UNIVERSITA' DEGLI STUDI DELL'INSUBRIA

DOTTORATO DI RICERCA IN BIOTECNOLOGIE, BIOSCIENZE E TECNOLOGIE CHIRURGICHE

Curriculum Biotecnologie Molecolari e Alimentari

XXXI CICLO

Developing of a novel enantioselective biocatalyst acting on L-amino acids of biotechnological interest through a "semi-rational design" approach supported by computational analyses

Sviluppo di un nuovo biocatalizzatore enantioselettivo di interesse biotecnologico attivo su L-amino acidi di interesse biotecnologico mediante un approccio di "semi-rational design" supportato da analisi computazionali.

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1. Introduction

1.1 Biocatalysis

Biocatalysis is the application of enzymes, cellular extract or whole cells in synthetic chemistry for the production of building blocks or high added-value chemicals [1]. Although enzymes have been utilized in fermentation processes as a means to produce and preserve foodstuffs (such as cheese, bread and wine) before their discovery for thousands of years, the purposeful use of biocatalysts in chemical transformations processes only started in the late 19th century [1,2]. In the first half of the 20th century researchers in academia and industry learned how to use whole cells, cell extracts or partially-purified enzymes in different biocatalytic processes [3]. One of the first examples of enzymatic biocatalysis, described by Rosenthaler in 1913, is the asymmetric synthesis of *R*-mandelonitrile by treating benzaldehyde with hydrogen cyanide in presence of a plant extract (containing an enzymatic mixture, which included oxynitrilase) [4]. Despite the fact, for example that oxynitrilases have been employed (as crude extract) since the early 20th century for chemical synthesis, only recently they have become broadly used in industrial applications. In present days, enantio-selective oxynitrilases are commercially available and they are used for the asymmetric production of various cyanohydrins, which are raw materials for the synthesis of different chiral compounds [5,6]. An early obstacle in the exploitation of enzymes in catalysis was the production of a sufficient amount of the biocatalyst.

Traditionally, enzymes were isolated from their native sources: fungi, insects, plants, or mammals. However, this process was often time-consuming and, in many cases, extraction yields were very limited [2,3]. This situation has radically changed thanks to advancements in the field of biotechnology. The development of the technology of recombinant DNA allowed the overexpression of the enzymes of interest in host organisms from different native species such as *E. coli* or *B. subtilis* [7]. In this way, large quantities of enzymes became accessible at a relatively low cost [3] and enzyme-based biocatalysis became an economically and sustainable alternative to chemical synthesis on an industrial scale [1].

The first wave of biocatalysis (1900s to1980s, Fig. 1) was based on the use of natural enzymes, such as the use of proteases in laundry detergents [8], the use of glucose isomerase for the conversion of glucose to D-fructose [9] and the use of penicillin G acylase to produce semisynthetic antibiotics [10]. The main challenge in these bioprocesses was the limited stability of the biocatalysts. The development of the immobilization techniques of enzymes improved their operational stability and facilitated also their recovery and recycling, enabling a significantly reduction of the costs of biocatalysis [1,11].

In the second wave of biocatalysis (1980s to 1990s, Fig. 1) companies such as Novozymes and Genencor were established with the aim to produce on large scale and commercialize bulk enzymes for the food, textile, detergent and chemical industry [12]. During this period, protein engineering based on structural information enabled the alteration of the enzyme' s substrate specificity, allowing the synthesis of unnatural intermediates [1]. Therefore, the use of the enzymes have been integrated in several industrial biotransformation reactions, in particular for the production of compounds used for the synthesis of pharmaceuticals, agrochemicals and fine chemicals [13,14]. By the 1990s many enzymes were commercially available [3] and in 2015, the global market of industrial enzyme was estimated to be worth $ 8.18 billion/year. Projections suggest that the market will reach $ 17.50 billion by 2024 [15]. An early example of an enzymatic process, developed and industrialized by DMS-Andeno, was the synthesis of a key chiral precursor for the production of diltiazem (a blood pressure drug). This process involves the asymmetric hydrolysis of a chiral glycidate ester by lipolase (a lipase from *T. lanuginous*), a commercial enzyme used in various industrial applications [16]. More recently, Pfizer used the same lipase in a chemoenzymatic treatment to produce the active compound of the anticonvulsant drugs Lyrica® with high yields [17]. Other examples are the use of hydroxy nitrile lyase for the synthesis of herbicides [18] or the use of nitrile hydratase for the production of acrylamide [19].

In the mid 1990's Pim Stemmer and Frances Arnold introduced the concept of the directed evolution of enzymes. This technology consists of iterative cycles of random mutagenesis, followed by screening of mutant libraries to identify variants with improved properties such as increased stability, altered substrate specificity or strict enantioselectivity [20]. Recently, the directed evolution approach and the rational design approach (supported by bioinformatic analysis) were integrated to improve the chance of success by creating "smaller but smarter" libraries [1]. The latest frontier of protein engineering is the "*de novo* design" of enzymes with new functions. This approach requires the exhaustive knowledge of the structure-function and dynamics-function relationships [21,22].

The wide application of enzymes as biocatalysts can be attributed to the several environmental and economic benefits of their exploitation. They possess several advantageous features such as a high chemo-, regio- and enantioselectivity and a high catalytic efficiency under mild conditions. Enzymes which act only on a single enantiomer of the substrate can be employed as biocatalysts in processes such as the deracemization or the dynamic kinetic resolution of racemic mixtures [23]. Their high selectivity prevent the accumulation of by-products during the bioconversion [2,24]. Moreover, it is relatively simple to set-up efficient catalytic enzymatic cascades, since most enzymatic reactions are performed under similar conditions. Biocatalysts can be used in transformations reactions without the need of blocking and deblocking steps or activation of functional groups required in canonical enantio- or region-selective organic chemistry reactions making enzymes an environmentally friendly alternative to conventional inorganic catalysts. The success of industrial biocatalysis eventually depends, on the cost-effectiveness of the whole process. Since, despite the progress of industrial biocatalysis, in many cases, the traditional chemical process remains economically competitive. The main challenge of the modern industrial biotechnology is the developing a broad range of cheap, robust and versatile biocatalysts that can be applied in chemical industry.

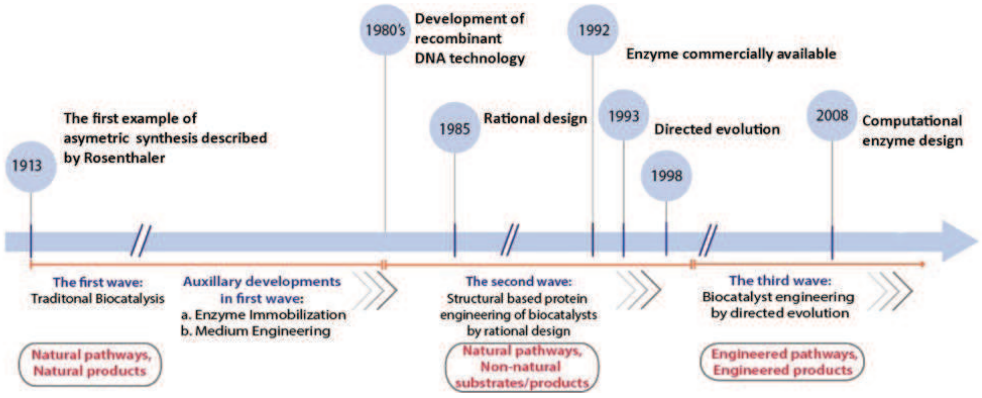

Figure 1. Time-line of the evolution of biocatalysis (Figure modified from [25]).

1.2 The development of the biocatalytic process

To fully establish biocatalysts in industrial applications, it is necessary to tailor enzyme properties so that they are optimal not only under laboratory conditions, but also in the context of the industrial process in which the catalyst will be applied [26]. The process optimization belongs to the field of biocatalysis engineering and consists in the development of enzymatic-based systems as a whole using different strategies (e.g., medium and protein engineering, biocatalyst formulation, down-stream processing) and it will play an important role in the next future [2].

The biocatalysis engineering, thanks to new technologies and to an increased understanding of basic biochemistry and bioinformatics, allowed the development of new and less expensive biocatalyst applications increasing the impact of enzyme technology in industry. Several key aspects have to be considered during the design and improvement of a biocatalytic process. The main ones are the optimization of the reaction conditions and of the *in vitro* evolution and production of the enzymes. Finally, biocatalysis engineering and downstream-processing enhancements facilitate product recovery and recycle of the biocatalyst; this two aspects drastically reduce the biotransformation costs [2,27]. The different steps in the development of a biocatalytic process and some of their major issues are shown in figure 2. As a general rule, the several aspects of the industrial process must be take into account using a rational approach. The

biocatalytic processes are improved in an iterative manner taking into account also the costs of the processes themselves [24]. The starting point of the biocatalysis design is the identification of a target reaction or of an industrial process product that could be optimized by enzymes technology. The next step is the selection of a suitable biocatalyst that can be commercially available or can be identified from the natural biodiversity (by screening of organisms or enzymes able to catalyze the desired biotransformation). The "natural" biocatalyst can be improved in order to acquire the desired properties and to match the desired applicative conditions. This can be obtained by metabolic pathway or protein engineering. A critical, and often under rated issue, is the identification of an effective formulation of the biocatalyst. For example, immobilization of the biocatalyst generally improves its shelf life under process conditions and facilitates its recovery and reuse [24,27]. Finally, since downstream processing represents a significant part of the cost of the whole bioprocess (up to 50% in several cases), an efficient and cost-effective recovery and purification procedure of the reaction product must be developed [27,28].

