Environmental biocatalysis: from remediation with enzymes to novel green processes.

Miguel Alcalde, Manuel Ferrer, Francisco J. Plou and Antonio Ballesteros

Departmento de Biocatálisis, Instituto de Catálisis y Petroleoquímica, CSIC, Cantoblanco, 28049 Madrid, Spain.

Corresponding author: Antonio Ballesteros (a.ballesteros@icp.csic.es).

Abstract

Modern biocatalysis is developing new and precise tools to improve a wide range of production processes, which reduce energy and raw material consumption and generate less waste and toxic side-products. Biocatalysis is also achieving new advances in environmental fields from enzymatic bioremediation to the synthesis of renewable and clean energies and biochemical cleaning of "dirty" fossil fuels. Among the obvious benefits of biocatalysis the major hurdles hindering the exploitation of the great repertoire of enzymatic processes are, in many cases, the high production costs and the low yields obtained. This article will discuss these issues, pinpointing specific new advances in recombinant DNA techniques for future biocatalyst development, as well as drawing the attention of the biotechnology scientific community to the active pursuing and developing of Environmental Biocatalysis, covering from remediation with enzymes to novel green processes.

Advances in both chemical catalysis and biocatalysis are determinant in reducing the environmental footprint of chemical processes and petroleum-based technologies. In the field of chemical catalysis (i.e. that using catalysts of non-biological origin), the subject *environmental catalysis* has been well established and accepted for decades: in the 60´s and 70´s it flourished an excellent research on how to decrease catalytically the amounts of contaminants in the fuels derived from petroleum, and in the 80´s and 90´s an increasing interest about Catalysis and Environment was pointed out in many scientific scenarios. However, leading scientists are currently expressing serious reservations about the long-term health and weather pattern problems (including global warming and pollution) that have been traditionally associated with chemical processes and "dirty-fuels". At that point, biocatalysis using either enzymes or whole cell microorganisms -now called white biotechnology-, is being implicated in many spheres of human activity in terms of

- environmentally friendly processes
- handful of opportunities for the production of renewable and clean energies
- remediation of many compounds that are unfriendly or even toxic for the environment by the present ecological standards of our societies.

In this context, biocatalysis fully participates in the "green chemistry" concept that was introduced in the 90's [1] and its impact on sustainability is now established beyond question. We would like to draw attention of the biotechnology community to the current developing concept of *Environmental Biocatalysis*, covering the three topics defined above. Moreover, we want to stress that *Environmental Biocatalysis* provides a different landscape compared with the well-known *Environmental*

Microbiology (e.g. the use of mixed cultures for environmental aims) [2], pointing specific questions on both the enzymatic remediation of contaminants for preserving the environmental health of our ecosystems [3], and the new advances in green chemistry for more benign processes for the production of new highly-value added compounds [4]. Although the benefits of biocatalysis for environmentally-friendly fine chemical transformations have been generally accepted, their critics claim several important issues about the economic utilization of enzymes for processes involving clean-energy production and bioremediation [5,6]. At this point, recent studies rely on modern molecular biology tools such as protein engineering, (meta)genomics and proteomics, for future biocatalyst development to reduce its cost and the use of chemicals, whereas at the same time decreasing overall costs and increasing yields and efficiency. In this review we discuss the growing field of *Environmental Biocatalysis*, paying especial attention to the needs and means by which enzymatic processes can be beneficial for the environment.

Biocatalysis platform for green processes

Green chemistry is defined as the design, development, and application of chemical processes and products to reduce or eliminate the use and generation of substances hazardous to human health and the environment [7-9]. Biocatalysts (either enzymes or whole-cells) constitute a greener alternative to traditional organic synthesis [1], offering appropriate tools for the industrial transformation of natural or synthetic materials under mild reaction conditions, low energy requirements and minimising the problems of isomerisation and rearrangement [10-13]. In addition, biocatalysts are biodegradable and may display chemo-, regio- and stereo-selectivity resulting in

