expression system, and a sensitive detection system are a prerequisites (Pera *et al*., 2005).

A typical example is the idea of expanding the functionality of lipase from *Rhizopus arrhizus* (RAL) using error-prone PCR and DNA shuffling methods to create RAL mutant with improved thermostability and high optimum temperature (Niu *et al*., 2006). The optimum temperature of the mutant lipase was higher by 10°C than that of the wild-type RAL (WT-RAL). In addition, thermostability of the mutant was also improved as result of

directed evolution. The half-life ($t_{½}$) at 50°C of the mutant exceeded those of WT-RAL by 12-fold. One amino acid substitution (E190V) that would contribute to improve the described function was identified by site-directed mutagenesis. The mutational effect was interpreted using a simulated structural mode of the mutant.

As an alternative to rational design and combinatorial engineering, it was proposed a data-driven approach that combining available knowledge about protein, such as alignment with homologous, to limit the library size without requiring the amount of detailed information necessary for rational design, such as the deactivation mechanism (Chaparro-Riggers *et al*., 2007). Data-driven protein engineering also limits a screening effort and still creates proteins with improved properties. Methods to detect which residues are important in a given template include structure, homology, experimental evidence, and computational algorithms. After identification, important residues can be mutated according to various schemes. The overall goal is to use available information to perform smarter protein engineering. As an example, Chordorge *et al*. (2005) screened an initial small-scale library and used statistical methods to determine the optimal level of mutation accumulation by balancing the number of inactive variants created with the probability of detecting an improved variant. A full-size library with that mutation level was then created and assayed for effect. After screening a total of 10,000 variants, the residual activity of *Candida Antarctica* lipase B was increased 7.5-fold after 15 min of incubation at 90°C.

6.- Reaction media

In order to enhance catalytic activity, selectivity and stability new concepts have been developed as reaction engineering area. Reaction medium engineering is to make the enzyme structure more stable by changing the surrounding media rather than the enzyme structure. This approach also involves the control of physicochemical reaction parameters. This often

includes a trade-off between maximizing the extent of the reaction and its specificity versus minimizing costs. Reaction engineering can be achieved either by the use of non-aqueous organic solvents and/or by changing the salt composition of an aqueous solution. Up to date, most of biotransformations in this type of systems are based on liquid, liquid-liquid, gas-solid, and liquid-solid phases. The gas phase is usually N_2 either with or without an inert component with an occasional volatile product. The liquid phase may consist of a reactant, product or a solvent. In most cases, the solid phase is a catalyst but in some cases, it is also a sparingly soluble reactant or product.

Solvent-free systems are highly concentrated media, economically, and operational interesting for industrial processes. In this kind of systems not only the cost of the solvent itself is avoided, but also its separation from un-reacted substrates and products, and the cost of recycle as well. Shah and Gupta (2006) have chosen this system for biotransformation of the non-edible *Jatropha* oil to biodiesel. A yield of 98% (w/w) was obtained by using *Pseudomonas cepacia* lipase immobilized on celite at 50°C containing 4-5% (w/w) water in 8 h reaction time. Also, the reaction yield does not decrease by switching substrate quality from analytical to commercial grade, suggesting a high reaction efficiency conversion. In addition, another advantage of the solvent-free system is that catalyst could be reused at least four times without losing activity.

Several advances in biocatalytic biphasic systems such as aqueous two-phase reactor using environmental sensitive polymers, two-phase aqueous-organic systems, reverse micelles, microemulsions-based organogels, and trapped aqueous-organic solvent continuous biphasic reactor were extensively reported in the literature, but mostly are systems at laboratory scale (Pera *et al*., 2003). In addition, as new trends the developments of ionic liquids and supercritical fluids systems as green non-aqueous media for enzymatic

transformations has been studied during the last decade (Mesiano *et al*., 1999; Yang & Pang, 2005).

