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Recent trends in biocatalysis engineering

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ABSTRACT

During the last 30 years the scope of biocatalysis has been expanding due to the advances in several technological fields. Diverse techniques as structural enzyme improvement (*e.g.* protein engineering, direct evolution), engineering approaches (*e.g.* ionic liquids, supercritical fluids) and physical stabilization (*e.g.* immobilization, CLEAS) have been developed, which in combination are powerful tools to improve biotransformation and to synthesize new products. In the present work, recent advances in biocatalysis are reviewed.

1. Enzyme properties: advantages and constraints

Enzymes are naturally tailored protein catalysts synthesized to perform under physiological conditions. However, biotransformations imply the use of enzymes under conditions that may depart significantly from physiological states. The challenge consists in building catalysts that preserve functional properties of enzymes but robust enough to withstand harsh process conditions. Enzymes are catalysts of exquisite specificity, being then well appreciated for the synthesis of pharmaceuticals and fine chemicals (Woodley, 2008). On the other hand, many enzymes are rather promiscuous catalysts since they are capable of catalyzing several reactions and/or transforming many substrates, in addition to the ones for which they are physiologically specialized, or evolved (Khersonsky and Tawfik, 2010). Enzymes possess complex molecular structures, which are labile and costly to produce. Besides, one of the main problems in enzyme biocatalysis is the low operational stability. Despite the enzyme constraints, many complementary strategies were developed to improve their performance. Enzyme engineering is based either on physical modifications of protein or DNA. In addition, recent developments in enzyme production by high-tech fermentation allow the production of cheaper and stronger enzymes for industrial use. This is opening up unprecedented opportunities beyond the traditional areas of food and detergents, such as in high added value processes for the production of pharmaceuticals, cosmetics, agrochemicals and fine-chemicals (Ran et al., 2008).

Enzymes can be produced from any living organism, either by extracting them from their harboring cells or by recovering from cell exudates. Microbial cells are excellent enzyme factories representing about 90% of the total biotransformation market. Microbial screening is a simple and frequently used method for finding new biocatalysts with required properties. At present, high-throughput-screening and metagenome analysis of uncultured microorganisms is utilized to take full advantage of the microbial diversity (Steele et al., 2009) and to produce new enzymes with outstanding properties. This is of particular interest for organic synthesis that usually requires non-conventional reaction media in which biocatalysts should be active and stable (Illanes, 2008). Additionally, the advances in molecular genetics and genetic engineering have made possible to clone and express virtually any gene into a suitable microbial host.

At the same time, microbes can be seen as Microbial Chemical Factories (MCFs) with metabolic pathways and enzymes that have

Abbreviations: B-FIT, B-Factor Iterative Test; CBM, Combinatorial Beneficial Mutagenesis; CLEs, crosslinking enzymes in solution; CLEAs, crosslinking enzymes aggregates; CLECs, crosslinking enzymes crystals; CM, cassette mutagenesis; Combi-CLEAs, co-aggregated enzymes; CoMFA, comparative molecular field analysis; CSD, consensus sequence design; DE, directed evolution; epPCR, error-prone PCR; FMO, fragment molecular orbital; IL, ionic liquids; ISM, iterative saturation mutagenesis; LTM, Look-Through Mutagenesis; MCFs, Microbial Chemical Factories; MD, molecular dynamics; MSA, multiple sequence alignments; NMR, nuclear magnetic resonance; RM, random mutagenesis; RD, rational protein design; SC-CO_2, supercritical-CO_2; SCSF6, sulfur hexafluoride; SDM, site-directed mutagenesis; SDSM, site directed saturation mutagenesis; SENs, single enzyme nanoparticles; SF, supercritical fluids; SPIONs, superparamagnetic iron oxide nanoparticles; StEP, staggered extension process; 3D-QSAR, three-dimensional quantitative structure-activity relationship.

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evolved along challenging environmental conditions for millenniums. *De novo* pathway engineering facilitates the expansion of microbially synthesized compounds beyond natural products. Protein engineering can be used for improving the selectivity and activity of enzymes and can be effectively complement conventional metabolic engineering approaches such as increasing the precursor supply by varying pathway enzyme expression levels or knocking out competing pathways to enhance productivity. Some recent studies focusing on the design, engineering and optimization of MCFs are reviewed previously (Dhamankar and Prather, 2011).

2. Biocatalysts: past, present and perspectives

According to the International Union of Biochemistry (IUB), enzymes are divided into six classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Hundreds of enzymes are used industrially, over half are from fungi, over one-third are from bacteria, with the remainder originating from animal (8%) and plant (4%) sources. Over 500 commercial products are made using enzymes. The industrial enzyme market reached U$ 1.6 billion in 1998 and in 2009, the market was U$ 5.1 billion. In the 1980s and 1990s, microbial enzymes replaced many plant and animal enzymes and they have found use in many industries including food, detergents, textiles, leather, pulp and paper, diagnostics, and therapy (Sánchez and Demain, 2011).

On the other hand, enzyme immobilization widened the scope of application allowing less stable, intracellular and non-hydrolytic enzymes to be developed as process catalysts and biocatalysis in non-aqueous media. This approach allowed to open up a vast field of enzyme applications in reactions of organic synthesis, with an exquisite selectivity, especially for the synthesis of pharmaceuticals and bioactive compounds (Illanes, 2008). Enzyme activity under mild conditions is also a valuable attribute for the production of labile compounds, having profound technological implications, significantly reducing the costs of equipment, energy and downstream operations. However, the use of enzyme catalysts in organic synthesis has been difficult to adopt by an industry not sufficiently acquainted to deal with biological materials. The bottlenecks of enzyme technology are their high cost, instability and poor performance under reactor conditions, narrow substrate specificity and requirements of complex cofactors, major hurdles for process development. Many of these constraints are in the way to be overcome both by advances in biocatalyst and medium engineering and by bioreactor design (Berenguer-Murcia and Fernandez-Lafuente, 2010; Dalby, 2011). Of remarkable technological potential is the use of robust and readily available hydrolases in reverse reaction of synthesis. As typical examples, proteases can catalyze the formation of a peptide bond (Kumar and Bhalla, 2005), carbohydrases can catalyze the synthesis of oligosaccharides by transglycosylation (Park and Oh, 2010) and lipases can catalyze esterification, interesterification and transesterification reactions (Hasan et al., 2006).