decreased by-product formation and avoiding the need for functional group activation, protection or deprotection. Large-scale industrial applications of biocatalysis include for example the thermolysin-catalyzed synthesis of the low calorie sweetener aspartame, the production of acrylamide and nicotinamide assisted by nitrile hydratases, the synthesis of isomaltulose –a non cariogenic sweetener- by sucrose mutases or, more recently, the production of biopolymers (e.g. polylactic acid) (Fig. 1) [14,15]. Good examples of the replacement of traditional organic processes by a "greener" biocatalytic alternative are the industrial synthesis of semisynthetic penicillins and cephalosporins [1], the transformation of natural and synthetic fibres [16], the pulp kraft-bleaching and recycling of paper [17], and the multi-step synthesis of polyketide and glycopeptide antibiotics [18].

To overcome competing chemical reactions, current frontiers for biocatalysis are protein activation and stabilization and reaction specificity. Among other examples, at the moment, by using protein engineering and high-throughput screening it is possible to create highly stereoselective enzymes with broadly extended substrate specificity useful in organic synthesis [19], stable hydroxynitrile hydrates for the synthesis of e.g. substituted (R)-mandelic acids [20], and hydrolases being able of enantioselective carbon-carbon bond formation or selective oxidation processes [19]. Moreover, in synthetic reactions involving oxidoreductases and expensive redox co-factors, where clearly whole cells are needed, biotechnological developments from up-stream (strain, cell and organism development) and mid-stream (fermentation and other unit operations) to down-stream processes, will benefit significantly the implication of white biotechnology in chemical transformations [21]. Additionally, combinatorial immobilization techniques are

providing effective methods for optimising the operational performance as well as the recovery and re-use of biocatalysts [22]. Although some enzymatic/microbiological processes are well established in industry, we strongly believe that biocatalysis is still in its infancy and its future is going to depend on the search for novel and versatile enzymes able to catalyse reactions that are difficult to perform by chemical methods. Indeed, metagenomics technology has revolutionized the possibilities of biocatalysis, since we can now have access to the genomes, genes and encoded enzymatic activities of unculturable microorganisms [23,24]. The success of metagenomics in finding new enzymatic activities has unequivocally demonstrated the power of this approach, showing that metagenomics is not a future opportunity anymore, it is a current reality for the production of new products and processes that were until recently hidden from us [25].

Bioremediation of persistent contaminants

Removal of anthropogenic and, widely dispersed, organic pollutants is considered one of the main concerns for a reasonable and sustainable development for Earth planet in this just-started new century. Comparing with traditional physico-chemical methods, bioremediation is generally the safest, least disruptive and most cost-effective treatment [2,3]. This biotechnological tool consists in the use of whole microorganisms, naturally occurring or introduced, or isolated enzymes to degrade persistent contaminants into non- or less-toxic compounds. In many cases bioremediation may be combined with complementary physical, chemical or mechanical processes to improve the reliability and effectiveness of detoxification. Just to give a recent example, since November 2002 microbial bioremediation (using

naturally occurring microbes) in combination with mechanical approaches are currently being used as a major mechanism of removing low and high molecular weight polycyclic aromatic hydrocarbons (PAHs) from the *Prestige* ship spill in the north coast of Spain. In fact, there are many possibilities in this area of biotechnology, either by *in situ* bio-stimulation (bioventing, natural attenuation) [26,27] or *ex-situ* technologies (biocells, landfarming) [28].

Microbial bioremediation

It is generally assumed that one of the major concerns of bioremediation with microbes, in particular in maritime affected areas, is that the microorganisms must be able to resist a variety of adverse conditions that are far away from the ideal conditions in which microbes were grown in the laboratory [2]. Additionally, in case of inoculated microorganisms, especially those genetically manipulated (GMOs) two major problems should be considered: the weakness and low level of fitness and growth of the inoculated microorganisms in competition with the indigenous population, and the possibility of altering a given ecosystem by introducing GMOs (international legislation is very strict in this sense) [2]. The controlled release of *Pseudomonas fluorescens* HK44, which possesses a naphthalene catabolic plasmid, represents the first and only genetically engineered microorganism approved for bioremediation testing [29]. Whatever the case and regardless the organisms are genetically modified or not, microbial bioremediation is limited by the mass transfer (low contaminant bio-availability), aeration, nutrient state of contaminant sites (requirement of bio-stimulation techniques) and thermal conditions problems

[26,30]. Therefore, in order to get maximum microbial growth to metabolize the toxicants all of these issues should be studied for every particular case.

