# Recent Advances in Cofactor Regeneration Systems Applied to Biocatalyzed Oxidative Processes

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**Abstract.** Nowadays, the design of sustainable processes applicable at industrial scale is highly desirable due to environmental reasons. The use of biocatalytic reactions to carry out oxidative transformations is one of the possible strategies framed in here due to the mildness and usually high selectivities achieved with these methods. Anyway, while implementing this type of system at industrial scale several drawbacks must be overcome in order to obtain efficient setups. Historically, one of the main issues has been the cofactor-dependency of these biocatalysts to be active. Herein, we will show the state-of-the-art concerning the recent efforts developed in the design of (potentially) efficient methods to regenerate the cofactor in oxidative transformations. Thus, the more studied enzymatic methods will be discussed to highlight some recent examples dealing with the co-expression of both oxidative and recycling enzymes in one host or the development of self-sufficient biocatalysts. Furthermore, novel applications of these systems to couple two productive synthetic reactions will also be reviewed. Subsequently, we will focus on some recent examples related with the employment of electrochemical, photochemical, and chemical strategies to carry out the nicotinamide coenzyme or the flavin/heme prosthetic group regeneration. In the case of biocatalysts that use NAD(P) as electron shuttle, these systems have also been employed to replace it, allowing the design of nicotinamide-free recycling setups. In all cases, the (dis)advantages that these methodologies present will be briefly discussed.

**Keywords.** Chemical recycling, Cofactor regeneration, Electrochemical recycling, Enzymes, Nicotinamide coenzyme, Oxidoreductases, Photochemical recycling, Prosthetic group

## 1. Introduction: the cofactor challenge

Oxidoreductases are emerging as an eco-friendly alternative applied to oxidative reactions due to their selectivity over the use of other chemocatalysts.[1-3] This feature is highly appreciated nowadays by the industry since the use of oxidative enzymes in organic synthesis can circumvent the protection and de-protection steps and minimize the by-product formation shortening the synthetic routes. The wide range of oxidative reactions catalyzed by oxidoreductases comprises oxidation of hydroxyl and carbonyl groups, including Baeyer-Villiger transformation, oxidation of primary and secondary amines, hydroxylation of non-activated hydrocarbons, heteroatom oxidation and double bond epoxidation.[4-6]

One challenge in the field of redox enzymes is their cofactor-dependency. Due to the high cost of the majority of cofactors, the *in situ* regeneration of a catalytic amount of the desired redox state has to be applied to design a feasible and scalable process. Traditionally, whole-cell biocatalytic systems have been used for this purpose due to their intrinsic ability to recycle the biological cofactor by simple addition of, *e.g.* glucose. This methodology presents several advantages like simplicity and low cost, nevertheless these systems have usually been used as non-growing (resting) cells since working with living organisms presents several drawbacks such as the low yields obtained due to the co-metabolism of substrates/products, the toxicity of reactants, the low global selectivities due to the action of several enzymes with different preferences, or the lower productivities due to the transport of the chemicals in and out of the cells.[7-9] In recent years, with the new development of enzymatic toolboxes and the advances in synthetic biology, some of the aforementioned problems can be overcome. Anyway, the development of novel systems for cofactor regeneration is in line with the increased applicability of oxidoreductases in preparative biocatalysis, especially from an industrial point of view. Since the majority of high-scale oxidation processes are based on cellular systems, some examples employing whole-cell biocatalysts will appear in this review, although the aim of this work is to summarize the state-of-the-art in cofactor regeneration for oxidative processes catalyzed by isolated enzymes. To accomplish this goal a broad range of enzymatic regeneration systems have been developed.[4,10] In the last decade, also a range of non-enzymatic regeneration methods have been described, including those using simple (electro/photo)chemical systems instead of ordinary nicotinamides.[11-13]

Oxidoreductases typically require (in)organic cofactors such as flavins, metal-ions, heme group or pyrroloquinoline quinone for their catalytic activity (Figure 1A-B). The binding of these cofactors to the enzyme is usually tight and they can be considered as a prosthetic group. These cofactors are part of the enzyme structure and their active form must be regenerated in each catalytic cycle by external redox equivalents. Conversely, cofactors that bind with a lower affinity, referred to as coenzymes, can be regarded as a substrate while acting as a redox mediator for different enzymes. In most cases, electrons are provided or accepted by natural nicotinamide coenzymes in a direct or indirect manner.[14] In nature, these nicotinamides exist in a phosphorylated (NADP) and non-phosphorylated (NAD) form according to their involvement in anabolic or catabolic pathways, respectively (Figure 1C). These molecules are indistinguishable in their mechanistic aspects; however, the only structural difference in the phosphate

group of the ribosyl moiety determines that most wild-type enzymes show strict (or highly favored) coenzyme specificity. On the other hand, NAD(P) exists in an oxidized [$NAD(P)^+$] and a reduced form [NAD(P)H], acting as an oxidant or a reductant, respectively. It is noteworthy that in many biocatalytic reactions NAD(P) is not directly involved in the enzymatic mechanism, but acts as a mediator transferring redox equivalents to and from the enzymatic redox reaction. So, the addition of NAD(P) can be omitted using cheaper (electro)chemical reducing agents and, therefore, a simpler and less expensive "nicotinamide-free" biocatalytic system can be developed.

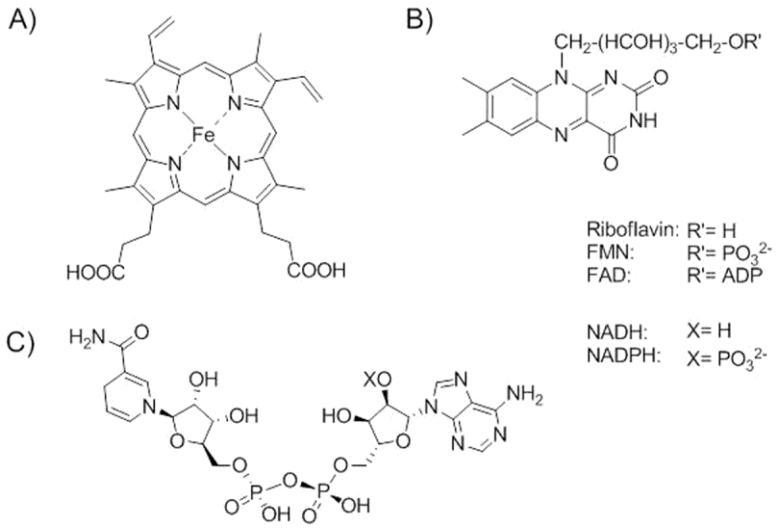



**Figure 1**. Structure of A) heme, B) flavins, and C) nicotinamide cofactors.

Taking into consideration the large number of publications about cofactor regeneration appeared in the last decade, this review will firstly focus on several innovative examples using different nicotinamide recycling systems for biocatalytic oxidations and possible synthetic applications (Section 2). Furthermore, regeneration of reduced and oxidized prosthetic groups in the absence of the NAD(P) cofactor when employing nicotinamide-dependent oxidative enzymes will also be discussed (Section 3), and finally some recent examples concerning the prosthetic group regeneration including peroxidases will be shown (Section 4).