Beyond organic synthesis, enzyme biocatalysis is playing an increasingly important role in the large-scale production of biofuels from renewable resources, since they are not associated with CO_2 production. Enzymatic hydrolysis of pretreated lignocellulose biomass is a key operation in the second generation bioethanol production. Thus, the development of more active and more stable cellulases is crucial. The main goal is to reduce its cost impact from about US$ 0.1 to about US$ 0.02 per liter of bioethanol by developing a front line technology (Gray et al., 2006). Biodiesel is a substitute diesel fuel produced from triglyceride sources, like vegetable oils, animal fats and even recycled cooking-oils and algal biomass. Biodiesel is considered a fossil diesel substitute that has been produced by chemical transesterification of triglycerides being, for the moment, the technology of choice. Nevertheless, enzyme catalysis is more specific and environmentally benign then, enzymatic transesterification with lipases is under intense research, being the cost of the enzyme the main drawback for performing the chemical synthesis (Ranganathan et al., 2008). In relative terms, enzyme biocatalysis applications related to energy and health are expected to have the most significant growth in the forthcoming decades, meanwhile conventional applications in food, detergents, textiles and leather are expected to grow at a lower rate.

3. Biocatalyst improvement

The availability of suitable enzymes with high activity and stability under process conditions, desired substrate selectivity and high enantioselectivity is required for the efficient application of biocatalysts. Many biocatalysts present high chemo-, regio- and stereo-selectivity at room temperature, making them superior to chemical catalysts. Recent progress in genetic-manipulation techniques allowed the improvement of the scaling up of the production of many enzymes at reasonable prices. For example, the change of a cofactor can increase enzyme activity, as it was reported for the novel NADH-dependent carbonyl reductase (Ye et al., 2010).

New enzymes with better performance can be isolated from living organism (biodiversity prospecting). Bioprospecting is relevant for the isolation in functional enzymes in extreme environments, such as organic solvents or extreme pHs, since their potential applications at industrial level. Wild-type enzymes, almost universally, exhibit low activities and/or stabilities (in the presence of these solvents) because they were not screened as organic solvent tolerant enzymes (Torres et al., 2009, 2011). Lately, various attempts have been made to screen enzymes from various microorganisms including organic solvent tolerant bacteria, thermophiles, halophiles and mesophiles. Nevertheless, the identification and characterization of new biocatalysts (*e.g.* screening of soil samples) does not always yield suitable enzymes for bioprocess reactions. Protein engineering using computational techniques and site-directed mutagenesis, or by directed (molecular) evolution techniques can be a powerful tool to produce enzymes with optimized features such as activity, selectivity (enantio-, regio- and chemo-), stability, substrate specificity, cofactor specificity, and solubility in cosolvents, pH optimum and cofactor requirements, among others. Those approaches are demonstrating to be successful for the biocatalysis production at large-scale.

3.1. Structural strategies: directed evolution and rational design

As can be pointed out above, the most widely used approaches to improve biocatalysts include: (1) directed evolution and (2) rational design.

3.1.1. Directed evolution

Directed evolution (DE) comprises a group of molecular biology techniques that allow natural evolutionary processes to be mimicked in the laboratory to optimize functional proteins, by creating a finite number of randomly distributed variants, radiating outwards in sequence space from parent enzymes that already have measurable activity (Dalby, 2011). DE involves the random mutagenesis (RM) of one or more starting enzymatic genes, followed by a screening or selection step to isolate or enrich for enzyme variants with improvements in one or more desirable properties. The process can be iterated until the desired change is reached, or until no further change is elicited. The most common strategies are error-prone PCR (epPCR) (Chen and Arnold, 1993) and DNA shuffling. The epPCR introduces random point mutations in a population of DNA products producing a high number of simultaneous mutations per gene, being useful for identifying

hotspots for site directed saturation mutagenesis (SDSM). DNA shuffling techniques allow random recombination typically between parent genes with more than 70% homology exploring chimeras of functional parent enzyme sequences retaining a high proportion of functional progeny (Stemmer, 1994). Another recombination technique is staggered extension process (StEP) that consists of priming the template sequence(s) followed by repeated cycles of denaturation and extremely abbreviated annealing/polymerase-catalyzed extension. In each cycle, the growing fragments anneal to different templates based on complementary sequences and extend further. The procedure is repeated until full-length sequences are formed (Zhao et al., 1998). Recombination methods create mutations that are mostly present in at least one of the functional parent sequences, while RM can create non-natural mutations. Thus, recombination methods tend to generate a greater proportion of sequences that retain native activity than RM, being the later more useful for acquiring novel function. Subsequent DE techniques accessed a wider range of amino acids through SDSM or cassette mutagenesis (CM) targeted to pre-chosen sites or at randomly distributed sites, and enabled the random recombination of non-homologous genes. Thus, SDSM implies the randomization of individual residues to all 20 amino acids; it was used to create single mutants at hotspots. Instead, CM produces simultaneous saturation mutagenesis at multiple adjacent target residues. Further techniques can create random insertions and deletions of codons, shuffle domains or exons, or loop regions, and produce a library of random truncations.

New DE methods were used to enhance thermostability as Look-Through Mutagenesis (LTM), which was developed as a method for rapid screening. LTM consists in amino acids mutations in protein sequence selected positions introducing favorable properties (Hokanson et al., 2011). In addition, Combinatorial Beneficial Mutagenesis (CBM) is a new method for identifying the best ensemble of individual mutations. For example, both methods were used to enhance the stability of wild-type GH11 xylanase 2 from *Hypocrea jecorina* (Hokanson et al., 2011). In addition, they could be applicable for quickly improving the physicochemical properties of enzymes at large scale.