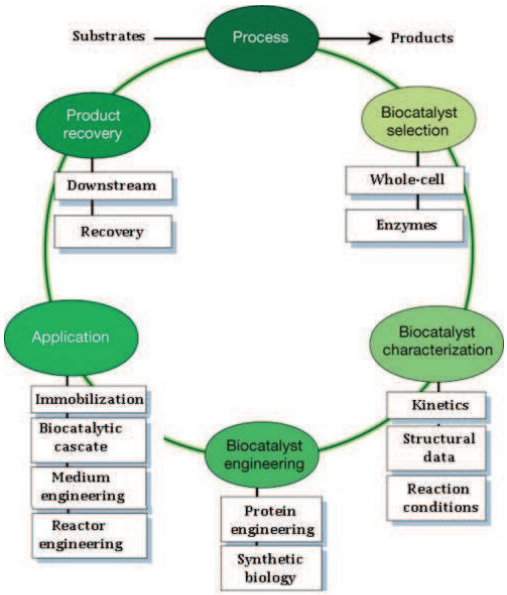

Figure 2. The biocatalysis cycle. Key steps and selected challenges in the development of a biocatalytic process (Figure modified from [24]).

1.3 Selection of the biocatalysts

Biocatalysis can be classified based on the biocatalyst exploited in the process, that is a whole cell or an isolated enzyme biocatalyst [29]. During the design and development of several biocatalytic processes, a critical point is the choice between an isolated enzyme or a whole-cell biocatalyst [30].

Processes developed before the 1960s were mainly based on whole-cell biocatalysis since the techniques for an efficient and cost-effective purification of the proteins were not yet available. However, through the 1960s and 1970s, several methods for effective and affordable purification of enzymes were developed allowing use of isolated enzymes as biocatalyst on a large scale. The subsequent introduction of recombinant DNA technology in the 1980s permitted to enhance protein expression which, together with the development of enzyme immobilization techniques, allowed the reuse of the catalyst resulting in an overall reduction of the enzyme costs [31].

The choice of the type of biocatalyst defines the architecture of the process. As a general rule, processes based on isolated enzymes require investments upstream of the reactor (i.e. the main cost is the preparation of the biocatalyst), while whole-cell processes require investments downstream (i.e. the main cost is the recovery and purification of the reaction products) [31]. In this respect, the economic sustainability of the whole process should be considered in order to select between the whole cell and the isolated enzyme biocatalysts.

1.3.1 Whole cell biocatalysts

In the whole cell catalysis approach, the cell growth phase, during which the enzyme of interest is produced, and the production phase, during which the resting cells convert the substrates into the desired products, are separated [32]. Accordingly, typical biotransformation processes consist into two stages: in the first one, cells are grown, and in the second one they are harvested and resuspended in the medium for the biocatalytic process. This step allows to remove unconsumed growth substrates as well as undesired metabolites that were

produced during cell growth, enabling a simpler and more efficient recovery of the products, this simplifying the downstream processing [33].

To produce effective whole cell biocatalysts, single or multiple enzymes must be expressed into host cells according to the complexity of the synthetic pathway required to produce the desired product(s). Indeed, to maximize the performances of whole cell biocatalysts, metabolic engineering and synthetic biology strategies can be used to optimize pathway flows and to enhance the productions of the target compound [34]. *E. coli* is the most promising microbial host to generate heterologous synthetic pathways (synthetic biology) on large-scale and at low-cost [34]. As a matter of fact, in recent years *E. coli* (or even yeast strains) have been engineered for the production of isobutanol [35], opiods [36] and antimalarial drug artemisinin [37].

Whole cell biocatalysts (derived from natural organisms or genetically modified expression hosts) represent the cheapest biocatalyst formulation since there is no need for the lysis of the cells and for the purification of the enzymes [34]. Moreover, the cellular endogenous metabolic pathways allow an efficient cofactor regeneration avoiding the addition of expensive external cofactors (e.g. NADH or NADPH), which are needed for complex biotransformation reactions, making the processes economically convenient [38,39]. In the whole cell system the recycling of cofactors can be achieved providing a suitable co-substrate and exploiting *in vivo* a secondary enzyme activity [31,39,40]. Several whole cell biocatalyst applications based on single step or multistep bioconversions to produce fine chemicals are reported in literature [34,39]. For example, the group lead by Dominguez de Maria used lyophilized *E. coli* whole-cell expressing carbonyl reductase from *C. parapsilosis* to produce *S*-phenylethanol starting from acetophenone and using n-propanol as a co-substrate for the cofactor regeneration. Under optimal conditions, the researchers reached a product titer of 300-500 g L^{-1} (with an e.e. > 99%) [41] (Fig. 3).

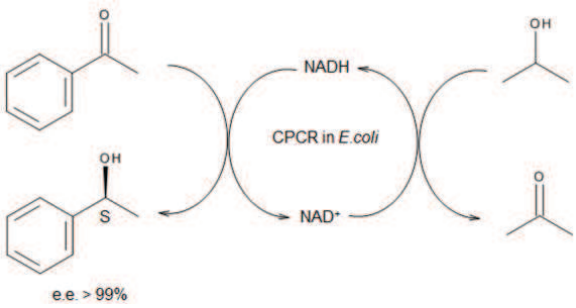

Figure 3. Biocatalytic reduction of acetophenone using *E. coli* cells with overexpressed carbonyl reductase (CPCR) (Figure modified from [41]).

Another relevant advantage of whole cell systems is the coexpression of multiple enzymes which allows to set up an efficient reaction cascade (due to the presence of different catalysts in a confined space) [39]. However, coexpression of multiple enzymes can results a high metabolic burden during cell growth, which may lead to an insufficient overexpression and thus unsuitable catalytic performance [42]. In addition, another disadvantage of whole cell biocatalysts can be the limitation in mass transfer [39]. The cell usually uptakes nutrients and natural substrates exploiting specific membrane transport mechanisms. However, bioconversions applied in industrial synthetic chemistry in general use non-natural compounds as substrates, whose mechanisms of uptake may not exist. Consequently, the reactants are transported into the cell by diffusion, a process that often becomes the rate-limiting step [31]. Classical methods to overcome this problem are the permeabilization of the cell wall by treatment with chemical agents (surfactants, chelating agents, or organic solvent) or the coexpression of specific membrane transporter (selective permeabilization) [39].

It must be highlighted that because of the high complexity of cell metabolism, the optimization of metabolic pathways for improving the product yield is often difficult and represents a main limitation of traditional *in vivo* metabolic engineering [12,43].

1.3.2 In vitro enzymatic biosynthesis

To address some of the major manufacturing problems of whole-cell biocatalysts and to address the high technical challenges of *in vivo* synthetic biology, purified (or partially purified) enzymes represent an alternative strategy for the set up of bioconversion reactions[12,43]. In cell-free systems, one or more enzymes and coenzymes can be used together *in vitro* to catalyze a specific enzymatic cascade for the production of desired compounds [44]. When using pure enzymes, the absence of the physical barrier formed by cell membranes allows the free and easy access of reactants to the catalysts, enables the maintenance of the optimal reaction conditions and facilitates the monitoring of the progress of the reaction [43,45]. In comparison to whole-cell biocatalyst, cell-free biocatalysts show also high product yield, fast reaction rate, high product titer and volumetric productivity, easy product separation and considerable high product quality (because of the absence of side reactions) [12]. Despite the advantages outlined above, application of free enzymes in industry requires to overcome several key challenges, which have been recently addressed by applied research:

1) the cost-effective production of the biocatalyst can be achieved by reducing the cost of the purification procedures. In this respect, it is even possible to use cell-free (crude) extracts to catalyze the desired reactions. In this case, no purification steps are required but, in the case of whole cell biocatalysis, metabolic engineering could be desirable to prevent undesired enzymatic side-reactions. Another approach to reduce the purification cost is the use of thermophilic enzymes (or better, stable enzymes), which can be purified by a simple heat treatment which eliminate the mesophylic enzymes by precipitation [43]. Thermophilic enzymes have been employed to produce a variety of products such as pyruvate [46], myo-inositol [47] and glutathione [48];

2) the enhancement of the enzyme stability by immobilization techniques, which also facilitate the recovery of the biocatalyst;

3) the achievement of economically sustainable cascades for cofactor recycling [43].

The main field of application of *in vitro* enzymatic biosynthesis is the fine chemical or pharmaceutical industry, where the additional efforts and costs for the preparation of the biocatalyst (mainly due to protein purification and/or immobilization) are compensated by a more strict and fine system control of the whole process (including reproducibility, control of the yield and increased optical purity) [49]. One of the most successful examples of the application of purified enzymes in the pharmaceutical industry is the development of a biocatalytic process for producing building-block used in the synthesis of drugs (such as in the case of atorvastatin or montelukast). These compounds were synthetized starting from a regio- and stereospecific hydroxylation of the first precursor catalyzed by a keto-reductase (KRED, from *L. kefir*) in combination with other enzyme(s) [50] (Fig. 4).