## 2. Nicotinamide cofactor regeneration

In the past years, effective methods have been developed for the regeneration of nicotinamide cofactors using enzymes and whole-cell based systems which are discussed in detail in recent overviews.[15-18] The requirements of oxidized or reduced nicotinamides will be used to classify the available cofactor recycling methods due to the existing connection with the diversity of biooxidative processes. While oxygenases perform the enzymatic reactions via reductive oxygen activation, therefore needing the reduced form of the nicotinamide cofactor [NAD(P)H], dehydrogenases achieve the direct substrate

oxidation employing the oxidized $NAD(P)^+$. Hence, recent methodologies involving the recycling of both reduced/oxidized nicotinamide cofactor applied to oxidative processes will be presented.

### 2.1. Enzymatic regeneration

The vast majority of examples in literature apply "*coupled-enzyme regeneration systems*" consisting of a secondary enzymatic reaction, which is thermodynamically highly favored, along with the primary synthetic process, so that depletion of the cofactor during the biocatalysis can be overcome using 'green' procedures (Figure 2). In this line, regeneration of reduced cofactors has extensively been studied due to the interest for producing enantioenriched alcohols by alcohol dehydrogenase (ADH)-catalyzed asymmetric reduction of prochiral ketones.[17,19] Moreover, many monooxygenases such as heme-dependent cytochrome P450 enzymes (CYP450s) or flavin-dependent proteins like Baeyer-Villiger monooxygenases (BVMOs), require NAD(P)H to carry on selective biooxidative processes, including hydroxylations, epoxidations, oxidations of heteroatoms, and Baeyer-Villiger reactions which can be used for the functionalization of non-activated alkenes, stereoselective desymmetrizations, and (dynamic) kinetic resolutions of racemic carbonylic substrates to obtain optically pure compounds.[14] On the other hand, regeneration of the oxidized $NAD(P)^+$ cofactor is essential for (stereoselective) functionalizations, desymmetrizations and (dynamic) kinetic resolutions of racemic alcohols and amines making use of, *e.g.* dehydrogenases.[17,20]

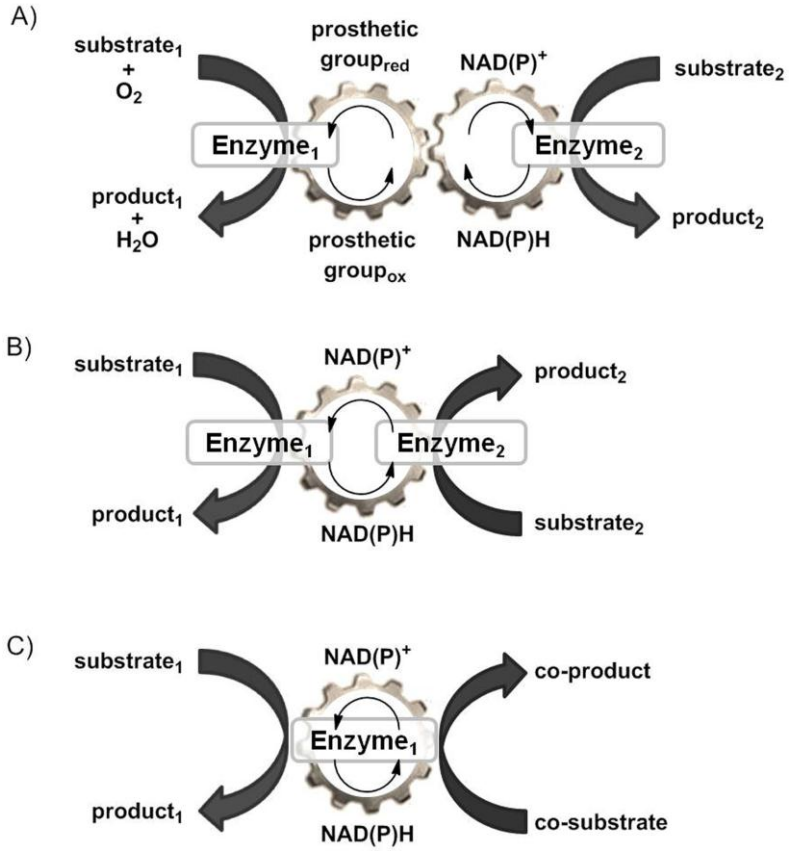



**Figure 2.** Enzymatic recycling of reduced (A) and oxidized (B) nicotinamide coenzymes by coupled-enzyme or (C) coupled-substrate regeneration systems.

*Regeneration of reduced NAD(P)H in biocatalytic oxidations*

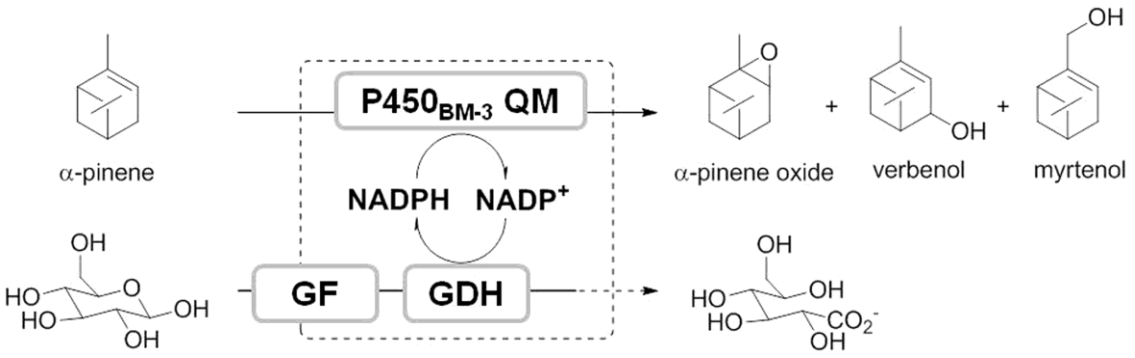
An enzymatic approach for cofactor recycling must satisfy certain requirements to be successfully employed for synthetic applications.[21] For instance, the regeneration reaction has to be thermodynamically favorable, enzymes and reagents of the secondary reaction should be inexpensive and should not interfere with the activity/selectivity of the main biocatalyst, and the formation of by-products (that would require additional purification steps) should be negligible. In this line, several dehydrogenases from a diversity of microorganisms are commonly used to regenerate NAD(P)H.