Traditionally, DE relies on an iterative two-step protocol, initially generating molecular diversity by random mutagenesis and *in vitro* recombination, then identifying library members with improvements in desired phenotype by high-throughput screening or selection. This approach can be problematic as even protein libraries with millions of members still sample only a tiny fraction of the vast sequence space possible for an average protein (Stefan, 2010). This problem can be solved by a semi-rational, smart or knowledge based library design which utilizes information on protein sequence, structure and function, as well as computational predictive algorithms to preselect promising target sites and limited amino acid diversity for protein engineering. The focus on specific amino acid positions translates into dramatically reduced library sizes while the consideration of evolutionary variability, topological constraints and mechanistic features to weigh in on amino acid identity can result in libraries with higher functional content. Together, these concepts offer promising predictors for altering protein features such as substrate specificity, stereo -selectivity and stability by enzyme redesign without modifying catalytic machinery as well as the creation of new functions by *de novo* design.

Despite that DE techniques have been extensively used for many years, it is required to be adapted to singular enzyme, and depends on the enzyme function or property to be improved. At present time, faster and more efficient DE strategies are necessary to be developed not only to overcome the enzyme function and properties but also to create catalytic functions not yet observed in enzymes.

3.1.2. Rational design

Rational protein design (RD) was the earliest approach for engineering enzymes. RD consists of a set of molecular biology techniques, such as site-directed mutagenesis (SDM), based on enzyme structural studies. The right identification of residues responsible for substrate-enzyme interactions, activity, specificity, docking and stability is essential for the application of RD in a directed manner. In some cases, it is necessary to perform alanine scanning or sequence homology of related species combined with biophysics data. Improvements in the prediction of the effect of SDM have been created thus, simultaneously the effect of mutation on stability, ligand affinity and pK(a) values can be evaluated as well as predictions for multiple mutants in one submission. For example, the active site Trp residues of the GH10 xylanase from *Cohnella laeviribosi* HY-21 play a key role in catalysis and/or substrate-binding by means SDM. Furthermore, mutants could be exploited as biocatalysts for enhanced synthesis of alkyl glycosides or xylooligosaccharides substituted with *p*-nitrophenol (Kim et al., 2010).

Since RD needs the knowledge of the structure of the enzyme of interest and/or its sequence in several and related species, the crystallography and spectroscopic analysis of many enzymes have been a powerful tool to use in computer modeling. This approach depends on the progress made in structure determination, improved modeling protocols and new insights into structure–function relationships. On the other hand, advances in modeling, especially calculations of free energy perturbation and molecular dynamics (MD) can predict the proper mutations for the improvement of enzyme enantioselectivity. Recent trends using mature enzymatic methods, mostly with α/β-hydrolase fold enzymes have been reviewed (Kourist and Bornscheuer, 2011). Several subclasses of enzymes which present that fold have a conserved motif GGG(A)X in their active site that was first identified by Bornscheuer's group as the motif necessary for the activity towards tertiary alcohols. By RD, the first Gly has been shown to be a key residue strongly affecting the enantioselectivity in esterase BS2 from *Bacillus subtilis* sp. In addition, molecular modeling methods in combination with high-throughput screening proved to be useful for the identification of key residues and changes in selectivity for esterase BS2 from *B. subtilis* sp. Besides, the creation of some structural databases as Lipase Engineering Database with annotated aligned sequences and superimposed structures of microbial lipases help to understand the functional role of individual amino acids on the enzyme structure (Pleiss et al., 2000).

Taking into account evolutionary constraints and energy contributions, computer simulation studies were developed for the improvement of enzyme catalysis. The desolvation hypothesis was considered and it was pointed out that mutation studies are inconsistent with ground state destabilization mechanisms (Warshel and Florián, 1998). Recently, in MD simulations, approximate free energy and hydrogen bond energy calculations were integrated to uncover the structure–activity relationships. Likewise, the integration of different structural prediction techniques can be applied in RD of enzymes as is the case of molecular docking, FMO calculation and 3D-QSAR CoMFA modeling (Zhang et al., 2008). Since, structural information is not always available, a new strategy as consensus sequence design (CSD) was developed. CSD is an appealing strategy for the stabilization of proteins, that exploits amino acid conservation in sets of homologous proteins to identify likely beneficial mutations and it does not depend on the availability of structural information (Jäckel et al., 2010). Data-driven CSD is based on the simple assumption that the frequency of a given residue in a multiple sequence alignments (MSA) of homologous proteins correlates with that amino acid's contribution to protein stability. Nevertheless, its success depends on the phylogenetic diversity of the sequence set available. The application of this method show that a phylogenetically unbiased CSD

can lead to substantial stabilization of secondary structural motifs in mesostable proteins. Optimizing the amino acid alphabet used for randomization with respect to structural propensities and functional diversity, and taking covariation into account in the design process are additional strategies that might be exploited to maximize stabilization while preserving activity.

Recently, RD by MD simulations was applied in thermostability improvement without reducing enzyme activity taking into account protein-surface properties instead on protein-core characteristics such as core packing and cavity filling (Joo et al., 2011). Thus, flexible surface residues tolerant to mutations are valid targets for thermostabilization and that local-interaction stabilization of cavity-lining residues using the MD method can be an effective alternative to the conventional cavity filling method.

Novel NMR relaxation dispersion experiments coupled to mutagenesis studies recently have been applied to the study of enzyme catalysis, effectively complementing "structure–function" analysis with "flexibility–function" investigations. NMR methods provide a powerful tool to help characterizing the effects of controlling long-range networks of flexible residues affecting enzyme function (Doucet, 2011).

Another new approach consists in the combination of SDM with immobilization on a support to improve the properties of immobilized biomolecules for use as biosensors or biocatalysts. Furthermore, SDM to control immobilization is useful for improving the activity, the stability and even the selectivity of the immobilized protein. Advances in support design and deeper knowledge of the mechanisms of enzyme–support interactions have allowed exploring new and better possibilities (Hernández and Fernández-Lafuente, 2011).