Enzymatic bioremediation

Admittedly, we should recognize that in the last few years, enzymatic bioremediation has risen as an attractive alternative to further support the bio-treatment techniques currently available, since enzymes are more simple systems than a whole organism [3,31]. Most xenobiotics can be submitted to enzymatic bioremediation, e.g. polycyclic aromatic hydrocarbons (PAHs) [32], polynitrated aromatic compounds [33], pesticides such as organochlorine insecticides [3,6], bleach-plant effluents, synthetic dyes, polymers and wood preservatives (creosote, pentachlorophenol) [17] (see chemical structures in Fig. 2). It is just a matter of searching the microorganisms capable to feed with a particular pollutant, and afterwards focusing the effort in finding out which enzyme(s) is(are) behind this behaviour. Historically, the most studied enzymes in bioremediation are bacterial mono- or di-oxygenases, reductases, dehalogenases, citochrome P450 monoxygenases, enzymes involved in lignin-metabolism (basically, laccases, lignin-peroxidases and manganese peroxidases from white-rot fungi), and bacterial phosphotriesterases [31]. Moreover, new developments in the design and application of enzymatic "*cocktails"* for bio-treatment of waste-waters have recently emerged due to the effort of many companies and administrations such as *American Industry Enzyme Technologies Inc*. and *Australian Orica Ltd*. From an environmental point of view, the use of enzymes instead of chemicals or microorganisms undoubtedly presents some advantages [6]:

- the biotransformation does not generate toxic side-products, as it is often the case with chemical and some microbiological processes, and after the treatment, the enzymes are digested on-site by the indigenous microorganisms
- the requirement of enhancing the bio-availability by the introduction of organic co-solvents or surfactants is much more feasible from an enzymatic point of view rather than using whole cells
- the possibilities to produce enzymes in a higher scale with enhanced stability and/or activity and at a lower cost by recombinant-DNA technology.

When performing enzymatic bioremediation, it is imperative that the enzyme is kept optimally during operational conditions. This requires cheap produced enzymes (i.e. heterologous expression) with high substrate affinity (K_m in the micromolar range), supporting thousands of product turnovers. At the same time enzymes should display robustness under an array of external factors and low dependency on expensive redox cofactors (i.e. NAD(P)H), which would be prohibitive in a commercial setting [3]. Many of these shortcomings have been successfully resolved by directed enzyme evolution (Fig. 3) or by semi-rational approaches (i.e. combinatorial saturation mutagenesis of several *hot-spot* residues) [34-36]. Obviously a convenient method of assessing bioremediation applications should be the improvement of several enzymatic properties at the same time (e.g. stability and activity). By directed (or *forced*) evolution, one can confer new features to enzymes that somehow do not have enough time to fit against novel or poorly degraded xenobiotics, including insecticides, herbicides, fungicides and mycotoxins [37-39]. A nice example is represented by the atrazine (2-chloro-4-ethylamino-6-

isopropylamino-1,3,5-s-triazine), a class of herbicide whose first appearance was in the 50´s. In 2001 Raillard and co-workers applied directed evolution to shuffle two highly homologous triazine hydrolases that utilized some triazines that originally were not substrates for parent types [37]. Directed evolution (two rounds of DNA shuffling) was also used to tailor a highly efficient phosphotriesterase towards organophosphates (OP) such as methyl parathion, a highly toxic neurotoxin used in insecticides and chemical warfare agents [38]. Sutherland and cols. through directed evolution and semi-rational approaches designed a phosphotriesterase capable of hydrolyzing aliphatic OP compounds, which were not degraded by natural enzymes [3]. A similar approach has been used to engineer a biphenyl dioxygenase that attacks polychlorinated dibenzofuran in the lateral position, proposing a new pathway of degradation of such molecule [40]. More recently, we have applied *in vitro* evolution procedures to obtain an improved version of a hexachlorocyclohexane dechlorinase (LinA), which is the primary biocatalyst under aerobic conditions to eliminate chlorine atoms from the molecule of γ-hexachlorocyclohexane (lindane), a still widely used and recalcitrant pesticide [41]. This may constitute a platform for lindane degradation schemes.