The advantages of commercial formate dehydrogenase (FDH) from *Candida boidinii* involve the use of innocuous formate as hydrogen donor for NADH regeneration and the formation of volatile $CO_2$ as co-product, which ensures quasi-irreversibility.[15] Whereas the wild type FDH only reduces $NAD^+$, a mutant enzyme for $NADP^+$ has also been described.[22] The use of a phosphite dehydrogenase (PTDH) from *Pseudomonas stutzeri* WM88 represents an alternative to regenerate the non-phosphorylated form of the reduced nicotinamide,[23] since this enzyme catalyzes the oxidation of phosphite to phosphate in the presence of $NAD^+$. In recent years, mutagenesis studies have been performed within this protein which has led to thermostable PTDHs active in organic solvents.[24] Also the affinity of PTDH for $NADP^+$ has been improved making possible its coupling with NADPH-dependent enzymes.[25] In addition to the engineered proteins that can accept $NADP^+$ instead of $NAD^+$, different biocatalytic approaches have been developed for coupling NADPH-dependent transformations with enzymes that originally prefer NADP. In this way, commercially available ADHs and, especially, glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides* [26] are classically employed for coupled-enzyme recycling of NADPH in biocatalytic oxidations.[27] Also glucose dehydrogenase (GDH) catalyzes the regeneration of either NADH or NADPH from the oxidized forms $NAD^+$ or $NADP^+$ with concomitant oxidation of inexpensive D-glucose to D-gluconolactone, which can be successfully applied not only coupled with the stereoselective reduction of ketones,[28] but also in oxidative processes catalyzed by NADPH-dependent oxygenases (Figure 2A).[29]

Another approach makes use of hydrogenases with molecular hydrogen as reducing agent. For that purpose, the thermostable hydrogenase I from *Pyrococcus furiosus* was originally proposed for NADP reduction.[30] However, its oxygen-sensitivity is counterproductive when biocatalytic oxidations are desired. Recently, an oxygen-tolerant $NAD^+$-reducing soluble hydrogenase from *Ralstonia eutropha* H16 has been described as a promising catalyst for cofactor regeneration.[31] Despite the initial application coupled to a reductive reaction, it is expected that its use in an NADH-dependent biooxidative process can therefore be considered.

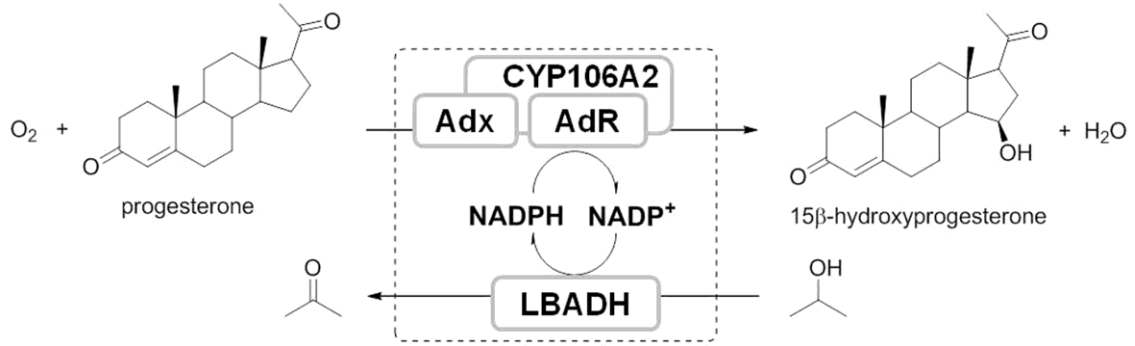
Although the "coupled-enzyme" system was typically applied with isolated enzymes, this strategy can also increase the productivity of overexpressed redox enzymes in whole-cell biocatalysts. In this regard, a cytochrome P450cam monooxygenase (CYP101) from *Pseudomonas putida* was coupled with the NADH regeneration enzyme glycerol dehydrogenase (GLD) in an *Escherichia coli* whole-cell system for oxidizing camphor.[32] Herein, the co-expression of CYP101 with GLD resulted in a nearly 10-fold increase in the hydroxylation reaction suggesting the potential of GLD applied to redox cofactor recycling

with other NADH-dependent oxidoreductases. Biocatalytic properties of the recombinant *E. coli* BL21 strain overexpressing a variant of a cytosolic P450 monooxygenase CYP102A1 from *Bacillus megaterium* ($P450_{BM3}$QM) have also been improved by integrating an heterologous intracellular NADPH regeneration system through co-expression of a glucose facilitator (GF) and a $NADP^+$-dependent glucose dehydrogenase (GDH), as shown in Scheme 1.[33] As a consequence, exogenous glucose was transported through the membrane of *E. coli* cells via GF and subsequently oxidized to gluconolactone (which spontaneously hydrolyzes to gluconate) by GDH, along with the NADPH regeneration needed for $P450_{BM3}$QM-catalyzed oxyfunctionalization of α-pinene to α-pinene oxide, verbenol, and myrtenol.




**Scheme 1.** Biotransformation of α-pinene by $P450_{BM3}$ whole-cell biocatalyst with improved glucose-driven cofactor regeneration.

In line with these studies, ADH from *Lactobacillus brevis* (LBADH) was recently employed for NADPH regeneration with 2-propanol in the cytochrome P450-catalyzed hydroxylation of steroids to the corresponding 15β-hydroxy products.[34] The soluble CYP106A2 from *Bacillus megaterium* ATCC 13368, previously co-expressed with the electron-transfer partners bovine adrenodoxin and adrenodoxin reductase, was also expressed together with LBADH in *E. coli*. To implement the cofactor regeneration system in the cells and the crude cell extracts, LBADH oxidized 2-propanol to acetone while reducing $NADP^+$ to NADPH (Scheme 2). Thus, after system optimization, productivities up to 5.5 g $L^{-1}$ $d^{-1}$ could be achieved.