3.1.3. Combined methods

For industrial biocatalysis it may be necessary to combine various DE strategies and techniques that may include the knowledge-based design of enzyme variant libraries. Computational methods can potentially guide the RM of proteins making DE more efficient. Alternatively, the utilization of molecular potential functions can be helpful to predict the effects of mutations on protein structure and stability for libraries of enzyme variants generated *in silico*. Several examples of the combination of DE and RD can be found in the literature to improve stability, cold adaptation, novel activities and products, substrate specificity utilizing sequence base enzyme redesign among others.

For rapid evolution of enzyme stability, a method known as iterative saturation mutagenesis (ISM) combines the randomization of SDSM with RD where the saturation was targeted at areas of the protein that are likely to create an enhanced phenotype based on structural or catalytic information (Reetz and Carballeira, 2007). In addition, ISM represents a "rapid" form of evolution in which the libraries created are small and focused and therefore do not require extensive screening programs.

Crystallographic B-factor data have also been utilized in recent examples, to identify sites at which RM could improve stability. The B-Factor Iterative Test (B-FIT) highlights the amino acids with the highest flexibility and thereby creates targets for mutagenesis. A successful engineering approach for enhanced thermostability using DE based on ISM in combination with the B-FIT method, showed besides, a significantly increased tolerance to hostile organic solvents (Reetz et al., 2010).

As can be appreciated protein engineering is a powerful tool and it is extensively applied in the study and improvement of enzymes to be used in bioprocess.

3.2. Environmental engineering approaches

Biotransformation engineering approaches involve the tuning of all physicochemical parameters of reaction media. Changes in the microenvironment of the biocatalyst not only are able to modulate the enzyme activity and stability but also to shape the enzyme selectivity. One of the main variables in biotransformations is the water content in the enzyme microenvironment. However, enzymes acting in bulk aqueous media are not generally useful in biotransformations. Therefore, the use of biocatalysts in synthetic reactions is constrained to environments in which water activity is restricted. The water activity is relevant since it involves the effect of water mass action on the chemical equilibrium (Castro and Knubovets, 2003). Besides, the role of water is complex and diverse, since water is able to participate directly as a substrate, and/or during the transitions states and/or as reaction product. Additionally, water can take part in the reaction not directly, but in an equally relevant role as "lubricant", providing solvation to polar residues of the biocatalyst and other intervening molecules in order to facilitate protein conformational changes during the biocatalytic process and to speed up the reaction. There are many different approaches to control water activity in biotransformations, but the control of media is the simplest one. The optimization of the biocatalytic process involves the knowledge of the enzyme source and their properties, the physical state of the biocatalyst and the reaction media characteristics.

The enzyme can be dissolved in the medium or added to the reaction media in different heterogeneous forms as powder or crystal or as microheterogeneous systems (*e.g.* liposomes). Different types of reaction media can be found and they can be classified, from a physicochemical point of view, in two main groups: continuous (without boundaries inside the medium) or discontinuous (with border inside the medium, or heterogeneous or multiphasic) as previously described (Davidson et al., 1997). Many substances and solvents, as organic solvents (with different physiochemical properties), gases (supercritical fluids) and ionic liquids can be mixed together in different proportions to fit in one of above -mentioned groups. Besides, it is important to remark that the behavior of water molecules in the vicinity of the enzyme will depend on the nature of the surrounding organic medium, protein intrinsic and extrinsic characteristics and other physicochemical parameters related to environmental conditions.

3.2.1. Solvents

Solvents can be categorized as: water-miscible (monophasic aqueous-organic systems, including some ionic liquid systems), nonaqueous (monophasic organic system), water-immiscible (multiphasic aqueous-organic systems, and most of ionic liquid systems described up to now), anhydrous systems (including solvent free systems), supercritical fluids and gas phase and reversed micelles. The last two, are out the scope of the present review, and are not further considered. The effect of organic solvents in the unfolding of enzymes is well described in the literature, and can be attributed to many causes. The effects of polar and non-polar solvents on enzyme activity are quite dissimilar; both reduce the enzyme activity for different reasons. In polar solvents, water stripping is the major, but not the only, cause of reduction of enzyme activity. Water stripping is referred to the ability of polar organic solvents to displace water molecules from the protein surface, to be replaced by solvent molecules which, rigidify the molecular structure of the enzyme and concomitantly affects the enzyme turn-over (Castro and Knubovets, 2003). Additionally, polar solvents can interfere with the ionic interactions of the protein and/or breaking polar interactions which induce at least a partial unfolding of the molecular structure, especially in enzymes with polar/dipolar transitions states and intermediates. High conversion efficiency in non-aqueous homogeneous biocatalysis can be achieved only with high enzyme solubility and stability in the water-organic mixture. Water-miscible polar solvents are considered as non-aqueous homogenous system when the solvent

concentration is higher than 20% v/v (Davidson et al., 1997). The rational of this 20% organic cosolvent in the water mixture is considered based on the reduction of 10% of water activity in the solution estimated for hydrophilic solvents like ethanol or glycerol by the Raoult's law. In the last three decades, several enzymes with high catalytic activity and stability in water-miscible organic solvents were reported. (Baigorí et al., 1996; Costas et al., 2008). However, in the presence of high concentrations of typically denaturing solvents (higher than 40% v/v), like dimethyl sulfoxide or dimethylformamide, the enzymatic activity is reduced drastically. On the contrary, at high concentrations (80% v/v or higher) of polyhydroxylated solvents such as 1,2 diethylenglycol and propylenglycol, enzymatic activity is at least kept unaltered for 12 h in the case of some lipases, proteases and lysozyme (Costas et al., 2008). In particular, spectroscopic studies confirmed the correct folding of lysozyme in 99% glycerol (Castro and Knubovets, 2003). Nevertheless, enzymes not only require an intact secondary and tertiary structure in the reaction media, but also conformational flexibility provided by water that it correlates with enzyme activity. Additionally, glycerol derivatives can be useful in order to develop new ionic and non-ionic solvents with novel properties (Díaz-Álvarez et al., 2011).