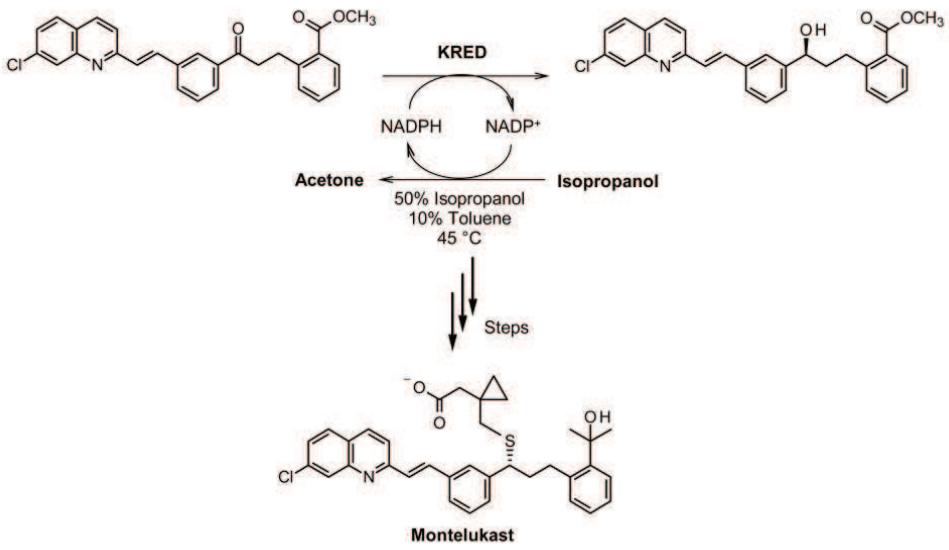

Figure 4. Regioselective reduction of a key intermediate in synthesis of montelukast by KRED (Figure modified from [50]).

Another interesting example is the enzymatic production of 7-aminocephalosporanic acid (7-ACA), which is used for the synthesis of semi-synthetic cephalosporins. The standard bioconversion is represented by two-step: starting from cephalosporin C, the D-amino acid oxidase (DAAO, EC. 1.4.3.3)

catalyze the oxidative deamination of the amino-adipyl group to give the corresponding α-ketoadypyl-7ACA, which spontaneous decarboxylate to produce glutaryl 7-ACA; this latter compound is hydrolyzed by glutaryl-7-aminocephalosporanic acylase (Gl-7-ACA, EC 3.5.1.93) to produced the final product (Fig. 5) [51–53].

Other applications of enzymes in cosmetic, textile and paper industry are reported in [54,55].

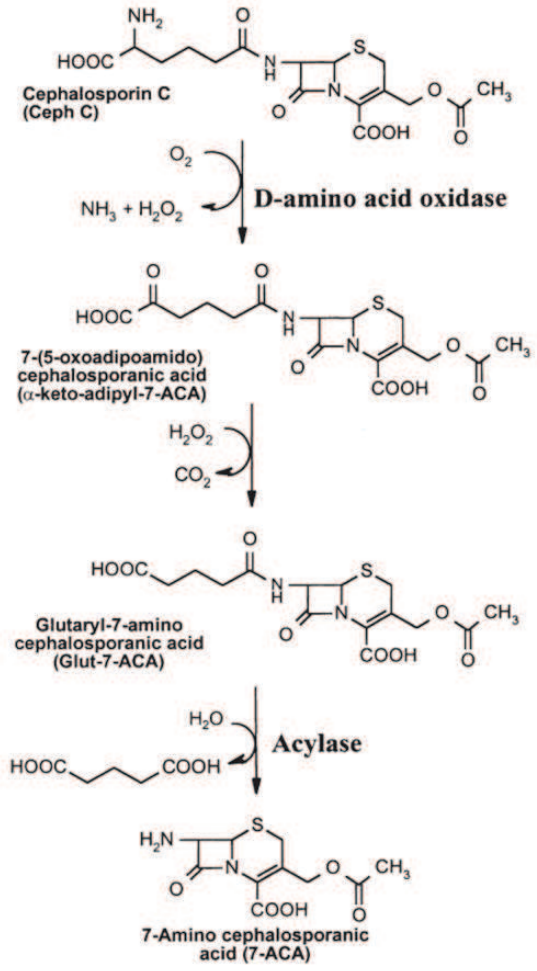

Figure 5. Two-step bioconversion of cephC to 7-ACA by D-amino acid oxidase and glutaryl-7-ACA acylase (Figure adapted from [51]).

Table 1. Whole cell and isolated enzymes biocatalyst: main advantage and disadvantages [56].

Whole cell		Isolated enzymes	
Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Better reaction control	Cofactor regeneration system necessary	Simple and cheap	Unwanted side reaction
No side reaction	Many enzymes non commercially available	Cofactor regeneration employing the cell metabolism	Mass transfer limitation
No limitation in substrate/product transport	Purification steps necessary	No purification steps	Hard *in vivo* metabolic engineering
High substrate/product concentration tolerance		Easy cascade reaction implementation	
Easy protein engineering			

1.4 Protein engineering

Present enzymes evolved over millions of years to operate most effectively on a narrow range of natural substrates under physiological conditions. In contrast, industrial biocatalysis, requires the biotransformation of synthetic substrates under harsh condition (e.g. elevated temperature or in the presence of organic solvents) [29,57]. Consequently, wild-type enzymes exploited as biocatalysts in synthetic organic chemistry and biotechnology, suffer from several limitations when used to transform unnatural substrates: poor catalytic efficiency, insufficient regioselectivity or stereoselectivity, narrow substrate scope and insufficient stability under operating conditions [3]. Hence, wild-type enzymes are often not suitable for biocatalytic processes. To increase the industrial productivity (space-time yields) and selectivity enzymes need to be re-designed [29,57]. The optimization of industrial enzymes is achieved by protein engineering [2,20] (Fig. 6).

The choice of the protein engineering approach for the improvement of the enzyme, a process also named *in vitro* evolution, depends upon the following criteria [58]:

1) the availability of structural information: namely, the availability of the high-resolution three-dimensional structure of the protein of interest or of a

reliable structural model built exploiting the sequence similarity with proteins of whose the 3D structure has been solved (homology modeling);

2) the availability of functional information: a deep knowledge in the structure-function relationships will facilitate/direct the selection of the mutagenesis approach;
3) the capacity of the screening step of the library variants: the size of variant libraries obtained after the mutagenesis step must be in the range of the capacity of the screening/selection method available. For example, *in vivo* selection assays allow the analysis of very large libraries (up to 10^{12} variants). Unfortunately, this method can be employed only to optimize the features of enzymes directly related to cell survival/growth [59]. Other high-throughput screening methods, such as the fluorescent activated cell sorting (FACS), allow the screening of up to 10^5-10^7 variants [60]. On the other hand, if a spectrophotometric assay is the sole technique available, only small libraries up to 200 clones can be screened [58];
4) the property of the enzymes that need to be optimized: changes in activity, selectivity or substrate specificity often involve mutations of residues close to the active site, while improvements in stability require modification of residues that in several cases are for away from the active site [61].

Two main different strategies are commonly employed to *in vitro* evolve a biocatalyst (Fig. 6): the rational design and the directed evolution approach. In addition, these two approaches can be combined in a semi-rational design approach (see below). Recently, thanks to the advances in computational methodology and of the improved knowledge of the physical principles that underlie protein folding, the *de novo* protein design approach is developing with an increasing pace. This approach aims to create new proteins that are not based on existing sequences (i.e. proteins design from scratch) [62]. However, despite the availability of highly sophisticated computational algorithms and of a growing number of 3D protein structures used as training set, the success rate of the *de novo* design is still low and limited [63]. As a proof of the concept, a successful

example is Top7, a 93-residue alpha/beta globular protein designed with a novel fold not observed in nature, described in *Kuhlman et al*., 2003 [64]. However, several main bottlenecks of *de novo* design have not yet been completely solved: the full understanding of the structure-function relationships, the protein folding mechanism, the detailed knowledge of protein dynamics and of the conformational changes induced by the interaction with the ligand. In addition a huge computational capacity is required to analyze a large data volumes associated to *de novo* design of proteins [65]. Thus, *de novo* design is still in its early days.

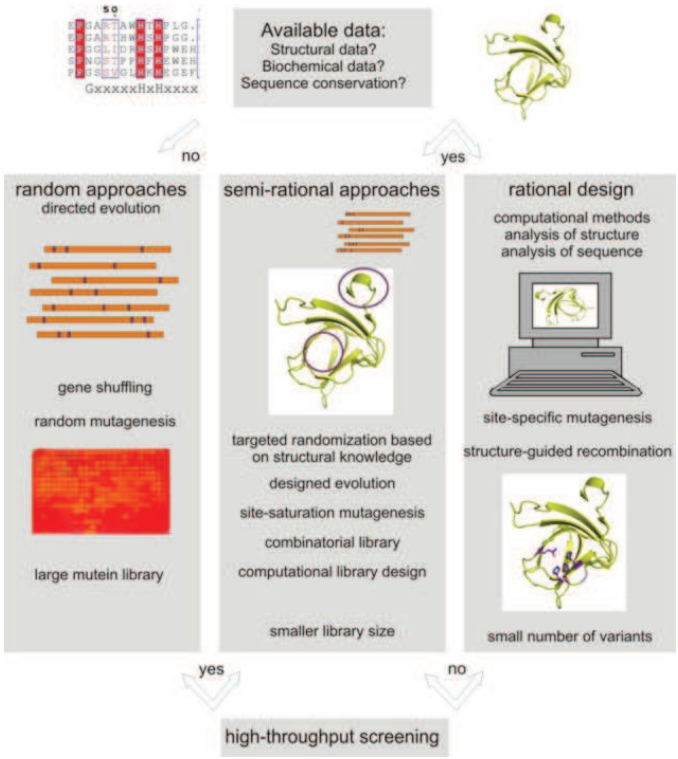

Figure 6. Overview of protein engineering strategies by ration design, directed evolution and combined methods [65].