Laccases (belonging to the multicopper blue family) have also been extensively investigated for new and challenging decontamination programs because they affect the oxidation of many aromatic compounds towards more benign and less toxic products. Indeed, laccases can be involved in the detoxification of phenols, trichlorophenols, organophosphorus pesticides, azo dyes, and interestingly, PAHs (i.e. benzo[*a*]pyrene), a class of highly mutagenic and carcinogenic xenobiotics widely distributed in terrestrial and aquatic environments [32]. Recently, we were

involved in the functional expression of a thermophilic laccase in *Saccharomyces cerevisiae* [42]. After ten rounds of laboratory evolution we improved the enzymatic activity up to 170-fold along with better performances at high temperatures. Now, this system can be tuned up for promising decontamination schemes.

Due to the hydrophobicity and low aqueous solubility of xenobiotics such as PAHs, enzymatic oxidations (e.g. by laccases) may be performed in the presence of organic solvents to minimize mass transfer limitations; however, laccases in organic solvents are fairly unstable ending up denaturated or inhibited [43]. Keeping this in mind, we have recently engineered a thermophilic laccase by *in vitro* evolution, to be highly active and stable in the presence of increasing concentrations of acetonitrile and ethanol [44]. Moreover, five rounds of error-prone PCR, *in vivo* shuffling and saturation mutagenesis, have led to the discovery of laccase mutants with several folds improvements in turnover rates at high concentrations of organic solvents (not published). It should also be noticed that to exert its remarkable action on PAHs or on lignin and lignin-related compounds, laccases need the presence of redox mediators (either synthetic or, more recently, from natural sources) [45]. Thus, specific thrust should be given in the following years to develop novel laccases with low dependency of redox mediators and/or higher redox potential (so far, ranging from 0.4 to 0.8 V) to convert this biocatalyst in an efficient environmental tool. In order to accomplish the goals described above, dramatic improvements in certain core technologies such as (meta)genomics from extreme environments (i.e. contaminated soils) will be needed to locate a greater repertoire of novel starting points genes and/or enzymes that can be used as parent types for directed evolution

in such a manner that, eventually, more robust and efficient biocatalysts can be tailored [25].

Enzymes for clean energy production

Nowadays exciting new opportunities for biocatalysis towards the production of renewable and clean energy sources, such as biodiesel, bioethanol and biohydrogen are rapidly emerging [5, 46-48]. Based on the premise that these alternatives can contribute to a cleaner environment, specially when using renewable agricultural products, the demand of these energies is increasing. In fact, today bioenergy covers about 15% of the world's energy consumption. We believe, for example, that in the next 20 to 40 years, we will be able to convert biomass for our transportation fuels [49]. According to the enzymatic platform and the biomass employed we can distinguish three main groups of clean-energies:

Biodiesel. The conversion of vegetable oils to methyl- or other short-chain esters in a single transesterification reaction using lipases has led to the production of high-grade biodiesel [50]. This technology overcomes the disadvantages of chemical transformation based on acid- or base-catalysts, because it reduces the consumption of energy and the separation of the catalyst from the reaction mixture, which is costly and chemically wasteful. However, it shows strengths and weaknesses. Firstly, biodiesel is renewable and has low emissions per volume, and besides biodiesel is exempt from diesel tax through special legislation in several European countries, which makes processes involving biocatalysis more competitive. Moreover, efficient solvent-free synthesis of oleic acid short chain alcohols esters have been achieved with immobilized lipases such as those from *Pseudomonas*

cepacia, Rhizomocur miehei and *Candida antarctica* [48]. Limitations include their relatively high production cost, moderate reaction yields and difficulties found during purification of the unreacted substrates, which obviously will require new future advances.