Ionic liquids are salts and therefore entirely composed of ions that are liquids below 100°C or close to room temperature. Their interest as green solvents resides in their high thermal stability and very low vapor pressure, which mitigates the problem of releasing volatile organic solvents onto the atmosphere. As compared to those observed in conventional organic solvents, enzymes in ionic liquids have presented enhanced activity, stability, and selectivity. Other advantages as reaction medium include their high ability of dissolving a wide variety of substrates, especially those highly polar ones, and their widely tunable solvent properties through appropriate modification of cations and anions (Yang & Pan, 2005). However, little attention has been paid to the intrinsic reactivity of these low temperature molten salts. Clues to the non-innocent nature of many ionic liquids are contained in the reports of altered reactivity of dissolved substrates, unexpected catalytic activity and unforeseen by-product formation (Chowdhury *et al*., 2007).

Bio-reaction in supercritical fluids is also an established new field of research in the area of clean technology. Supercritical carbon dioxide has emerged perhaps as the most promising non-aqueous green solvent. It is toxicologically harmless, not inflammable, readily available and inexpensive. Besides, the easily tunable solvating power of supercritical carbon dioxide facilitates a relatively easy separation of reactants, products, and catalysts after reaction.

In connection to solid-fluid heterogeneous biocatalysis, an interesting approach was recently reported by Laudani *et al* (2006). They successfully carried out the synthesis of n-octyl oleate in supercritical CO_2 by immobilizing *Rhizomucor miehei* lipase in a continuous high-pressure packed-bed reactor. The system stably operated for at least 7 weeks by intermittent washing with CO_2. Productivities of fatty acid ester on the order of kg/l-reactor

day were achieved. Due to its intrinsic characteristics, solid-gas biotransformations appear to be a promising technology for basic research and the development of new cleaner industrial processes. The use of enzymes or whole cells at solid-gas interface now appears concurrent to liquid processes and presents some very interesting features since total thermodynamic control of the system can be achieved easily. Moreover, from a technological point of view, solid-gas systems offer very high production rates for minimal plant sizes, allow important reduction of treated volumes and simplified downstream processes. The advantages of the system are based on the ability to precisely control all thermodynamic parameters influencing not only the kinetic of reactions, but also the stability of the biocatalysts. As an example, a continuous gas phase reactor was used to study the effect of organic molecules on alcoholysis kinetic of methyl propionate with 1-propanol catalyzed by immobilized *Candida Antarctica* lipase B (Létisse *et al*., 2003). In this reactor, a solid packed enzymatic sample is percolated by nitrogen (carrier gas) containing gaseous substrates, and if necessary other gaseous components can be added or removed from the reactor (including products). One of the main advantages of this type of system is the possibility of setting thermodynamic activity of all gaseous components at the desired values independently. Particularly, it is feasible to study the influence of an extra added component at constant thermodynamic activity value, contrary to classical solid/liquid systems (in which large variations of thermodynamic activity are observed when an additional component, e.g. solvent, is added). Therefore, by performing alcoholysis in gas/solid system, it was possible to infers that the dissociation constant between enzyme and acyl substrate, and the alcohol inhibition constant (K_I) are strongly enhanced by polar compounds like water, and fairly enhanced by a moderately polar compound like 2-methyl-2-butanol (Graber *et al*., 2003). On the contrary, an apolar compound like hexane has no effect on K_I at a thermodynamic activity equal to 0.3. Besides, the feasibility of the enzymatic enanantioselective acylation through transesterification of

methyl propionate and R-2-pentanol in a solid/gas reactor catalyzed by CALB was also reported (Leonard *et al.*, 2004). On the other hand, biotransformation of halogenated compounds by lyophilized cells of *Rhodococcus erythropolis* in a continuous solid-gas biofilter was recently described (Erable *et al*., 2005). *Rhodococcus* species are well known to play a significant role in the biodegradation of volatile organic compounds and could play a relevant role for the development of next generation bioremediation processes. The main advantage is that *R. erythropolis* can utilize a wide range of 1-haloalkanes as sole carbon source. Short chained 1-chloroalkanes (C_2 to C_8) appear to be metabolized by the initial action of hydrolytic dehalogenase (E.C. 3.8.1.5) to form the corresponding alcohol. At the present, haloalkane dehalogenases are the only enzymes able to dehalogenate halo-compounds without cofactors (coenzymes or oxygen) and are thus attractive catalyst for biotransformation of volatile halogenated organic compounds. This new type of biocatalyst is a very promising tool for bioremediation processes.