1.4.1 Rational design

Rational design by site-directed mutagenesis (SDM), introduced by Smith and coworkers in the late 1970s [66], involves the introduction of site-specific mutations that allow the replacing a particular amino acids with one of the other

19 canonical ones into the coding sequence of the protein of interest. In order to improve the enzymatic properties (such as, substrate specificity, activity) of the protein of interest by SDM, a detailed knowledge of the relationships between structure and function in the target enzyme must be available [2,20]. This information can be achieved from analysis of the 3D structure(s) of the protein of interest by *in silico* studies (such as molecular dynamic, docking analysis). For example, amino acids that hinder substrate access to the active site or release of product can be identified by the analysis of the 3D structure and subsequently substituted with the aim to improve the enzyme performance [67,68].

The availability of an increasing number of protein experimental structures (or reliable 3D models), of bioinformatic methods and of biochemical data, spurs the use of rational design approaches. One of the advantages of this approach is the possibility to introduce potentially beneficial mutations in an enzyme without the need to generate variant libraries (shotgun); this is a desirable condition when no high-throughput assay system is available [65]. However, it is difficult to predict all potential effects of mutations. Therefore, it is not surprising that often inserted substitutions do not produce the expected effect [65,69].

1.4.2 Directed evolution

Directed evolution is an effective method of protein engineering, which reproduces the natural Darwinian evolution in a test tube [3]. The major advantage of directed evolution is that no structural information is required. Traditionally, directed evolution involves an iterative protocol to create genetic diversity by random mutagenesis or *in vitro* recombination, followed by the screening (or selection) variant libraries (Tab. 2), in order to identify improved enzyme variants showing the desired phenotype [3,70].

Several strategies for creating genetic variability have been developed based on random mutagenesis such as error prone PCR (ep-PCR) [71], DNA shuffling [72] and several homology-independent *in vitro* recombination approaches (i.e. Staggered

Extension Process, StEP [73] or, Incremental Truncation for the Creation of Hybrid enzyme, ITCHY [74]).

An example of directed evolution is based on ep-PCR is the generation of variants of the protease subtilisin E possessing improved stability in presence of organic solvents such as dimethylformamide (DMF) [75]. Although, early applications of directed evolution were focused to increase the enzymes stability under specific reaction conditions, more recently this approach has been exploited to improve other important enzyme properties. In 1997, as a proof of concept, Reetz and coworkers enhanced the enantioselectivity of the lipase from *P. aeruginosa* used as biocatalyst in the hydrolytic kinetic resolution of the chiral ester rac-16 [76]. The main issue in the application of directed evolution is the generation of large libraries of variants; this requires the development of a robust, fast and cheap high-throughput screening (or, possibly, a *in vivo* selection method) to identify the desired interesting [65]. Indeed, its success is based on the integration of an efficient library construction with a reliable and high-throughput screening assay [77]. It is necessary to underline that, although using suitable experimental approaches libraries composed by millions of variants can be analyzed, only a tiny fraction of the vast possible sequence space for an average-size protein can be sampled [70]. To reduce this sequence space (increasing the change to identify a desired variant), the semi-rational design was introduced.

Table 2. Exploited selection and screening methods used in directed evolution [78].

Strategy	Library size	Advantage	Disadvantage	Examples
1. Selection	$\sim10^9$	Yields desirable variant only	Only possible if activity gives growth advantage	
Growth on appropriate antibiotic		High sensitive. HTP-screening possible	Not feasible. Can generate highly resistant strains	Increased moxolactame resistance using *Cephalosporinase* genes
Complementation		Very specific. HTP-screening possible	Depends on metabolic products	Identification of tryptophan producing mutants
Display method coupled with detection (e.g. FACS)		Ultra sensitive. HTP-screening	Difficult to detect improved variant for existing activity	Identification of protease
2. Screening methods				
Agar plate screening	$\sim10^5$	Simple to operate	Limited dynamic range	Conversion of a β-galactosidase into a β-fucosidase
Microtiter plate screen	$\sim10^4$	Very sensitive, low background signal. HTP-screening apllicable. Detection of improved properties	Synthesis required. Surrogate substrate used	Identification of more stereoselective lipase/esterase variants. Fingerprinting of various enzymatic activities
Cell-in-droplet screen	$\sim10^9$	Large libraries	Fluorescent detection and DNA modifying enzyme only	Droplet-based microfluidic technology that enables HTP-screening of mammalian cells
Cell as microreactor	$\sim10^9$	Large libraries	Fluorescent detection only	
Cell surface display	$\sim10^9$	Large libraries	Fluorescent detection only	Displaying antibody libraries on the surface of *E. coli* and binding with fluorescent label antigens resulted in antibodies with high affinities
mRNA display	$\sim10^{12}$	*In vitro* selection and evolution of proteins. Search of *de novo* proteins that are presumably in library	Dependent on puromycin	Extensively used to isolate binding proteins and peptide

1.4.3 Semi-rational design

Semi-rational design combines the directed evolution with the rational design approach; this approach results into the generation of small (but smart) libraries of variants [70,79]. Typically, in the "semi-rational" design approach, the knowledge derived from the analysis of protein sequences, biochemical or structural data and predictive computational algorithms is used identify amino acid positions in the protein, which are preselected as promising targets for mutagenesis [70,80]. The most efficient strategy to introduce genetic diversity is the site-saturation mutagenesis (SSM) [81,82], a technique in which all the 20 naturally amino acids is randomly introduced in one or more specific position of the protein. This is obtained by using a mutagenesis primer containing one or more degenerated codons(NNN) or a reduced subset of them based on the degeneracy of the genetic code (e.g., NNK and NDT where N: adenine/cytosine/guanine/thymine; K: guanine/thymine and D: adenine/guanine/timine) [82–84]. Next, interesting variants can be identified by functional library screening. Semi-rational design allows the generation of small, high-quality libraries and avoid the need for high-throughput screening methods for library analysis [70,80].

A popular semi-rational strategy used to improve substrate scope and/or enantioselectivity is the Combinatorial Active Site Saturation Test (CASTing), which uses the information derived from structural data to select amino acid residues in the active site pocket. These residues are then exhaustively mutated by site-saturation mutagenesis one by one and in combination in order to observe potential synergistic effects that can be missed when only one residue at time is mutated. The first example of this approach, is reported in *Reetz et al., 2001* [85]: the stereoselectivity of *Pseudomonas aeruginosa* lipase (PAL) was enhanced by SSM at four residues using mutagenic oligonucleotides containing the NNK degenerated codon by subjecting to screening only 5000 variants. A variant of CAST strategy that can be employed to enhance the thermostability or resistance to chemical denaturation of enzymes is the B-Factor Iterative test (B-FIT) [82,86]. In this case site-saturation mutagenesis is focused on highest flexibility sites, which

are identified from the analysis of the distribution of the B-factor from X-ray data. Another variant of the CAST approach is the Iterative Saturation Mutaghenesis test (ISM) in which SSM is applied in a systematic manner [82,87]. ISM is a rational extension of the original CAST approach, which can be applied to improve several catalytic parameters [88]. In this strategy, the chosen sites of randomization (e.g., A, B, C, in Fig. 7) are independently subjected to SSM, creating the first generation of libraries. Subsequently, a variant from each library is used as a template for a second step of mutagenesis at the remaining three sites (e.g., B, C, D), and the process is continued until all sites have been mutated once in a given upward pathway (Fig. 7).

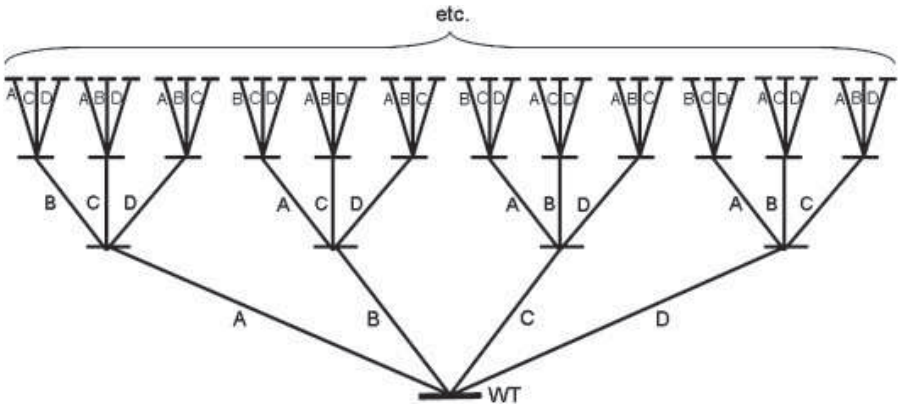

Figure 7. Scheme of iterative site-saturation mutagenesis at four sites (A, B, C and D). The vertical axis symbolizing an improved property [88].