Bioethanol. Prior to the discovery of petroleum, natural carbohydrates were used for the production of food, clothing and energy. Ethanol fuels can be derived from renewable resources: agricultural crops such as corn, sugar cane, and sugar beet or from agricultural byproducts such as whey from cheese making and potato processing waste streams [51-54]. Ethanol can be used as a 100% replacement for petroleum fuels or as an extender, since it can replace the toxic oxygenate, methyl *tert*-butyl ether. The current best available technology for conversion employs an acid hydrolysis of the biomass into sugars; however, the enzymatic alternative using enzymes such as α-amylases, glucoamylases, invertases, lactases, cellulases and hemicellulases to hydrolyze starch, sucrose, lactose, cellulose or hemicellulose into fermentable sugars is growing up. These sugars can be further fermented with bacteria, yeasts and fungi to produce ethanol [52], avoiding the use of strong acids and resulting in a cleaner stream of sugars for fermentation and fewer by-products [54]. Again the environmental benefit is the greater utilization of natural, renewable resources, safer factory working conditions, reduced harmful automobile emissions, and the consumer benefit as a safer alternative to existing supply of liquid fuel, i.e., gasoline.

Biohydrogen and biofuel cells. The possibility of using molecular hydrogen as a fuel is also gaining attention as a renewable, efficient and pollution-free energy source. Hydrogen is colorless, odourless, tasteless and non-toxic, making it different

from every other common fuel we use today because on burning it yields only water [55]. Hydrogen from biomass has the potential to compete with hydrogen produced by other methods (from natural gas which include catalytic conversion of hydrocarbons, electrochemical or photochemical water splitting). Although certain microbes such as *Rhodobacter spheroides* have been successfully used in production of hydrogen from fruit and vegetables waste [5,56], the process is currently still at the laboratory stage, and work needs to be done on increasing cost efficiency and application. For this reason, most research has concentrated on the employment of hydrogenases for the production of hydrogen, e.g. by using fermentations of sugars or, more attractively, from waste [5]. However, at present typical production ranges are only 0.37 to 3.3 moles hydrogen per mole of glucose, and these low yields may explain why hydrogen is not our primary fuel (both the cost and low production). This has prompted the search of new hydrogenases by using genome database mining [57] and also metagenomes, although the latter has not been exploited so far for such enzymatic screening.

Hydrogenases, laccases and other redox enzymes find also broad application as electrocatalysts, especially in the development of biofuel cells. In this field, recent investigations have demonstrated that hydrogenases, which convert hydrogen to generate an electric current, posses similar energy conversion efficiency that noble metal based commercial methods [58,59]. In this context, an enormous effort is being done to incorporate laccases in the design of biofuel cells (laccases are one of the few enzymes that can accept electrons from the cathodic compartment of a biofuel cell). Over the next few years we will see the growing use of enzymes in biological hydrogen and energy production, and this would require the collaboration

of biologists, chemists and engineers to integrate their knowledge to achieve more efficient enzymes.

Conclusion

Robust biocatalysis methods now exist for chemical synthesis and transformation, bioremediation of contaminants and clean-energy production confirming and reinforcing the potential of this technology for environmental purposes. The main advantages of using enzymes in these scenarios are their favourable unique properties, basically their biodegradability and high chemo-, regio- and stereo-selectivity resulting in low by-product formation; all together is allowing their progressive implementation. However, a more extensive effort is required to overcome several bottlenecks: high enzyme cost, low activity and/or stability under given conditions, low reaction yields and the low biodiversity screened so far (it has been estimated that more than 10^{30} microbial species are still unexplored). The relatively recent introduction and development of novel recombinant DNA technologies such as (meta)genomic and directed evolution, have had and will have a profound positive effect in the expression and production of larger and larger amounts of recombinant proteins (grams to kilos, which means more competitive prices), with new- or tailored catalytic activities. Results obtained to date suggest that the engineering of virtually any old or new enzyme for a commercially acceptable price and catalytically optimal properties could be achieved, suggesting a promising future for *Environmental Biocatalysis*.