The discovery of ionic liquids (IL) in 2000 opened new perspectives in all research areas including biocatalysis. Ionic liquids are organic salts which remain liquid at room temperature, without vapor pressure, but having a higher viscosity than water. According to their chemical structure, IL can be grouped on four types: alkyl-3-methylimidazolium, alkylpyridinium, tetraalkylammonium and phosphonium ions; meanwhile, there are more choices for the counterions, going from inorganic anions like Cl^- to organic molecules (*e.g.* citrate, tosilates). Consequently, the physicochemical properties of IL depend on both, the type of cation/anion pair and the alkyl chain of the ions, and this is because IL are regarded as *designer solvents*. Particularly, many IL systems have been described in the biocatalysis area, going from monophasic hydrophilic to hydrophobic and multiphasic systems (Quijano et al., 2010). The huge variety of ion choices allows manipulating the combination of substrates, products and effectors. Accordingly, many enzyme classes have been successfully worked on IL, going from the classical work-horse hydrolases (*e.g.* lipases, proteases) to dehydrogenases, nucleases, peroxidases, among others, following almost the same mechanism than in aqueous media (Quijano et al., 2010). IL biocatalysis is equally as significant as in organic solvents, presenting some advantages such as low volatility and high thermostability, plus the possibility of fine tuning some physicochemical properties such as polarity, hydrophobicity, thermostability, viscosity, and miscibility, just by modifying the appended lateral chains. In addition, biocatalysis in IL maintain the regio-, enantio- and stereo-selective properties of the enzymes required in biotransformations for the synthesis of complex molecules. It is a common assumption that enzymes dissolved into IL are inactive because of losing the secondary structure leading to protein unfolding, but some exception were also reported with soluble enzymes on IL showing high thermostability and long-term storage stability. Nevertheless, some disadvantages should be mentioned: enzymes present unpredictable solubility behavior in aqueous and nonaqueous IL systems, and the toxicity could be, in some cases, higher than in molecular organic solvents (Quijano et al., 2010).

In nonpolar solvents, enzymes are insoluble but the solubility of hydrophobic substrates and products is improved. The enhanced solubility of substrates and products in nonpolar solvents implies the stabilization of ground states of the molecules, and consequently the decrease of the biocatalytic reaction rate. Additionally, nonpolar solvents tend to rigidify the protein structure, but a delicate balance in the dynamics of the protein structure between protein flexibility and stiffness of the protein shell structure is required. The leading work of Halling (1994) described the addition of salt hydrates to the reaction medium in order to keep water activity at constant levels allowing some enzyme flexibility in neat apolar solvents to achieve the enzyme activity enhancement. These results were recently confirmed by NMR spectroscopy, which showed high activation of the protease subtilisin Carlsberg, in the presence of highly mobile water (Eppler et al., 2006). Experimentally, salts are commonly added as salt hydrates or saturated salt solutions to the samples during the manipulation of the enzyme and prior to start the reaction in order to reduce ionic disruption. However, the role of salts on enzyme activity not only depends on the individual ions, but also on salt concentration. At low ionic strength ranging from 10 to 30 mM salt concentrations, the major effect on biocatalyst surface is produced by electrostatic interaction (dipole moments). The electrostatic interaction occurs at a distance less than 0.50 nm from the protein surface. At lower salt concentrations it seems that the bulk water is not affected. On the contrary, at high salt concentrations, the effect on enzyme structure is also related to the polarizable groups of the enzyme, including the solvent layer (water) on the protein surface and the surrounding water. Additionally, present ions are able to interact with substrates, products, and reaction intermediates changing the kinetic and the thermodynamic parameters of biocatalysis. From the thermodynamic point of view, the contribution of salts to biocatalysis process is almost entropic and related to the degrees of conformational freedom along the transition states, with no enthalpic role (Eppler et al., 2006).

3.2.2. Salts

Historically, salts have been classified in a lyotropic series (or Hofmeister series) of ions based on their ability of ions to change water structure by ionic hydration (hydrogen bonds). Kosmotropic ions are able to establish hydrogen bonds; meanwhile chaotropic ions just break them. The same effect on proteins is related to the salting in or salting out phenomena, and subsequently on the stability of secondary and tertiary structures. In opposite sides, ions were classified as protein structure stabilizers or disruptors, as illustrated in the following series, from kosmotropic to chaotropic for anions: $F^- \approx SO_4^{2-} > HPO_4^{2-} > CH_3COO^- > Cl^- > NO_3^- > Br^- > ClO_3^- > I^- > ClO_4^- > SCN$, and for cations: $NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} >$ guanidinium.

Empiric studies determined that anions appear to have stronger effect on proteins than cations, because anions are more polarizable (Grossfield et al., 2003). There are several coefficients used to predict the ion-protein-water interactions, but the most accepted one is the viscosity B coefficient in the Jones–Dole equation. The B coefficient is associated with the ion tendency to establish H-bonds, building or breaking structured water and consequently changing viscosity of water. The Jones–Dole equation can be expressed as follows:

$$\frac{\eta}{\eta_o} = 1 + Ac^{\frac{1}{2}} + Bc + Dc^2$$

where η and η_o are the viscosities of salt solutions and pure water under experimental conditions, respectively and c is the salt concentration; the constant A is related to long-term electrostatic interactions, the constant B to ion–solvent interactions, and the constant D is considered only at very high salt concentrations. Kosmotropic ions have positive B values and display strong interactions with water. Besides, the B coefficient in chaotropic ions is negative and showed weak interactions with water (Ru et al., 2000). Since water plays a crucial role in protein-medium H-bonding, ions mimicking the water role in accepting or donating H-bond facilitate conformational changes and functioning of enzymes. In addition, the effect of kosmotropic ions on the water surrounding hydrophobic residues of

the protein is associated with the increase in the water molecular order. At the same time, kosmotropic ions enhance the strength of the hydrophobic interactions providing a more closed protein shell structure and therefore increasing stability (Dill, 1990). Furthermore, the amino acid sequence of the protein must be considered in the biocatalysis landscape since polarizable groups are participating in the reaction process. By using the same criteria for salts, it can be distinguished kosmotropic entities in the protein like carboxylates (*e.g.* glutamyl and aspartyl residues) and chaotropic entities such as amide and amine residues (*e.g.* tyrosyl, asparagyl, arginyl residues) (Sedlak et al., 2008). The delicate balance between the lyotropic aminoacyl residues of the protein and the lyotropic series of ions, salts and IL, affects the solubility of solutes in the surface of the protein and determines folding/unfolding and stability of the protein.