**Scheme 2.** Steroid hydroxylation with cytochrome P450 monooxygenase CYP106A2 coupled with LBADH in *E. coli*.

As mentioned before, the use of whole-cells can be exploited taking advantage of the intrinsic coenzyme recycling ability of the host or through the co-expression of all enzymes needed in a single recombinant

organism.[35] Nevertheless, these cellular approaches exhibit some limitations such as different expression levels of both biocatalysts.[7] But, would it be possible to combine the catalytic oxidative activity of an oxidoreductase with concomitant coenzyme regeneration in a single recombinant protein? This system, where only the addition of the co-substrate would be mandatory, was recently achieved by fusion engineering as reported by Torres Pazmiño *et al.*[36] As a result, different NADPH-dependent BVMOs were covalently fused to a NADPH-regenerating PTDH for obtaining self-sufficient redox biocatalysts for oxidative synthetic applications, the so-called CRE-BVMOs (CRE; Coenzyme Regeneration Enzyme). The applicability of these novel biocatalysts was tested with cell-free extracts and whole-cell biotransformations by carrying out stereoselective desymmetrization reactions over prochiral substrates as well as regiodivergent oxidations of fused bicyclic ketones. In a subsequent communication, the authors obtained a second generation of bifunctional enzymes (CRE2-BVMO) by using a codon-optimized PTDH gene for better expression in *E. coli* encoding a thermostable mutant of PTDH as fusion partner for effective coenzyme regeneration and a polyhistidine affinity-tag at the *N*-terminus that facilitated its purification, which improved the stability and efficiency of the system to be applied in biocatalytic processes.[37] This newly developed expression vector was also used for producing a self-sufficient flavin containing monooxygenase (FMO), recently explored for the preparation of chiral sulfoxides and indigo derivatives as valuable dyes or precursors of bioactive compounds.[38]

*Regeneration of oxidized NAD(P)$^+$ in biocatalytic oxidations*

Although oxidized nicotinamides are more stable in solution than the reduced forms, synthetic schemes within efficient coupled-enzyme regeneration of NAD(P)$^+$ are less developed (Figure 2B). Nevertheless, several enzymes have been described for the regeneration of oxidized nicotinamides. Classical examples of these oxidoreductases comprise L-lactate dehydrogenase that catalyzes the reduction of pyruvic or glyoxylic acid into L-lactic or glycolic acid, respectively, with concomitant oxidation of NADH,[39] glutamate dehydrogenase to regenerate NAD$^+$ as well as NADP$^+$ coupled with reductive amination of α-ketoglutarate,[40] and promising NAD(P)H oxidases (see below). Dihydrolipoamide dehydrogenases (LPDs), belonging to the flavin-containing pyridine nucleotide disulfide oxidoreductase family, catalyze the NAD$^+$-dependent dehydrogenation of dihydrolipoamide.[41] However, *in vitro*, LPDs also display diaphorase activity that is able of transferring electrons from NADH to a variety of electron acceptors including lipoamide, quinones, and synthetic dyes such as 2,6-dichlorophenolindophenol (DCPIP) and nitrotetrazolium blue (NTB). Hence, LPDs could also be considered as an enzymatic system to regenerate NAD$^+$ from NADH in a biooxidative process. As demonstrated by Kurokawa *et al.*, by coupling LPD from *Microbacterium luteolum* (MluLPD) with a laccase and using 2,5-dimethoxy-1,4-benzoquinone (2,5-DMBQ) as hydrogen acceptor, the system was suitable for regenerating the coenzyme that is required by an NAD$^+$-dependent ADH to catalyze the oxidation of 2-octanol (Figure 3).

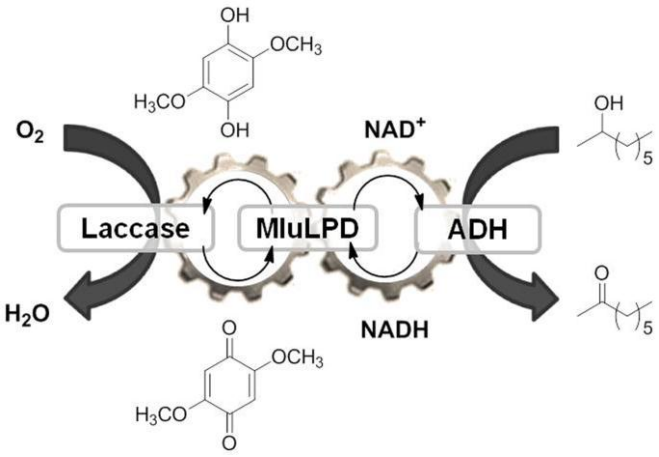
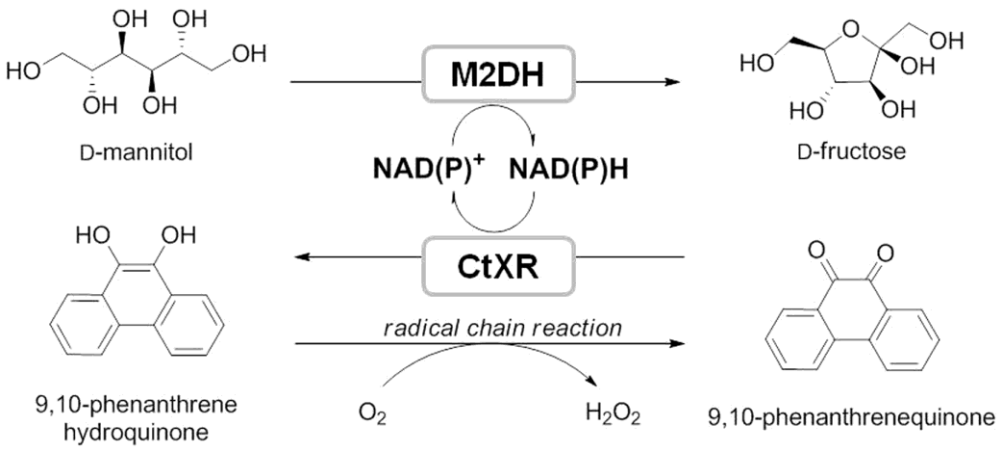



**Figure 3.** Coupled reaction of MluLPD and a laccase using 2,5-DMBQ as hydrogen acceptor for the biocatalyzed oxidation of *rac*-2-octanol.