7.- Web sites

Additional information about enzymes, biocatalysis and biotransformations and including general information on the web is listed alphabetically on Table 4.

8.- Concluding Remarks

The area of biotransformation, including biocatalysis, is going to play a key role in our societies by changing most of the processes for synthesis and degradation of materials to produce “clean” goods at large scale. Benefits of biotransformations include synthesis of novel compounds non-available by chemical tools, high material recovery and yields, low energy consume, and eco-friendly processes.

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Table 1. Relevant enzyme properties

High specific conversion rates of substrates on products.
Enzymatic reactions can be performed on mild experimental conditions.
Very high substrate selectivity in complex mixtures.
Regioselectivity, defined as the enzyme capability to distinguish a specific chemical group location in the substrate molecule.
Functional group selectivity, defined as the ability of enzyme to catalyze the reaction on one functional group selectively in the presence of the same functional groups located in other part of the molecule.
Stereoselectivity, defined as the capacity of an enzyme to act selectively on a single enantiomer or diastereomer.
Absence of side reactions.
Enzymes are not bound to their natural role (unnatural substrates can be used)
Some enzymes are requiring cofactors for catalytic function (e.g. NAD^+/NADH).
Enzyme activity can be modulated by the presence of activators/inhibitors.
Biocatalysts are environmentally friendly (Green chemistry).

Table 2. Enzyme classification

Class (E.C.)	Enzyme Type	Reaction type	Examples of biotransformations
1	Oxidoreductases	Redox reactions. Oxygenation or hydrogenation of substrates	Serra *et al*., 2005
2	Transferases	Transfer of group(s) from one molecule to another	Breuer and Hauer, 2003; Daines *et al*., 2004.
3	Hydrolases	Cleavage of group(s) in a molecule	Pandey *et al*., 1999; Daines *et al*., 2004; Serra *et al*., 2005.
4	Lyases	Addition of double bond(s) to a molecule	Breuer & Hauer, 2003
5	Isomerases	Several types of isomerization	Maru *et al*., 2002
6	Ligases	Formation of bonds (synthetases)	Turner, 2000; Daines *et al*., 2004.

Table 3. Common bulk enzyme applications in industry

Enzyme	Product	Purpose
Alpha amylase	Sweeteners Detergents	Starch liquefaction Weaning foods Viscosity reduction Stain removal
Alpha galactosidase	Legumes	Flatulence reduction
Beta-amylase	Sweeteners Beer	Saccharification Malting
Cellulases	Oils Detergents	Oil extraction Laundry
Chitinase		Antimicrobial activity
Cutinase	Koji Fish sauces Protein hydrolysis	Polyester oligomer removal
Glucoamylase	Starch	Saccharification of liquefied starch
Glucose oxidase + catalase	Fruit juices	Flavour and colour preservation Removal of oxygen
Hydantoinases (amidases)	Processed food	aminoacids change in food tastes
Laccase	Juices	Removal of oxygen
Lactase	Milk	Degradation of lactose
Lactoperoxidase	Milk	Antibacterial activity
Laminarase + hydroxy nitrile lyase	Cassava	Elimination of toxic compounds
Lipases	Oils	Modification of oils

	Detergents	triglyceride synthesis; transesterification of fatty acids;
Mannanase		Degradation of galactomanan
Naringinase	Citrus	Juice debittering
Oxidoreductases	Detergents	Bio-bleaching and disinfection
Papain	Fish sauces	Production of fish protein hydrolysates
Pectic enzymes	Fruit and vegetable juices	Maceration, depectinization; improved extraction, bast fiber processing
Phospholipase	Natural oils	Oil Degumming
Proteases	Silk Milk	Silk Degumming, Biofuels protein degradation of plant residues Milk low allergenic dairy products
Pullulanase	Sweeteners	Saccharification