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Fig. Legends:

Fig. 1 Examples of molecules manufactured using biocatalysts.

Fig. 2 Some xenobiotics amenable of enzymatic bioremediation.

Fig. 3. Typical experiment of laboratory evolution. After a few rounds of *in vitro* evolution, tailor-made enzymes with improved properties are ready for trials or field applications. The development of the high-throughput technology along with the application of computational methodologies will push further the engineering of enzymes by directed evolution and semi-rational approaches.

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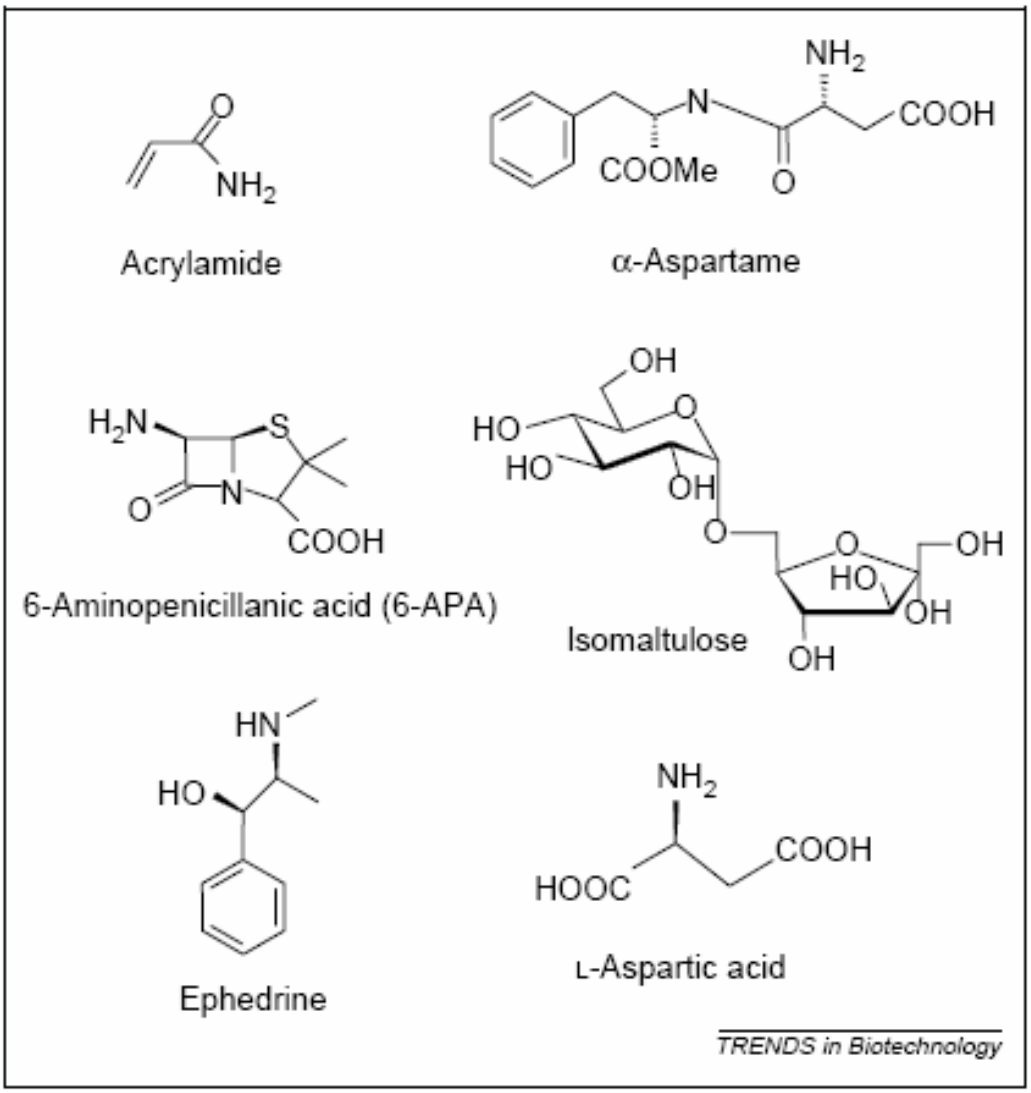

Figure 1. Examples of molecules manufactured using large-scale industrial applications of biocatalysts.