The rational of lyotropic series is still unclear and some reports showed some mismatch or reverse order of the series, which can be probably associated to enzyme structural characteristics and folding. Even so, lyotropic series and the *B* coefficient are commonly accepted, as general criteria, as a good predictor in heterogeneous biocatalysis. However, the main challenge of scaling-up heterogeneous biocatalysis to industrial level is to obtain a high operational stability of the biocatalyst and to validate the system under optimized conditions.

3.2.3. Supercritical fluids

The studies on supercritical fluids (SF) started during the 90's and were mostly based on supercritical carbon dioxide (SC-CO_2). One of a main attribute of SC-CO_2 is the solubility that present some materials in this fluid which, is similar to liquids and with comparable low viscosity and high diffusivity close to ordinary gases. SC-CO_2 has the advantage of working at low critical pressure (7.36 MPa) and mild temperatures (31.6 °C), besides being non-flammable and nontoxic. The high performance of many enzymes such as hydrolases, oxygenases and dehydrogenases in SC-CO_2 compared to organic solvents used for hydrolysis and synthesis purposes were well documented and recently reviewed (Wimmer and Zarevúcka, 2010). However, in order to reduce water droplets instability, new systems were developed for biocatalysis working in near critical SF. In some cases SC-CO_2 was replaced by other compounds like sulfur hexafluoride (SCSF6) (Celia et al., 2005). Alternatively, lipases from different sources performed on near critical conditions using gases like methane, ethane, or propane with high reaction rates (García et al., 2005). Additives like polymers and hydrophilic molecules were incorporated into the system by the addition of fluorocarbon surfactants (Holmes et al., 1998) and water-miscible cosolvents (methanol, ethanol, and others) were used to modulate the hydrophilicity/hydrophobicity of substrates, intermediates and products (Paljevac et al., 2007).

Several advantages of biocatalysis in SF can be enumerated: (1) the tunable hydration of the enzyme makes possible a fine control of the reaction kinetics by changes of pressure and temperature and without producing interference into the system and (2) easy purification of products and intermediates since, SF are gases at room temperature. However, this system is still not scalable to production level and the reuse of the enzymes is hampered by the high inactivation produced in the pressurization/depressurization process.

3.2.4. Semisolid systems

The use of solvents in biocatalysis is rather contradictory since it departs from the concept of Green Chemistry, which is a prominent advantage of enzyme catalysis. A similar result can be obtained in an aqueous medium if working at very high substrates concentration *i.e.* the use of substrates concentrations beyond the limit of solubility (Youshko et al., 2004), or even in solid-state (Basso et al., 2006). Thus, semisolid systems stems as a very promising technology that avoids the use of obnoxious chemicals and allows obtaining very high product concentrations. Enzyme catalysis in nearly solid or semi-solid systems has been studied, in which the reaction mixture consists of solid reactants suspended in a comparatively small volume of liquid phase (Ulijn et al., 2003). That liquid phase, aqueous or organic, becomes saturated with substrates thus, the reaction ensues and the formed product precipitates out from that liquid phase. A definite advantage of solid systems is the extremely high volumetric productivity attainable since, at the end of the reaction, virtually the whole content of the reactor is composed by the product. Other salient features are its environmental innocuousness, high conversion yields in reversal of hydrolytic reactions and high enzyme stability. Nevertheless, mass transfer limitations and mixing problems may represent an important drawback, especially when scaling up to production level is required (Erbeldinger et al., 1998).

The use of high concentrations of substrates in aqueous media is a greener alternative than the most non-aqueous media. The aqueous solution precipitation consists in keeping a saturated concentration of the substrate throughout the reaction by repetitive additions. It has been successfully applied to the synthesis of ampicillin with penicillin acylase by repetitive additions of the nucleophile (6-amino penicillanic acid), with a conversion yield over 97% (the highest reported up to now) have been obtained. Working under substrate supersaturation, significant increases in yield have been obtained for ampicillin and cephalexin with respect to homogeneous systems (Youshko et al., 2004). Substantial improvement has been obtained in the kinetically controlled synthesis of galacto-oligosacharides with β-galactosidase at very high and also at supersaturated lactose concentrations (Huerta et al., 2010).

3.3. Physical stabilization

3.3.1. Immobilization

For technical and economical reasons, most chemical processes catalyzed by enzymes require the stabilization, re-use or continuous use of the biocatalyst for a very long time. From an industrial perspective, simplicity and cost-effectiveness are key properties of immobilization techniques, but the long term industrial re-use of immobilized enzymes also requires the preparation of very stable derivatives having the right functional properties for a given reaction (Cao, 2005). The immobilization methods can be broadly divided into two categories: carrier bound or carrier free depending on the inclusion of an inert matrix. Table 1 shows the principal characteristics of the most relevant immobilization methods.

Among many systems for immobilization to solid inert supports, multi-point covalent attachment, where the enzyme is linked to the support porous (glass, polyacrylamide, cellulose, agarose and so on) through several amino acid residues is particularly interesting since very high stabilization can be attained (Tran and Balkus, 2011). A scheme of multi-point covalent attachment is presented in Fig. 1.

The generation of a number of attachment points between every immobilized enzyme molecule and the support exerts very strong stabilizing effects thus, in this sense heterofunctional supports have been successful (Mateo et al., 2007).