Another strategy to identify "hotspot" of functionality in the protein sequence is the use of evolutionary information. Multi sequence alignments (MSAs) and phylogenetic analysis (e.g., using the web-platform Consurf [89]) have become standard means to evaluate amino acid conservation and ancestral relationships between sequences and structure of homologous proteins [70,80]. This approach allows to identify residues of the target protein that are poorly conserved during evolution. These positions represent evolvable residues that can be substituted without altering the stability of the protein. Consensus design, the one used in like ancestral protein reconstruction approach, has been used to improve the thermostability of several proteins [65]. For example, Bommarius and his group,

combining this approach with structural data, were able to increase the thermal stability of a glucose dehydrogenase and penicillin G acylase from *B. subtilis*. In both cases, due to the core full selection of the mutagenesis sites, about 50% of the variants showed improved thermostability [90,91]. Another example was the use of this approach by *Conti et al*., *2014* with the aim to alter the enzyme substrate scope of cephalosporin C acylase. The best variant is H57βS-H70βS-L154βY glutaryl acilase VAC variant, which shows the highest production of 7-amino cephalosporanic acid (3.5 fold higher than wild-type) [92].

1.5 Formulation of the biocatalyst: enzyme immobilization

After the identification of an enzyme suitable for the desired biotransformation, the optimization of its properties (such as substrate scope, stability, enantioselectivity) by protein engineering and its production in large amounts at relatively low cost, the following step is the set up of an effective biocatalyst formulation. Since enzymes are soluble in water their recovery from the reaction mixture is challenging. Consequently, many enzymes can be only employed as single-use biocatalyst [20,29]. The enzyme cost per kg of product can be drastically reduced through the development of an easily recoverable and reusable heterogeneous biocatalyst. This kind of biocatalyst can be obtained by protein immobilization [11]. The immobilized biocatalyst results in simplification of the biocatalysis process, in the improvement of the product quality and in the achievement of a cost effective process. Importantly, enzyme immobilization can enhance the stability of the protein toward chemical or thermal denaturation [2,20,29].

Basically, the methods for enzyme immobilization can be categorized into three classes: binding to an inert support (carrier), entrapment (encapsulation) or cross-linking (Fig. 8) [11].

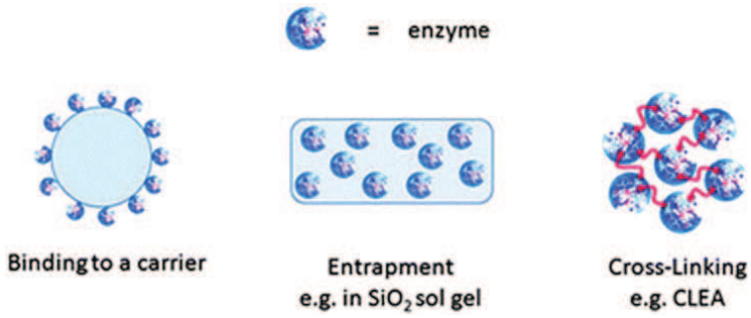

Figure 8. Different techniques for enzyme immobilization [11].

1.6 Stereoselective biocatalysis

The increasing demand of optically pure chiral compounds in the pharmaceutical industry is due to the restrictions applied by international regulation agencies. Unsurprisingly, almost 60% of the 400 new active substance entities approved world-wide between 2002 and 2011 are formed by enantiopure compounds while racemates represent only 11% of the total amount [93]. The enantioselective synthesis of chiral compounds is a challenge in organic chemistry. The creation of a specific chiral center in organic synthesis is not obvious, although approaches based on organometallic and organocatalytic methods, which used complex catalysts to induce asymmetry in selected transformations have been developed. The intrinsic chirality of enzymes and they ability to catalyze a wide range of chemical reactions, make them powerful tools for the development of stereoselective biotransformation [94]. The use of enzymes for enantioselective biocatalysis turned out to be a competitive approach both in terms of yield and cost in comparison to the classical asymmetric synthesis due to the possibility of developing highly stereoselective biotransformations in a sustainable manner [23]. The synthesis of single enantiomers of pharmaceutical and drug building blocks is of special interest, not only because of side effects caused by the opposite drug enantiomer but also due to the low affectivity when administrating racemates [94,95].

1.6.1 Kinetic resolution

The biocatalytic kinetic resolution (KR) has enjoyed a considerable growth in the past few decades and is become a favorite industrial production method nowday for the synthesis of bioactive natural product and derivates. In the KR of racemic mixtures, stereoselective biocatalysts react with only one of the two enantiomer of a racemic solution allowing to obtain an optically pure product and leaving an unreacted optically pure substrate behind. Hence, KR represents a useful preparative method in research and industrial applications, despite its maximal yield is 50%. Esterases, proteases and lipases are often applied in kinetic resolution owing to their ease of production and application at industrial scale. Also, a large number of hydrolases have been used to resolve racemic mixtures of esters, amides, lactones, epoxides, hydantoins and nitriles (exploiting the hydrolytic reaction) as well as, for kinetic resolutions of amines and alcohols (exploiting the reverse reaction) [96]. For instance, *P. cepacia* lipase commercialized by Amano (Amano PS) has been successfully used to catalyze the resolution of aristelegone B a bioactive natural tepenoid-derivated. Using 5 molar equivalent of vinyl acetate as a acyl donor and acetone as a solvent, after 36 h at 25 °C the (+)-acylaristelegone B (46% yield and 96% ee) and (–)-aristelegone B (54% yield and 94%) were obtained [97]. The same enzyme has been applied in the first step of the production of the (+)-artabotriol, a the precursor for the synthesis of several bioactive product such as (-)-tulipalin B (Fig. 9) [98].

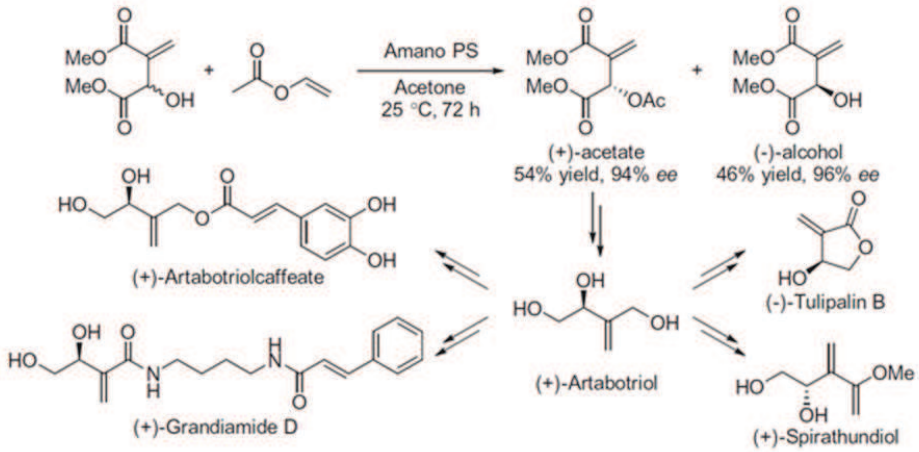

Figure 9. KR of racemic dimethylenesuccinate for the of (+)-artabotriol by *P. cepacia* lipase [94].

1.6.2 Deracemization

Dynamic kinetic resolution (DKR) and stereoinversion reaction are the two most common strategies to obtain the racemic mixtures deracemization (Fig. 10). DKR is, in principle, a KR process in which the non-catalyzed enantiomer is racemized *in situ*. During an enantioselective process the biocatalyst promotes the transformation of one enantiomer into the product, while the other enantiomer is racemized (at a comparable rate), restoring the racemic mixture [99]. The racemization step can be performing by enzymes (e.g., racemase) or non-enzyme catalysts (e.g., transition metal) [23].

While the maximal yield of KR is 50% of the product, in DKR a 100% conversion can be reached following the optimization of the two reactions [96,99].

Biocatalytic stereoinversion is a process in which one enantiomer of the racemic substrate is enantioselectively transformed into an intermediate (typically, non-chiral) that is, in turn, transformed (by non-enantioselective catalyst or by an enantioselective catalyst with the opposite stereo-preference catalyst) giving the starting racemic compound and accumulating the opposite enantiomer. The reiteration of the non-enantioselective reaction allows the complete deracemization of a specific racemate.

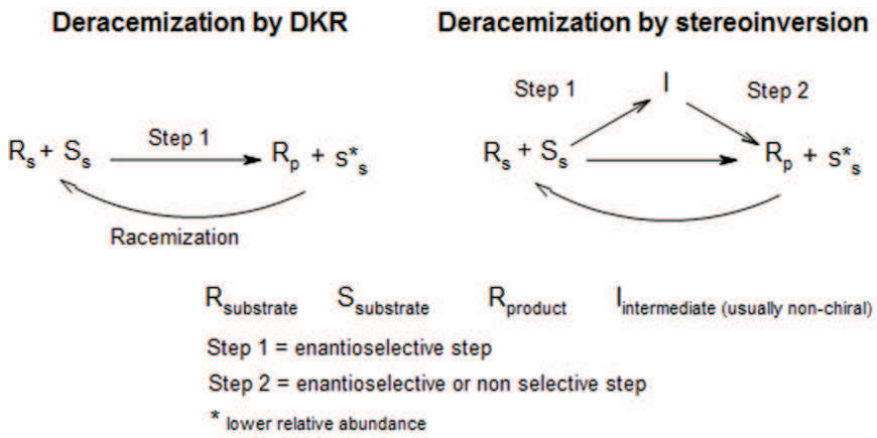

Figure 10. DKR and stereoinversion strategies (Figure modified from [99]).