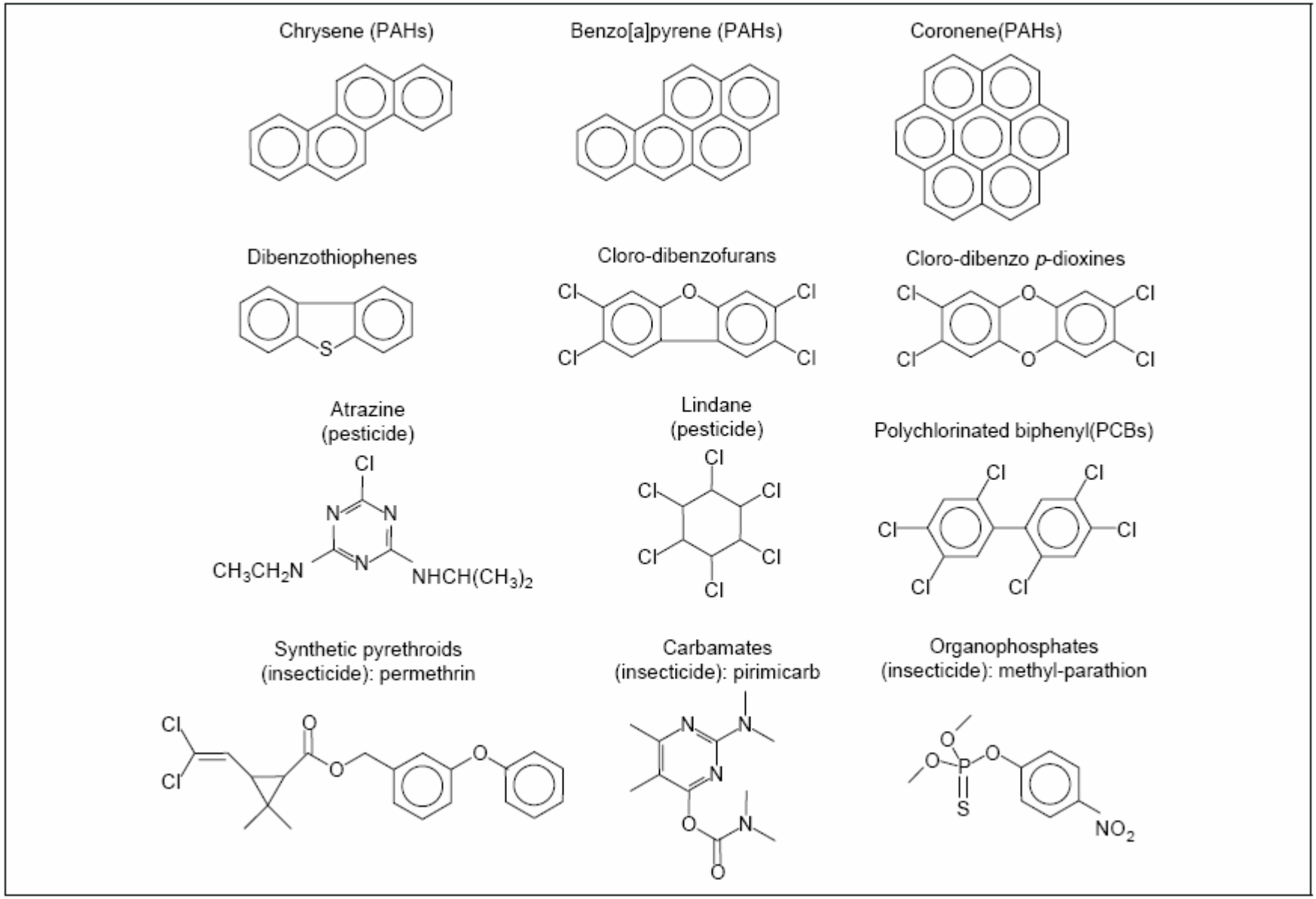

Figure 2. Some xenobiotics amenable to enzymatic bioremediation.