Carrier-free biocatalysts are a novel type enzyme catalysts bearing the advantages of the high concentration of active enzyme within the biocatalyst particle and the reduced cost since, no inert solid matrix is required as support, which is sometimes even more expensive than the enzyme itself (Roessl et al., 2010). In this case, the enzyme protein constitutes its own support so that concentrations close to the theoretical packing limit are obtained (Cao, 2005). Therefore, carrier-free immobilized enzymes are advantageous as catalysts in processes where high productivity and yield

Table 1
Methods of enzyme immobilization.

Principle	Main characteristics	References
Immobilization on inert matrix (carrier-bound)		
Covalent immobilization	High operational stability, quite flexible, so that directed immobilization can be done to suit the particular characteristics of the process	Mateo et al. (2007)
Non-covalent immobilization	Simple method, the carrier can be easily recovered after enzyme activity exhaustion by promoting protein desorption. Immobilization yields are usually high and no obnoxious reagents are involved. Main drawback: the enzyme can be easily desorbed from its carrier by subtle changes in the reaction medium	Mateo et al. (2000)
Immobilization by entrapment	Consists in confining the enzyme within the inner cavities of a solid polymeric matrix compact enough to retain the enzyme molecules within it. Most popular matrices for gel entrapment: alginate, polyacrylamide, polyurethane, polyvynil alcohol and κ-carrageenan	Tran and Balkus (2011)
Membrane retention	Containment by ultrafiltration membranes is relevant for processes involving coenzyme-requiring enzymes. Both the enzyme and the derivatized coenzyme are retained	Liu and Wang (2007)
Immobilization without support (carrier-free)		
CLEs cross-linked solution enzyme	Considered for some industrial purposes some decades ago, at present are no longer used mainly because of their poor mechanical properties and severe mass transfer limitations	Cao (2005)
CLECs cross-linked enzyme crystals	Are endowed with excellent properties: high stability under harsh conditions, resistance to autolysis and exogenous proteolysis and extremely high volumetric (and specific) activity, relevant for the rather slow reactions of synthesis. CLECs of several enzymes have been produced, the main drawback is the high cost of the biocatalyst due to the high degree of purity that is required for enzyme crystallization	Roy and Abraham (2004)
CLEA cross-linked enzyme aggregates	Present better mechanical properties than CLEs, also higher yields of activity and are simpler and much cheaper to produce. No purified crystal protein is required as starting material and straightforward protein precipitation methods are used prior to cross-linking. Types:	Sheldon (2011)Roessl et al. (2010)
	• By co-aggregation of enzyme and polymer and also by encapsulation on gel particles	Wilson et al. (2006)
	• CLEAs of multimeric enzymes with increased stability by preventing subunit dissociation	Wilson et al. (2004)
	• Combi-CLEAs allowing multiple non-cascade or cascade reactions	Dalal et al. (2007)Sheldon et al. (2007)

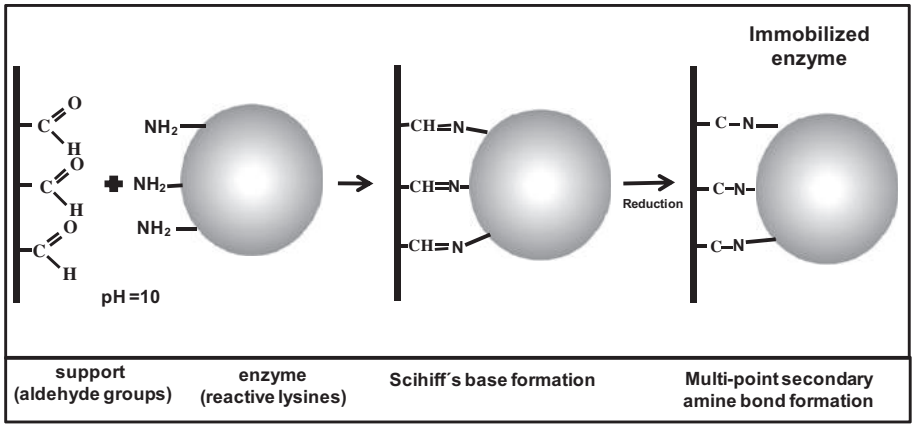

Fig. 1. Schematic representation of immobilization of enzyme on glyoxyl agarose support by multi-point covalent attachment.

is required or in the case of labile enzymes that cannot be properly stabilized by conventional immobilization to solid supports (Illanes et al., 2009). Carrier-free immobilized enzymes are prepared by direct chemical crosslinking of the protein containing the enzyme, using mainly glutaraldehye as crosslinking agent. This strategy has been applied to enzymes in solution (CLEs), to enzyme crystals (CLECs) and more recently to enzyme aggregates (CLEAs). Even though, many of the advantages of this kind of catalysts is due to the existence of a high density of protein within the catalyst, this promotes diffusional restrictions to substrates and products inside the enzyme matrix. CLEAs have the advantages over CLEs of better mechanical properties and higher yields of activity and are simpler and much cheaper to produce than CLECs, which require a purified crystal protein as starting material.

CLEAs are produced by cross-linking enzyme protein aggregates produced by conventional protein precipitation techniques, like salting-out, solvent or polymer precipitation as shown in Fig. 2. This method represents a major contribution to biocatalysis because it combines the properties of non-supported (carrier-free) biocatalysts with simplicity and low production cost (Sheldon, 2011). However, no general guidelines have been proposed for the preparation of CLEAs, particular conditions have to be determined and optimized for each enzyme (Wilson et al., 2006; Roessl et al., 2010). It has been reported that CLEAs of multimeric enzymes present increased stability preventing subunit dissociation (Wilson et al., 2004). CLEAs produced by coaggregation of enzyme and polymer and also by encapsulation on gel particles allowed the creation of a proper microenvironment with respect to the substrate nature and enhance the mechanical properties of the biocatalyst (Wilson et al., 2006). Co-aggregated enzymes (combi-CLEAs) allow multiple non-cascade (Dalal et al., 2007) or cascade (Sheldon et al., 2007) reactions. Due to its potential, CLEAs of many industrially important enzymes have been produced in recent years and special reactor configurations have been proposed for the recovery and handling of CLEAs (Sorgedrager et al., 2008).