Deracemization by stereoinversion can be used, for example, for the interconversion of chirals alcohols through the corresponding ketones, as well as for the interconversion of amines/amino acids through the corresponding ammines. This can be achieved through the combination of an enantioselective oxidative step with a non-selective reducing agent [23,99]. In figure 11 is reported the stereoinversion reaction to obtain the production of optically pure L-2-naftilalanine (L-2-Nal) from the corresponding racemate by an enzymatic system formed by three different enzyme working in the same reaction condition. The bioconversion is almost complete since the yield is 95% of L-2-Nal and the e.e. 99.5% [100].

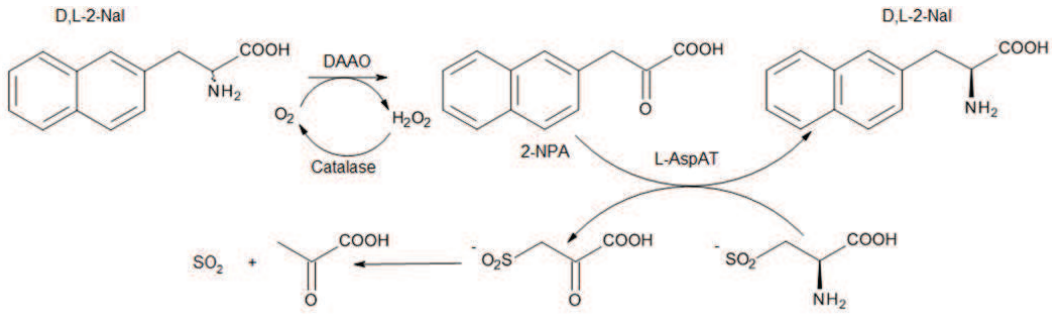

Figure 11. Stereoinversion of D,L-2-Nal by an enzymatic cascade (Figure modified from [99,100]).

1.6.3 Desymmetrization

Desymmetrization reactions are used for the production of optically pure compounds starting from non-chiral reagents such as meso- or prochiral compounds. As in the case of the deracemization process, the desired enantiomer can be obtained with a theoretical yield of 100%. The main advantage of this approach is the production of high amounts of enantiopure compounds by a simple reaction. This is in contrast to KR or stereoinversion reactions, where iteration or combination of different steps are required [96]. For instance, [(1*R*,6*S*)-6 (hydroxymethyl)cyclohex-3-en-1-yl]methyl acetate is used as building block for the production of (–)-alloyohimbane and (–)-yohimbane, antihypertensive and antipsychotic drugs. The enantioselective synthesis of this compound is obtained by the hydrolysis from the meso-diacetate precursor catalyzed by the porcine pancreas lipase (PPL, EC 3.1.1.3). The desired monoacetate intermediate is produced with a 84% yield and a >95% e.e. (Fig. 12) [101].

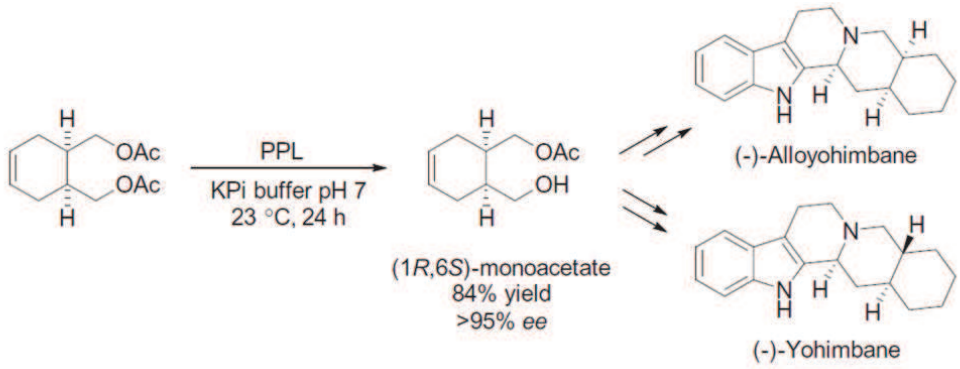

Figure 12. Chemo-enzymatic synthesis of (–)-alloyohimbane and (–)-yohimbane involving the hydrolysis of a diacetate precursor catalyzed by PPL [94].

1.7 Industrial applications of D-AAs and α-keto acids

D-AA and α-keto acids are high value added compounds for the fine chemistry, since they are used for the synthesis of pharmaceutical drugs, food additives and insecticides [102,103].

1.7.1 D-amino acids

D-AAs are commonly used for the synthesis of semi-synthetic antibiotics (such as semi-synthetic penicillins and cephalosporins) or therapeutic peptides. Drugs containing D-amino acids assimilated and processed more slowly than other drugs in humans. For example, aspoxicillin which contains D-Asp has a more extended half-life and a lower tendency to bind serum proteins to amoxicillin [104] while the substitution of D-His and D-Cys in the Pro-His-Ser-Cys-Asn peptide (ATN-161) determines a potent inhibition of invasion and lung colonization effects in human prostate cancer [105]. In addition, D-AAs can enhance the activity of antimicrobials against biofilms [106]. In the food industry, D-AAs are used for the synthesis of food additives such as of the sweetener alitame (L-Asp-D-Ala dipeptide), which has 10-fold higher sweetening potency than aspartame and it is used in the diets of diabetic patients. Furthermore, D-AAs themselves can be used as food additives and flavor agents. Finally, D-AAs can be added in cosmetic preparations [102].; it has been reported that D-Asp has antioxidant effects and D-Ser reduced ultraviolet damage.

1.7.2 α-Keto acids

α-Keto acids are used in various applications such as in pharmaceutical, in fine chemical, in food and animal feed industries. As replacement of the protein amino acids, the α-keto acids such as α-ketoisovalerate and α-ketoisocaproate can be used as nitrogen-free substitutes for essential amino acids in patients with liver and kidney diseases to avoid the accumulation of nitrogen-containing metabolites [103,107]. Also, they are used to treat patients with chronic glomerulonephritis and

hepatitis B virus infection [108]. Another important application of α-keto acids is their use as building blocks in organic chemistry, owing to the presence of the carbonyl group. For example, they are precursors of the biofuel n-butanol [35], or can be used for the production of L- and D-amino acids [109].

The main α-keto acids and their application are summarized in Table 3.

Table 3. Summary of the major α-keto acids and their applications [103].

Keto acids	**Application**
Pyruvate	Improveexercise endurance capacity Weigh-control supplement Nutraceutica Antioxidant Procursor of pharmaceutical chemicals
α-ketoglutaric acid	Ammonium ion receptor Precursor of chemical compounds Improve athletic performance The poly α-KG used in tissue scaffolding and therapeutic delivery
α-ketoisovalerate acid	Precursor of 1-butanol Hepatitis B virus infection and chronic glomerulonephritis Feed of lamb, pig, chickens
α-ketoisocaproate acid	Precursor of biofuel Hepatitis B virus infection and chronic glomerulonephritis Depression of silenced tumor with organoselenium compounds Stimulate insulin secretion Increase muscular power Promote the milk production and composition
Phenylpyruvate	Diet sweetener aspartame, indole-3-acetic acid
α-keto-γ-methylthiobutyric acid	Poultry industry, anti-cancer drug
2,5-diketo-D-gluconic acid	Precursor of 2-keto-L-gulonic acid
2-ketobutyric acid	Precursor for chemical synthesis of pesticide, spice and food additive
Oxaloacetic acid	A key metabolite in tricarboxylic acid cycle
2-oxoadipic acid	A key metabolite of tryptophan and lysine
4-hydroxyphenylpyruvate	Important methabolite in tyrosine degradation pathway
N-acetyl-L-2-amino-6-oxopimelate	Metabolic intermediate in L-lysine synthesis
L-2-amino-6-oxoheptanedioate	Lysine biosynthesis

1.8 Synthesis of optically pure D-amino acids (D-AAs) and α-keto acids

D-amino acids (D-AAs) and α-keto acids can be produced by chemical synthesis, fermentation or enzymatic biotransformation. Bio-based catalysis can provide D-AAs and α-keto acids with high a degree of purity and with a high productivity by green processes [102,103].

1.8.1 D-AAs enzymatic production

The enzymatic production of D-AAs has been performed exploiting hydrolases, oxidoreductases, and aminotransferases. Some examples are shown below:

1. D-Hydantoinase (EC. 3.5.2.2) and D-carbamoylase (EC. 3.5.1.77)

D-Hydantoinase coupled with D-carbamoylase is an efficient system for producing D-AAs in industry. Several natural D-AAs, including D-Trp, D-Phe, D-Val, D-Ala, and D-Met are produced by this method [110]. In addition, also several unnatural amino acids are produced in huge amounts (several thousand tons of product per year). In particular, the most significant are D-phenylglycine and D-*p*-hydroxyphenylglycine, which are used as building block for the synthesis of semisynthetic penicillin and cephalosporins.