NAD(P)H oxidases (NOXs) catalyze the oxidation of reduced nicotinamides at the expense of the molecular oxygen provided from the atmosphere. As a consequence of the two-electron or four-electron reduction of molecular oxygen, hydrogen peroxide or water are formed, respectively, as by-products.[17] Among the $H_2O$-forming NOXs, those originating from *Lactobacillus brevis* [42] and *L. sanfranciscensis* [43] were employed for the oxidative kinetic resolution of *rac*-1-phenylethanol by coupling it with (*R*)-specific LBADH. Also NOX from *L. brevis* has been used for $NAD^+$ regeneration coupled with L-amino acid dehydrogenase-catalyzed oxidations of L-*tert*-leucine [44] and L-methionine [45]. These enzymes are preferred over $H_2O_2$-forming NOX, since the hydrogen peroxide frequently causes enzyme deactivation. However, a recent study has demonstrated the usefulness of a novel thermostable NOX belonging to this class for the regeneration of both $NAD^+$ and $NADP^+$ at 30ºC and higher temperatures.[46] This biocatalyst (TkNOX) from the hyperthermophylic archaeon *Thermococcus kodakarensis* KOD1 was applied in the oxidative kinetic resolution of *rac*-1-phenylethanol coupled with two ADHs, the NADP-dependent (*R*)-selective ADH from *L. kefir* (LkADH) and the thermostable NAD-dependent (*S*)-selective ADH from *Rhodococcus erythropolis* (ReADH). This system entails several drawbacks that must be overcome such the different optimum pH for both enzymes, the suitable reaction temperature for carrying out the redox process without thermal decomposition of the nucleotide coenzymes, or the presence of $H_2O_2$ in the reaction medium. Despite these inconveniences, (*R*)-1-phenylethanol was completely oxidized at 30ºC and neutral pH by LkADH employing TkNOX, and (*S*)-1-phenylethanol was fully converted by the coupled system ReADH-TkNOX at 45-60ºC using 0.2-2 mM $NAD^+$ and a concentration of the racemate of 53 mM after 1 h. Under these conditions, the total turnover number (TTN) value achieved was of 136.

The acquired knowledge about $H_2O_2$-forming NOX for coenzyme regeneration has served as inspiration for a novel enzymatic system that combined a D-mannitol 2-dehydrogenase from *Pseudomonas fluorescens* (M2DH) and a xylose reductase from *Candida tenuis* (CtXR).[47] In this approach, the oxidation of D-mannitol to D-fructose was used as a model reaction while the $NAD(P)^+$ required for M2DH was regenerated by CtXR that utilized NAD(P)H to reduce 9,10-phenanthrenequinone (PQ) to 9,10-phenanthrene hydroquinone ($PQH_2$), which was rapidly reoxidized by $O_2$ (Scheme 3).



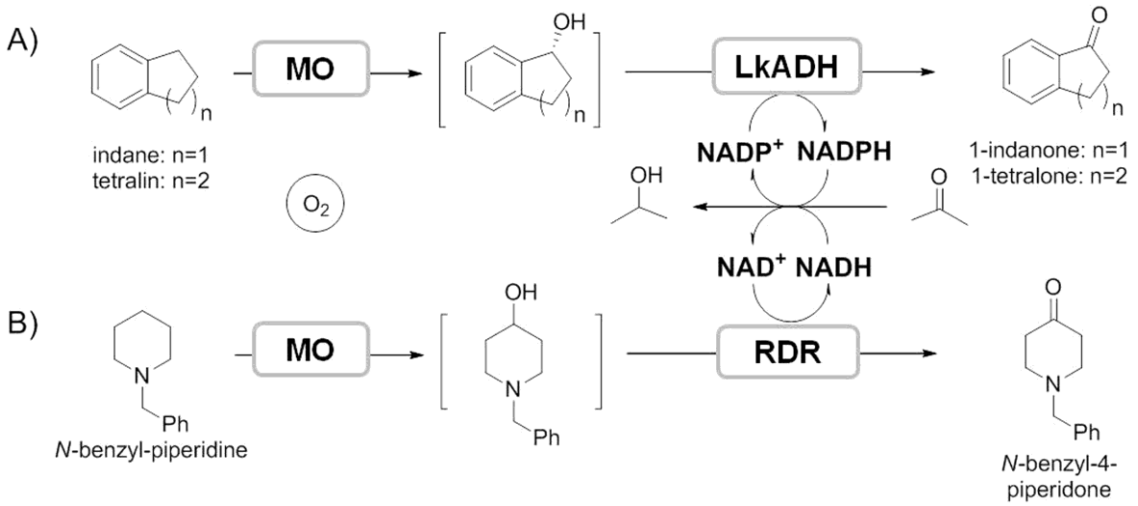

**Scheme 3**. Regeneration of NAD(P)$^+$ during the D-mannitol 2-dehydrogenase-catalyzed biooxidation of D-mannitol with isolated xylose reductase and 9,10-phenanthrenequinone.

The most advantageous approach for enzymatic cofactor recycling would be, by its elegance and simplicity, the use of a single enzyme that could simultaneously oxidize the target substrate reducing a second one (co-substrate) that would be cheap, harmless for the enzyme, and would not affect the outcome of the process in order to regenerate the cofactor (Figure 2C).[20] In this sense, the "*coupled-substrate regeneration system*" has widely been used with ADHs for the oxidation of *sec*-alcohols. Thus, an ADH can be used together with auxiliary co-substrates such aldehydes or ketones that serve as terminal electron acceptors in a hydrogen transfer fashion for NAD(P)$^+$ regeneration. A drawback of this approach is the need of an excess of the co-substrate to overcome thermodynamic limitations, which sometimes leads to enzyme inhibition. Therefore, many dehydrogenases cannot be used with this setup because highly stable enzymes are required for this purpose. In this way, the (*S*)-selective NAD$^+$-dependent ADH from *Rhodococcus ruber* DSM 44541 (ADH-A) exhibited an exceptional tolerance towards elevated concentrations of organic substances, including solvents and other reactants.[48] This fact made ADH-A [49] a highly efficient biocatalyst and a versatile tool for organic synthesis as previously shown for the asymmetric preparation of up to 126 g L$^{-1}$ of (*S*)-sulcatol in the presence of 50% v/v isopropanol. Also, the kinetic oxidative resolution of racemic *sec*-alcohols was possible by employing this microbial alcohol dehydrogenase system in the presence of up to 20% v/v acetone,[50] including the gram-scale oxidation of the natural (*S*)-rhododendrol to obtain the flavor and fragrance additive raspberry ketone [4-(*p*-hydroxyphenyl)butan-2-one].[51] Another enzyme that has widely been employed under these conditions to perform the oxidative kinetic resolution of several *sec*-alcohols is ADH from *Thermoanaerobacter ethanolicus* (TeSADH).[52] Although less employed, acetaldehyde has also been utilized as co-substrate to achieve the whole cell-catalyzed oxidation of primary alcohols.[53,54]