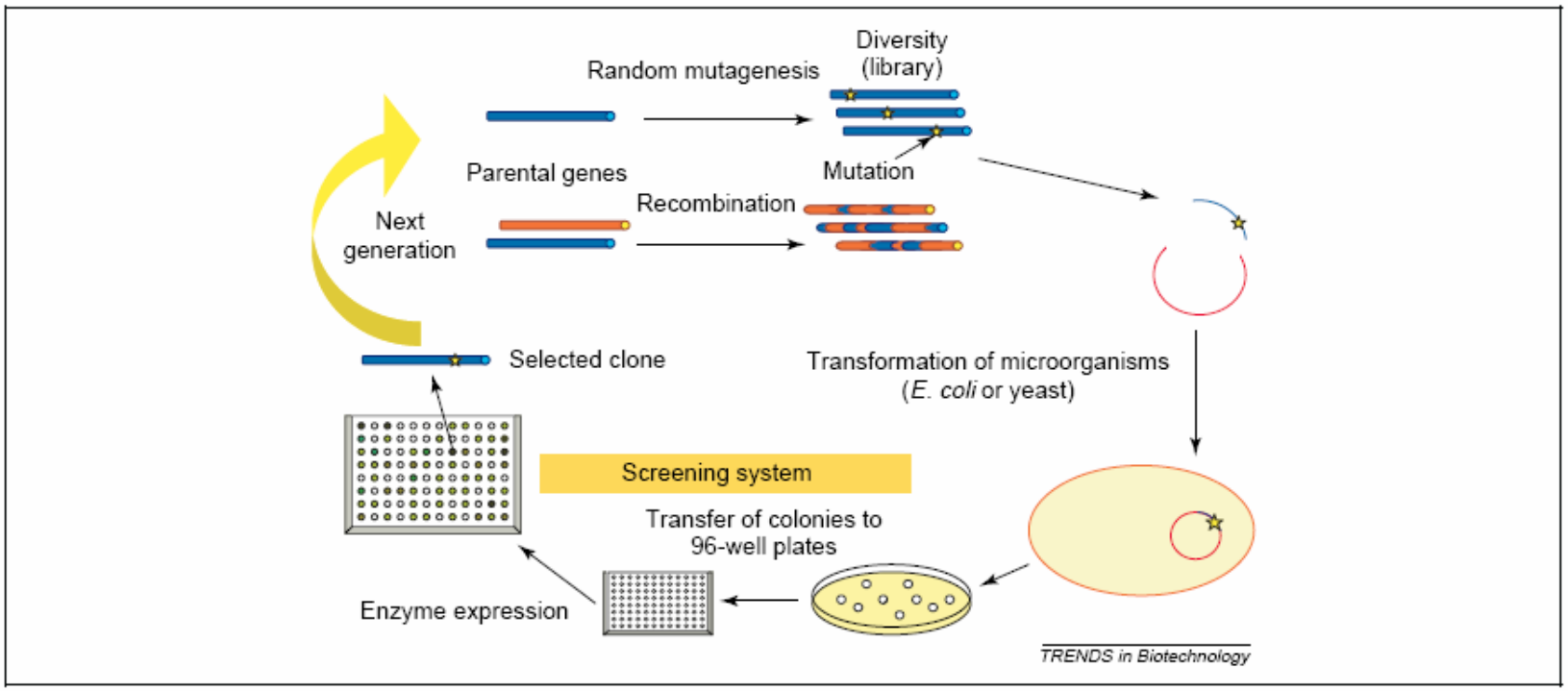

Figure 3. Typical experiment of laboratory evolution. After a few rounds of *in vitro* evolution, tailor-made enzymes with improved properties are ready for trials or field applications. The development of the high-throughput technology, along with the application of computational methodologies, will further push the engineering of enzymes by directed evolution and semi-rational approaches.