This enzymatic biocatalytic process utilizes D,L-5-substituted hydantoins as starting substrate and is formed by two steps: D,L-5-substituted hydantoins are first hydrolyzed into their carbamoyl derivatives by the strictly enantioselective D-hydantoinase and then into corresponding D-AAs by a decarbamoylation catalyzed by the D-carbamoylase [102] (Fig. 13A).

2. D-Stereospecific amidohydrolase

D-Stereospecific amidohydrolases, such as *N*-acyl-D-amino acid amidohydrolase, D-amino acid amidase and D-peptidase, can be used for dynamic kinetic resolution of racemic amino acid amides to produce D-amino acids, starting from mixture of amides of D,L-amino acid derivatives producing the corresponding D-AA and fatty acid. The D-amidohydrolases reacts with the D-enantiomer of the N-acyl-D,L amino acid. The *in situ* racemization can be

performed by an L-amino acid amide racemase (or by a chemical reaction starting from the unreacted N-acyl-L-anino acid. The racemic amides could be hydrolyzed to D-amino acid with a theoretical yield of 100 %. D-AAs produced using this approach include D-Arg, D-Leu, D-Ala and D-Leu [111] (Fig. 13B).

3. D-Amino acid aminotransferase (EC 2.6.1.21)

D-Amino acid aminotransferase catalyzes the transfer of the amino group from D-amino acids to α-keto acid to yield the corresponding D-amino acids. For example, the D-AAT from *L. salivarius* can be exploited as biocatalyst, to aminate several α-keto acids (e.g., α-ketobutyrate, glyoxylate and indole-3-pyruvate) to produce their corresponding D-amino acids [112] (Fig 13C).

4. D-Amino acid dehydrogenase (EC 1.4.99.1)

D-AADHs are NADPH-dependent oxidoreductases, which can catalyze the stereoselective reductive amination of α-keto acids into their corresponding D-amino acids. However, D-AADHs are only marginally applied for the industrial D-amino acid production because they are rare in Nature and they are usually membrane-bound proteins [102] (Fig. 12D).

5. L-Amino acid oxidases/deaminase (EC 1.4.3.2)

L-Amino acid oxidase/deaminases (LAAO/LAAD) catalyze the oxidative deamination of L-amino acids with a strict enantioselectivity, providing a simple method to produce pure D-AAs from a racemic solution by dynamic kinetic resolution [107,113] (Fig. 13E). It is important to highlight that the D- substrates are more expensive than the corresponding L-form (e.g., D-Phe costs 4-fold more than L-Phe). LAAO from *Rhodococcus opacus* shows a broad substrate specificity, therefore this enzyme is used to produce a wide variety of D-AAs such as D-Glu, D-Arg, D-citrulline D-homoserine [114].

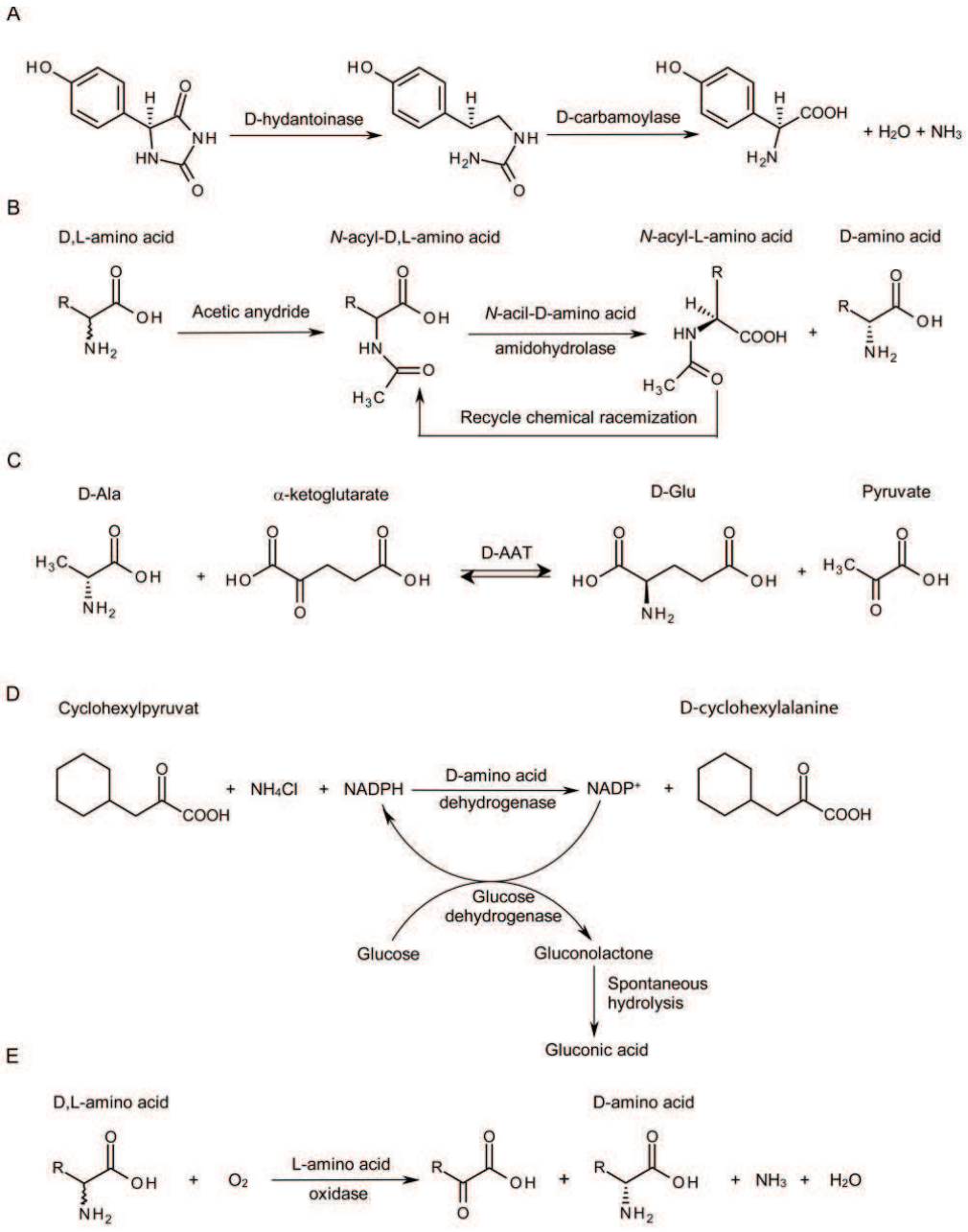

Figure 13. Examples of production of D-AAs by enzymatic synthesis. A) Enzymatic route for the production of D-*p*-hydroxyphenylglycin ; B) Commercial process for the production of D-AAs by N-acyl-D-amino acid amidohydrolase; C) D-Ala production by D-amino acid aminotransferase; D) Enzymatic system for the synthesis of D-cyclohexyalanine by D-amino acid dehydrogenase BC621 variant; E) Production of D-AAs by LAAO starting from racemic mixture [102].

1.8.2 Enzymatic production of α-keto acids

Several enzymes such as dehydrogenases, aminotransferases, DAAOs and LAAOs or LAADs can also be used for α-keto acids production from α-amino acids. However, it is important to highlight that the reactions catalyzed by aminotransferases or dehydrogenases require two substrates or an extra cofactor respectively, while DAAOs require expensive substrates (the D-enantiomers). Interestingly, the oxidation of L-amino acids by LAAO or LAAD requires only a single L-amino acid as a substrate (which is less expensive than the corresponding D-form) and do not require without extra cofactors; this makes LAAOs and LAADs the ideal enzymes for α-keto acids synthesis [103]. For instance, LAAO from *Rhodococcus* sp. is used for the bioconversion of L-DOPA into the corresponding α-keto acid in presence of catalase to avoid the decarboxylation of the product [115]. Similarly, LAAO from *P. alcalifaciens* and *T. viride* have been to convert N-□-carboxy(CBZ)-L-Lys to its α-keto acid [116].

1.9 Amino acid oxidases

Amino acid oxidases (AAOs,) are enzymes that oxidize amino acids releasing ammonium and hydrogen peroxide. Based on the chirality of the amino acid used as substrate, these enzymes can be divided into two groups. D-amino acid oxidases (EC 1.4.3.3) are flavoenzymes showing strict specificity for D-amino acids [117]. L-Amino acid oxidases (EC 1.4.3.2) are flavoproteins, with the exception of lysine oxidase from *M. mediterranea* which use cysteine tryptophylquinone as a cofactor [118], that oxidize L-amino acids releasing the corresponding α-keto acids in addition to ammonium and hydrogen peroxide.

In particular, in this thesis I focus my attention on the flavoenzymes active on L-AAs to generate compounds with which have potential application in medicine as antitumorals, antimicrobials [52,113].