On the other hand, some advances have been developed to overcome the large molar excess of the required co-substrate, thereby reducing the generated waste and increasing the atom efficiency [55] of the biocatalytic oxidation reaction. To minimize this drawback, an interesting work was reported by Kroutil and co-workers.[56] Since α-chloro ketones can be reduced to 1-chloro-2-alcohols, but the latter cannot be reoxidized, the use of chloroacetone as oxidant instead of acetone would result in a quasi-irreversible system. Then, by employing activated ketones presenting electronegative groups at α-position, only a

slight excess of the oxidant would be required due to the quasi-irreversibility of this secondary reaction. In this way, several racemic aliphatic and aromatic *sec*-alcohols were oxidized to the corresponding ketones by ADH from *Sphingobium yanoikuyae* (SyADH) at elevated substrate concentrations (30 g $L^{-1}$) using 1.5 equivalents of chloroacetone as hydrogen acceptor. Also the preparative oxidation of *rac*-2-octanol (0.2 g, at 30 g $L^{-1}$) with just 1.1 equiv. of chloroacetone using lyophilized cells of *E. coli* containing this over-expressed non-selective ADH resulted in complete conversion (>99%) after 24 hours. Moreover, it was possible to perform the kinetic resolution at high concentrations of *rac*-2-octanol (up to 100 g $L^{-1}$) by using the (*S*)-selective ADH-A from *Rhodococcus ruber* in the presence of 0.6 equiv. of chloroacetone.

In the last few years, novel methodologies employ (catalytic) multistep chemical synthesis to avoid the tedious purification and isolation techniques that increase processing time and costs, and lower the overall process yield.[57] A recent example has been shown by Li and co-workers, where the coupled-substrate approach has been used for the selective concurrent oxidation of methylenes in compounds such as tetralin, indane and *N*-benzyl-piperidine to the corresponding 1-tetralone, 1-indanone and *N*-benzyl-4-piperidone in one-pot (Scheme 4).[58] This novel tandem system involved a microorganism containing a monooxygenase (MO) that catalyzed the selective hydroxylation of the methylene group into the alcohol, plus an isolated alcohol dehydrogenase that catalyzed the subsequent oxidation of this intermediate to the corresponding ketone with internal cofactor recycling. In this case, acetone was used as the coupled substrate for LkADH and a $NAD^+$-dependent alcohol dehydrogenase (RDR) for efficient regeneration of $NADP^+$ or $NAD^+$, respectively. These systems gave rise to clean reactions with around 80% yield and >99% regioselectivity.




**Scheme 4**. Oxidation of methylene groups into ketones in a one-pot system.

Soluble pyridine nucleotide transhydrogenases (STHs), which catalyze the transfer of reducing equivalents between $NADP^+$ and NADH or $NAD^+$ and NADPH, have appeared as a new class of promising regenerating enzymes. In this regard, a nice work was developed for the biological production of the semisynthetic opiate drug hydromorphone.[59] By applying STH from *Pseudomonas fluorescens*, both cell-free and whole-cell systems prevented the cofactor depletion resulting from the activities of

NADP-dependent morphine dehydrogenase and NADH-dependent morphinone reductase. Authors also demonstrated that the ratio of these three enzymes was critical for reducing the formation of the undesired by-product dihydromorphine while increasing yields of hydromorphone up to 84%. This STH strategy was recently applied in a multienzymatic cell-free system consisting of a NADPH-dependent cytochrome P450BM3 (CYP102A1) and a $NAD^+$-dependent glycerol dehydrogenase (GLD, Figure 4).[60] While NADH was generated by GLD using glycerol as a sacrificial co-substrate, the reducing equivalents were transferred to $NADP^+$ by STH. As a result, the reduced NADPH required for the oxidative CYP102A1-catalyzed process was regenerated. It was shown that the transhydrogenation catalyzed by STH was the rate-determining step. Moreover, complete conversion to *p*-nitrophenoxydecanoic acid could be achieved in the presence of only 5 μM of oxidized $NAD^+$ and $NADP^+$, instead of 50 μM NADPH required by the system lacking the two-step cofactor recycling, rendering a potent system to regenerate NADPH in oxidoreductase-based processes.

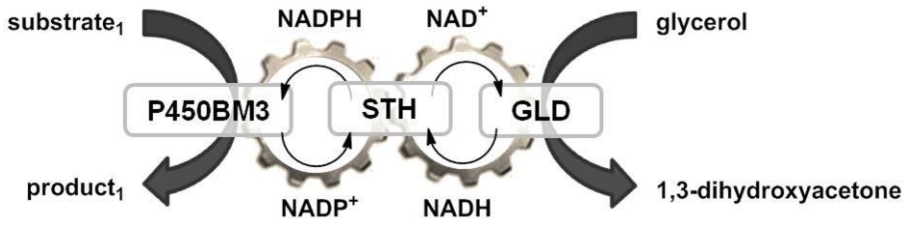
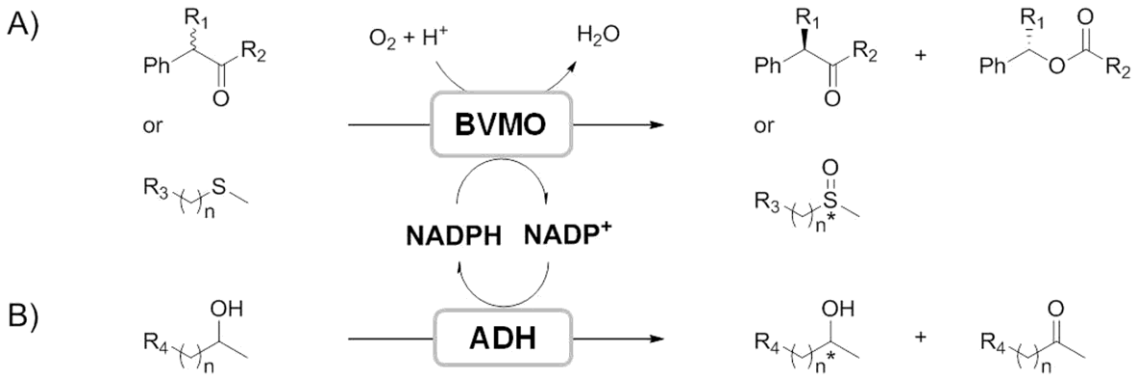