Table 4. Biocatalyst information on the web

Name - Institution	Web site
Biocatalysis and biodegradation data base - University of Minnesota	http://umbbd.ahc.umn.edu
BRENDA - Cologne University Bioinformatic Center	http://www.brenda.uni-koeln.de
ChemLin (virtual Chemistry library)	http://www.chemlin.net/chemistry/enzyme_chemistry.htm
EBI PDB sum - European Bioinformatics Institute (EBI)	http://www.ebi.ac.uk/thornton-srv/databases/enzymes/ http://www.ebi.ac.uk/intenz/
EXPASY - Swiss Institute of bioinformatics - Enzyme nomenclature database	http://www.expasy.ch/enzyme http://www.expasy.ch/sprot
Frontiers of Biosciences (restriction enzymes)	http://www.bioscience.org/index.html
KEGG - Kyoto Encyclopedia of genes and genomes	http://www.genome.ad.jp/kegg http://www.genome.ad.jp/dbget/ligand.html
Lipase Engineering - University of Stuttgart	http://www.led.uni-stuttgart.de
Metalloprotein database and Browser - The Scripps Research Institute	http://metallo.scripps.edu
RCSB protein data bank	http://www.pdb.org

Legend of Figures

Figure 1. Scheme of optically active nonsteroidal anti-inflammatory drug Naproxen from his precursor by catalyzed carboxyl esterase (Cretich et al, 2001).

Figure 2. Scheme of acrylonitrile biotransformation into acrylamide by nitrile hydratase from *Rhodococcus rhodochrous* J1 cells

Figure 1.

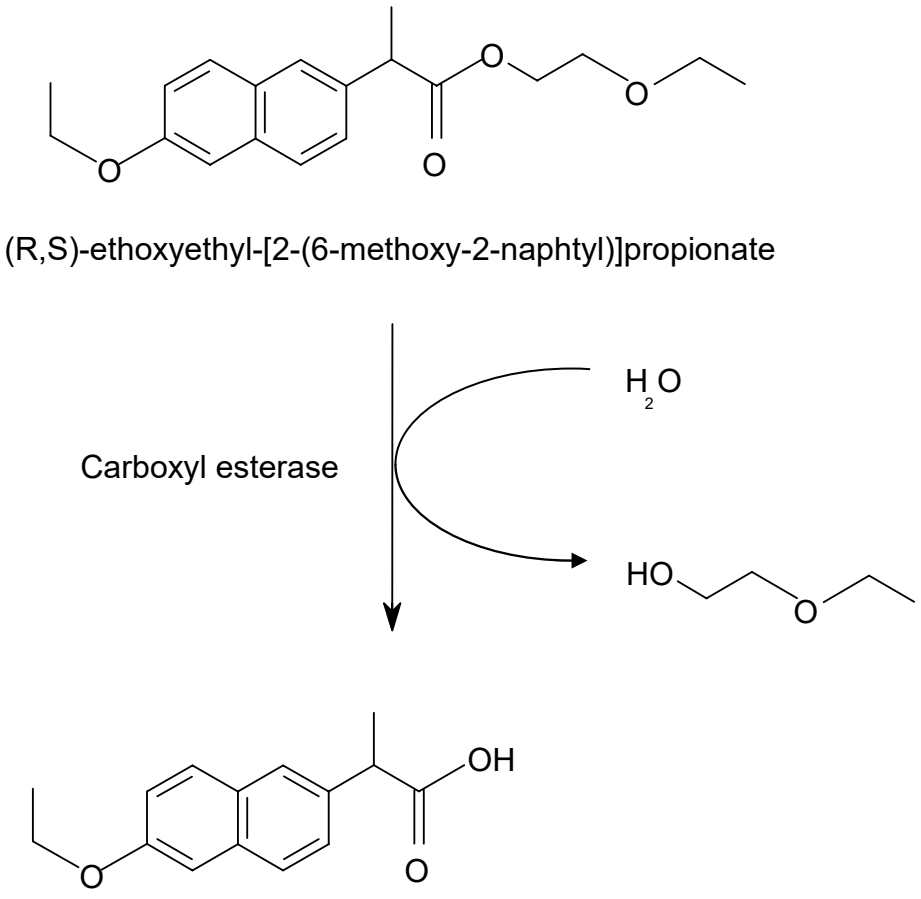
O
O
O
O
(R,S)-ethoxyethyl-[2-(6-methoxy-2-naphtyl)]propionate
Carboxyl esterase
H2O
HO
O
OH
O
O
(S)-(+)-Naproxen

Figure 2.