1.10 L-Amino acid oxidase (LAAO)

L-Amino acid oxidases, described by Zeller and Maritz (1944) [119] are flavoenzymes, which belong to the class of oxidoreductases. LAAO catalyzes the oxidative deamination of L-amino acids with a strict stereospecificity to give the corresponding α-keto acids with the production of ammonia and hydrogen peroxide via an imino acid intermediate (Fig. 14).

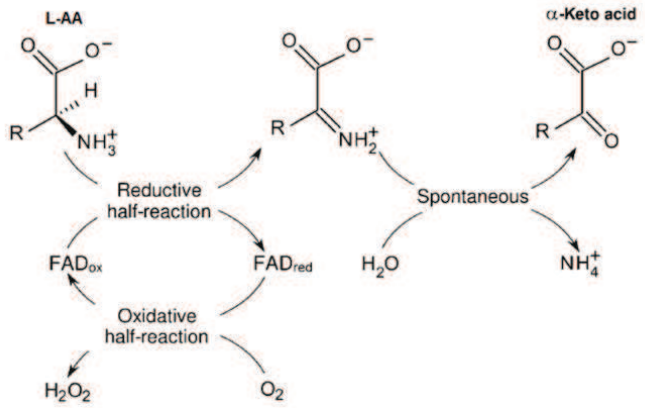

Figure 14. Catalytic cycle of a generic L-amino acid oxidase (Figure modified from [113]).

The ability of LAAO to discriminate between L- or D-forms of the substrate lies in the architecture of their substrate binding pocket that conforms to the catalytic model proposed by Mesecar and Koshland [120]. According to this model, the enantioselectivity of LAAO is based on the presence of three different binding interactions between the substrate and the active site and the particular orientation of the α-hydrogen of the substrate towards the N5 of the isoalloxazine ring of the cofactor. The main anchorage points are represented by a salt bridge interaction between the α-carboxylate of the amino acid (negatively charged) and the guanidinium group of an arginine of the active site (positively charged) that is located near the isoalloxazine ring. The second and third interaction are respectively represented by a hydrogen bond between the α-amino group of the substrate and the main protein chain belonging to a small residue (for example, glycine or alanine) and the hydrophobic region of the active site to which the

lateral chain of the substrate is located, a structural determinant responsible for the different substrate specificity in various LAAO [113] (Fig. 15).

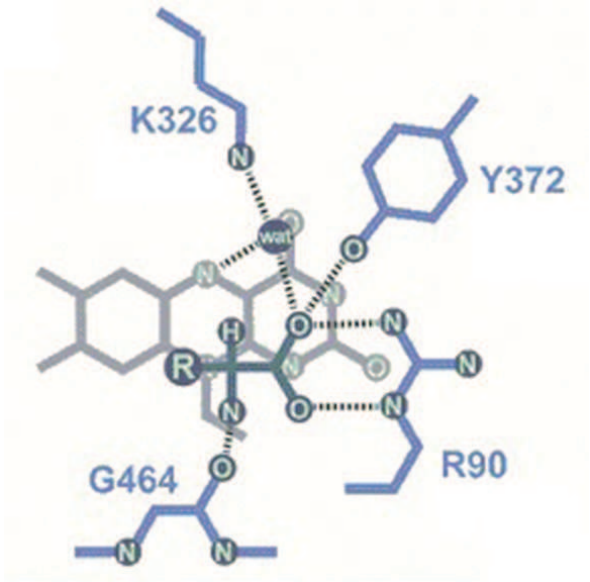

Figure 15. Binding modes and interactions between the α-amino and α-carboxylate group of the substrate and the active site residues of a "generic" LAAO (R = side chain of the substrate) (Figure modified from [113]).

LAAO activity is broadly distributed in nature, across diverse phyla from bacteria to mammals; (the major source of eukaryotic LAAO is the enzyme from venous snakes) [121]. In different organism LAAOs differ both in cellular localization (intracellular, extracellular or membrane bound) and in biological function (which is related to the oxidation of L-amino acids and to the production of reactive oxygen species [122]). Since LAAOs are able to produce hydrogen peroxide during catalysis, they play a main role in antimicrobial or cytotoxic/apoptotic processes such as the competition related to biocontrol between microbial species [123]. Moreover, LAAOs isolated from prokaryotic organisms are involved in the production of ammonia and α-keto acids starting from free L-amino acids, which are used as a source of nitrogen and carbon for the growth of the microorganism itself [124].

From a biochemical point of view, LAAOs from different sources distinguished for molecular mass, substrate specificity and post-translational modifications. This diversity suggests that LAAOs underwent enormous evolutionary changes by divergent evolution from a putative ancestral protein [124]. Most LAAOs show broad substrate specificity with a preference for hydrophobic substrates (e.g., L-Phe, L-Leu, L-Trp), but there are several members that exhibit a very strict preference for a specific L-amino acid, such as L-aspartate oxidase or L-phenylalanine oxidase [113].

All LAAOs whose 3D structures were solved are present in solution as homodimers, with a molecular mass between 50 and 300 kDa. LAAO monomers fold in two domains: an FAD-binding domain and a substrate-binding domain which also hosts the amino acid residues that form the entrance funnel of the active site [113]. In particular, the access to the active site of LAAOs is allowed by the presence of two different channels: a large channel connecting the *re*-face of the FAD to the solvent which is used by the substrate L-amino acid and the release of the product α-keto acid, and a small channel that connects the *si*-face of FAD to the solvent which is used by the molecular oxygen [125]. The superimposition of the 3D structures of the LAAO present in the PDB database reveals a high conservation of the overall tertiary structure (RMSD of 2.0 - 2.2 Å), despite of the low amino acid sequence identity (e.g., RoLAAO and CrLAAO share less than 23% of sequence identity). This indicates that these enzymes have been probably evolved through divergent evolution from a common ancestral protein giving rise to a family of evolutionarily-related LAAO enzymes [113].

All LAAOs are subjected to post-translational modifications. Eukaryotic LAAOs need a high level of glycosylation for a correct maturation, secretion, and function. On the other hand, prokaryotic LAAOs are synthesized as inactive pro-enzymes, which require a proteolytic cleavage to become active. The proteolytic activation, which is performed by an endopeptidase, occurs after the secretion of

the protein and represents a strategy to prevent cellular apoptosis due to H_2O_2 production and depletion of intracellular L-amino acids [113,121].

From the applicative point of view, LAAOs have important potential biotechnological applications: they are component of biosensors for a rapid and precise determination of L-amino acid concentrations in several biological, medical and food production applications. For example, a potentiometric biosensor based on LAAO [126] has been used in the food industry to control the nutritional quality of different products by their L-amino acids content. Although there are some drawbacks of LAAO biosensors (for example, their different sensitivity of detection for different amino acids) their applicability at industrial scale is much simpler and cost-effective in comparison to other analytical methods (e.g., HPLC-chromatography or spectrophotometric analysis) [127].

In addition, snake venom LAAOs are known to induce antibacterial effects and apoptosis mediated by the hydrogen peroxide produced during the catalysis. These enzymes can be useful as antitumor and as therapeutic drugs against bacteria and leishmaniasis infections [121,128]. For example, LAAO (ACTX-6) from *D. acutus* demonstrates cytotoxicity *in vitro* and inhibits HeLa cervical tumor *in vivo* by inducing apoptosis in a concentration and time-depending manner. For this reason, ACTX-6 is a potential anti-cancer drug.

As previously reported, LAAOs can be used as catalysts in biotransformation [113,127]. However, the use of these enzymes on large-scale is rather limited. The production of recombinant enzymes (in particular for industrial biocatalysis) is essential to ensure the availability of large quantities of the biocatalyst at a low cost and to improve its biochemical properties through protein engineering. Currently, no LAAO with a wide substrate specificity has been successfully expressed in a recombinant form in prokaryotic hosts. The main bottleneck is represented by the cytotoxicity caused by the intracellular production of hydrogen peroxide from the overexpressed enzyme [113]. LAAO from *Rhodococcus* sp. has been expressed in *B. subtilis* and *S. lividans*, but at low yields [127].

1.11 L-amino acid deaminase

A suitable alternative is represented by L-amino acid deamminases (LAADs, EC 1.4.99.B3). LAADs are membrane flavoenzymes that catalyze the stereospecific oxidative deamination of L-amino acids to the corresponding α-keto acid and ammonia. In this case, the electrons of the reduced cofactor ($FADH_2$) are transferred to the membrane electron transport chain by a specific electron acceptor (probably a cytochrome b-like protein) without producing hydrogen peroxide.[129].

Unlike L-amino acid oxidase, several LAADs have been expressed as a recombinant protein in *E. coli* and have been biochemically characterized: type-I LAADs from *P. mirabilis* (PmirLAAD) [130] and from *P. myxofaciens* (PmaLAAD) [109] and type-II LAADs from *P. vulgaris* (PvLAAD) and from *P. mirabilis* (Pm1LAAD) [131]. The 3D structure of PvLAAD [132] and PmaLAAD have been recently solved [129,132].

In literature are reported several examples of LAAD applications as whole-cell [133,134] or enzymatic biocatalyst[135]. Accordingly, there is an increasing in both scientific and practical interest to these enzymes.

The review "Breaking the mirror: L-amino acid deaminase, a novel stereoselective biocatalyst" will give an overview of the structural and biochemical properties of LAADs and will summarized the advances that have been made in biocatalytic processes based on these enzymes.