**Figure 4**. Nicotinamide recycling mediated by a soluble pyridine nucleotide transhydrogenase.

*Recent synthetic applications*

In nature, redox transformations take place concurrently by sharing electron acceptors/donors such as $NAD(P)^+$/NAD(P)H to connect oxidative and reductive reactions in an effective metabolic network. It is increasingly common to find examples in the literature trying to mimic such evolved and efficient processes.[57,61] Among them, those in which both the main reaction and the cofactor recycling reaction give rise to synthetically useful products should be highlighted. Herein, some recent examples with synthetic applications will be commented. In this way, an elegant scheme of a one-pot tandem system was developed by Bisogno *et al*.[62] Although the final objective was to obtain simultaneously two different enantiopure *sec*-alcohols using a single enzyme and a catalytic amount of cofactor, actually this biocatalytic system includes a second valuable transformation, resulting in a one-pot process combining the quasi-irreversible ADH-reduction of activated ketones with the oxidative kinetic resolution of racemic *sec*-alcohols. Another approach to maximize the redox economy of the oxidative processes consisted of coupling two enzymatic systems (BVMO- and ADH-catalyzed oxidations) through internal cofactor (NADPH) regeneration for producing two or even three enantioenriched products concurrently (Scheme 5). This methodology named PIKAT (from Parallel Interconnected Kinetic Asymmetric Transformation) allowed to minimize the reagents employed, to carry out the selective oxidation of a ketone into an ester catalyzed by the BVMO and the oxidation of a *sec*-alcohol into a ketone by the ADH in a parallel and concurrent fashion.[63] This system was also used in the BVMO-catalyzed asymmetric oxidation of different sulfides coupled with the ADH-catalyzed oxidative kinetic resolution of secondary alcohols.[64]

Moreover, by the proper selection of both biocatalysts, it was possible to obtain all possible enantiomers in a one-pot process.




**Scheme 5**. PIKAT methodology for the selective BV-oxidation of racemic ketones or the asymmetric oxidation of prochiral sulfides (A) coupled to the oxidative kinetic resolution of racemic *sec*-alcohols (B).

The relevance of the cofactor dependence has been shown in other synthetic approaches involving concurrent oxidation and reduction processes in the same vessel. It is worth mentioning the work developed by Kroutil and co-workers in the field of the deracemization of *sec*-alcohols by using two enantiocomplementary ADHs with two independent cofactor-recycling systems,[65] or the deracemization of *rac*-mandelic acid into L-phenylglycine *via* a redox-neutral biocatalytic cascade using D-mandelate dehydrogenase and an L-amino acid dehydrogenase, internally recycling NADH due to the action of both processes.[66] In these cases, the concurrent oxidative and reductive half-reactions allowed to obtain the final enantiopure product in quantitative yield.

### 2.2. Electrochemical regeneration

The simplest way to regenerate the oxidized or the reduced form of the nicotinamide cofactor would be the employment of electrochemistry.[12,13] In fact, the transfer of two electrons between $NAD(P)^+$ and NAD(P)H lays around 900 mV versus the saturated calomel electrode (SCE). This methodology (Figure 5) has several advantages because just the electrons would be delivered maximizing the atom [55] and the redox efficiencies.[67] At first, no additional co-substrates and auxiliary catalysts might be employed, leading to cleaner processes that would facilitate the downstream processing. Furthermore, electrons are among the cheapest redox equivalents available.

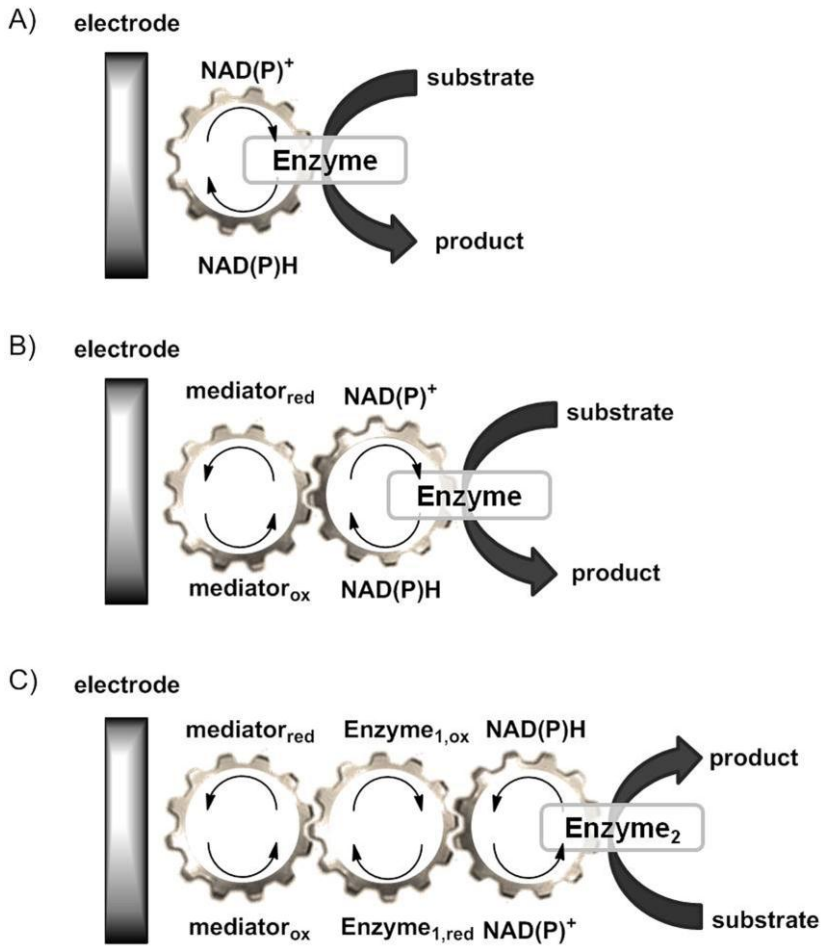



**Figure 5.** Electrochemical regeneration of oxidized nicotinamide coenzymes: A) by direct interaction with the electrode; B) in the presence of a mediator; C) in the presence of a second biocatalyst.

However, not many examples have appeared coupling the electrochemistry and the enzyme catalysis applied to preparative synthesis, although the combination of isolated enzymes with electrochemical reactions is well established in biosensor techniques for analytical purposes.[68,69] Apart from the special bioreactor design (*i.e.*, electrochemical cells) and the expertise in both biotechnology and electrochemistry required for these reactions, other important drawbacks must be taken into account. Firstly, due to the high overpotentials needed, this method can be only used for reactions involving substrates that are stable under these conditions to avoid side-reactions. Secondly, when the cathodic reduction of $NAD(P)^+$ is involved, this process is usually hampered by the low selectivity of the two-step electron donation and protonation pathway. The $NAD(P)^{\bullet}$ intermediate radical can dimerize, yielding enzymatically inactive dimers and even the protonation step is rather unselective, affording significant amounts of the enzymatically inactive regioisomer 1,6-NAD(P)H.[70] In the case of the anodic oxidative regeneration of $NAD(P)^+$ this drawback is not present, but anyway, there are not many examples of direct electrochemical regeneration of $NAD(P)^+$ (Figure 5A) probably due to low mass- and electron-transfer yields. One of the most remarkable contributions in this field was given by Moiroux and co-workers that employed the direct anodic regeneration of $NAD^+$ with the concomitant cathodic reduction of pyruvate to deracemize or perform the stereoinversion of DL- or L-lactate into D-lactate, respectively.[71,72]

Recently, a promising tin (IV) oxide electrode has been made using an anodization and annealing method and was used as a working electrode in an electrochemical cofactor regeneration reaction.[73] The main advantage of this material with regards to others was its large surface area and the flexibility of its morphology by changing the preparation conditions. Employing this electrode, the mediator-free electrochemical coenzyme regeneration was feasible. It was demonstrated that it could oxidize both NADH and NADPH over a broad range of pHs and temperatures. As a model reaction, alcohol dehydrogenase from *Thermoanaerobium brockii* was used for the oxidation of 2-propanol to acetone.