3.3.2. Nanobiocatalysis

Nanobiocatalysis is considered the merging area of biotechnology and nanotechnology, which started in the 90s. Structured nanomaterials having well established characteristics such as pore

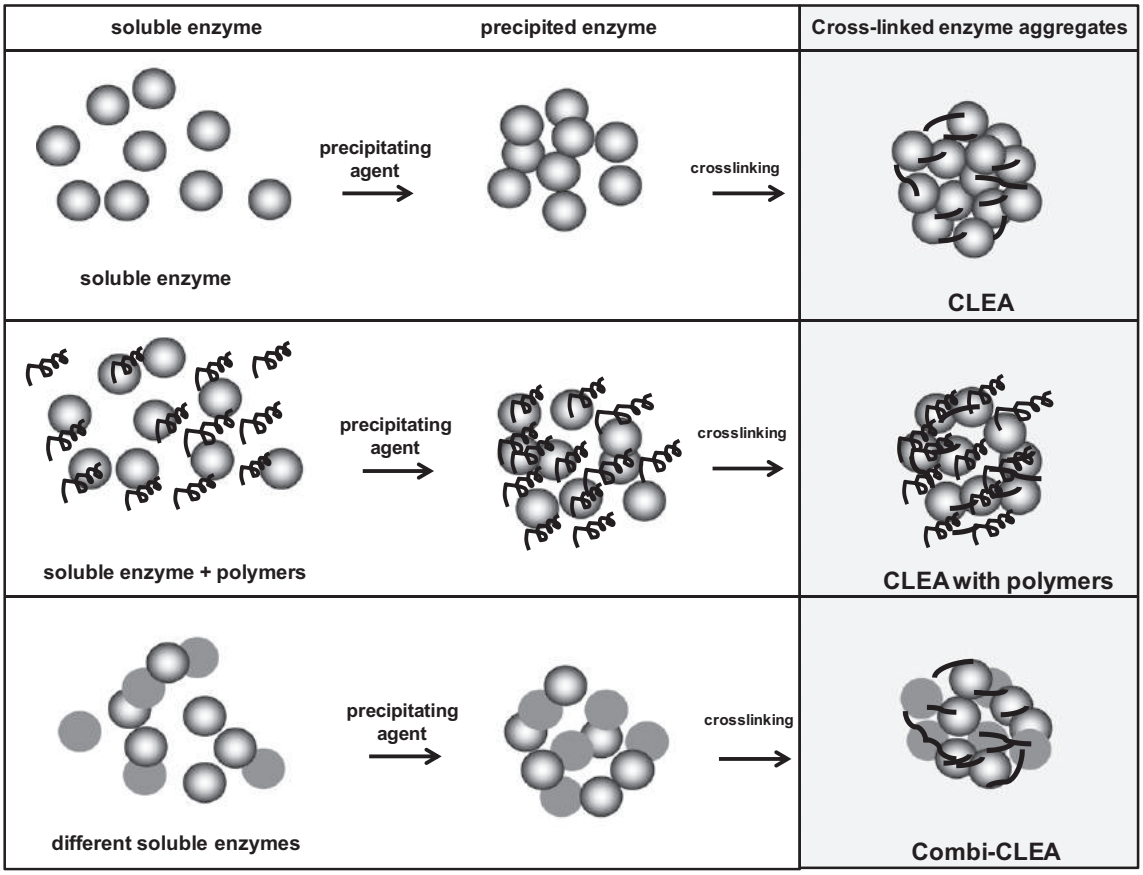

Fig. 2. Schematic representation of production of CLEAs.

diameter (5–100 nm roughly), defined geometry, hardness, hydrophobicity/hydrophilicity ratio, magnetic properties, conductivity, and so on, allowing to design robust biocatalysts. Many structures and materials for biocatalysis were successfully developed and explored under one or more concepts from nanoparticles, nanofibers, nanotubes (Wang et al., 2011). The main advantage of nanobiocatalysts is the high surface/volume ratio of the nanoobjects that enhances the exposure of the biocatalyst to reaction media as the size of the nanocarrier decreases. Two main approaches have been used: enzyme adsorption into the material surface with or without further covalent linkage, and enzyme encapsulation and entrapment in defined materials, generally using the methodology of self-assembly (Kim et al., 2006; Zhao et al., 2011).

Among the advantages of using nanocarriers for enzyme immobilization, it can be mentioned the possibility of fine tuning the biological activity by designing specific material for the required use, and the high surface area allowing a high enzyme loading. A typical example in nanobiotacalysis technology is the use of self-assembly nanotechnology approach for the development of single enzyme nanoparticles (SENs) based on the deposition of a hybrid polymer built-up on α-chymotrypsin surface that provides similar kinetic constant but with the advantage of enzyme shield (Kim et al., 2008). Another attractive model is the use of enzymes attached to superparamagnetic iron oxide nanoparticles (SPIONs) with different non-allosteric enzymes (Kim et al., 2006). Interestingly, a complex system composed by NADH-dependent allosteric enzymes as glutamate dehydrogenase, glucose dehydrogenase and the cofactor separately immobilized on SPIONs was developed. Because of the particle Brownian movement produced by alternating the magnetic field, the cofactor could be regenerated and reused, and the reaction rate can be sped up 1.8 folds (Zheng et al., 2011).

Nanobiocatalysis is new promising and exciting area to understand and developed novel biotransformations process by the manipulation of the complex molecular interactions at atomic level between the environment and all reaction components.

4. Conclusions

Biocatalysis is becoming one of the most powerful tools in biotechnology, having a profound social impact on health, food supply, environmental protection and sustainable fuel production. Biocatalysis is gaining a prominent place in the present and new scenarios of White Biotechnology within the framework of Green Chemistry, being nurtured by the advances in several fields as genetics, molecular biology, fermentation technology, bioinformatics, nanotechnology, material sciences, advanced spectroscopy and others. Major challenges are referred to the production of robust enzyme catalysts at reduced price, both being properly addressed by a wide spectrum of technological developments that have flourished in recent decades.

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