Some of the problems mentioned before can be minimized at some extent employing another substance that can act as an electron shuttle between the electrode and the cofactor (mediator, Figure 5B). An appropriate mediator should avoid the *in situ* formation of NAD(P)-radicals transferring two electrons at once, having a more anodic potential than the first reduction step of $NAD(P)^+$, and should exclusively produce the enzymatically active 1,4-NAD(P)H regioisomer form. Furthermore, it should be robust and active in a broad range of conditions, should perfectly diffuse in the reaction medium and should not interfere with the biocatalyst employed. There are more examples of the indirect electroenzymatic methodology applied to oxidative synthetic processes than using only an electrode. Historically, the first mediators successfully utilized for this purpose were phenantroline [74] and phenantrolinedione complexes [75,76] with alcohol dehydrogenases. Afterwards, $[Cp^*Rh(bpy)(H_2O)]^{2+}$ was used to regenerate NADH in the oxidation of 2-hydroxybiphenyl catalyzed by 2-hydroxybiphenyl-3-monooxygenase (HbpA). However, the productivity of this setup was lower than the one observed for the chemical regeneration method using $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and formate (see Section 2.3).[77] Other mediators such as 2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonate (ABTS) have shown to be suitable derivatives.[78,79] Thus, the oxidative kinetic resolution of phenylethane-1,2-diol catalyzed by glycerol dehydrogenase from *Cellulomonas* sp. was achieved using a graphite electrode and ABTS. In this case, to circumvent the product inhibition, a membrane reactor with solvent extraction of the lipophilic product was applied.[79] In a very recent contribution by Bornscheuer and co-workers,[80] two aldehyde dehydrogenases (ALDHs) from *Escherichia coli* BL21 and *Deinococcus geothermalis* were cloned and overexpressed in *E. coli* to oxidize glyceraldehyde into glyceric acid. The best results in terms of activity and selectivitiy were achieved with the ALDH variant of *D. geothermalis*. Furthermore, this ALDH allowed the kinetic resolution of glyceraldehyde employing an indirect electrochemical coenzyme recycling by means of a foam glassy-carbon electrode and ABTS, leading to D-glyceric acid with 88% *ee*.

In some cases, a more sophisticated system (Figure 5C) using a second auxiliary enzyme to improve the electron-transfer kinetics between the nicotinamide cofactor and the mediators have also been successfully designed. Thus, among the proteins employed, artificial mediator accepting pyridine nucleotide oxidoreductases (AMAPOR) [81] or diaphorases [82] can be highlighted.

### 2.3. Chemical regeneration

Another alternative to promote the *in situ* regeneration of NAD(P) is the use of chemical reagents. This approach is similar to the previously mentioned 'coupled-substrate' approach, but in this case the chemical agent directly regenerates the nicotinamide coenzyme without the biocatalyst intervention, in

contrast to, *e.g.* the acetone mediated regeneration of $NAD(P)^+$ in ADH-catalyzed oxidations. With regards to electrochemical methodologies, the homogeneous nature of these approaches is a clear advantage so diffusion limitations frequently observed with electrochemistry are circumvented. In many cases, the chemical reagents employed are the same than the mediators previously shown for the indirect electrochemical regeneration methodology.

Historically, the earliest example of a chemical NAD(P)-regeneration system comprised the use of flavins in excess functioning as hydride acceptors from the reduced nicotinamide coenzymes.[83] Its efficiency however was limited due to the slow hydride-transfer kinetics from NAD(P)H to the oxidized flavin. Most of the examples in this field make use of pentamethylcyclopentadienyl rhodium bipyridine complexes $[Cp^*Rh(bpy)(H_2O)]^{2+}$, a versatile reagent for chemical regeneration of oxidoreductase cofactors due to its interesting properties (Figure 6).[13,84] When reducing $NAD(P)^+$ into NAD(P)H, it produces exclusively the enzymatically active 1,4-NAD(P)H regioisomer, is equally active with NAD or NADP, and operates in a broad range of medium conditions.

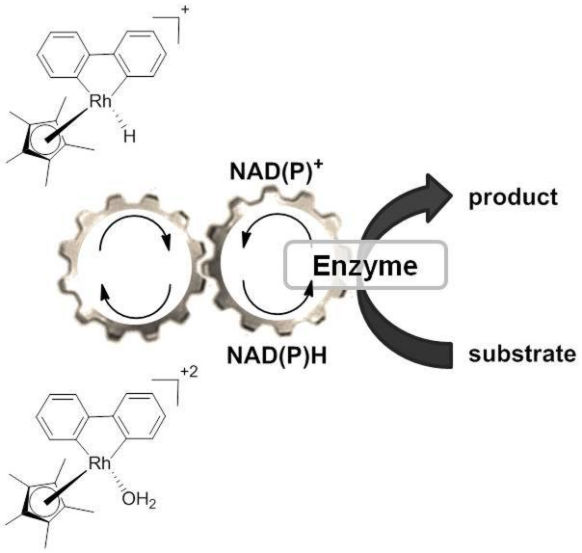



**Figure 6.** Employment of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ for chemical-mediated cofactor regeneration.

$[Cp^*Rh(bpy)(H_2O)]^{2+}$ is usually employed in catalytic amounts with formate as final reducing agent and it has been applied to the ortho-hydroxylation of α-substituted phenols catalyzed by 2-hydroxybiphenyl-3-monooxygenase [77] or the stereoselective sulfoxidation of several sulfides catalyzed by Baeyer-Villiger monooxygenases.[85] It has been described the use of this metal complex together with flavin to achieve an ADH-catalyzed oxidation. In this case, molecular oxygen was the terminal electron acceptor.[86]

### 2.4. Photochemical regeneration

In this approach a chemical compound (photosensitizer) mediates the electron transfer from the nicotinamide coenzyme to the terminal electron acceptor. These methods are based either on the light-induced excitation of this mediator enabling it to oxidize NAD(P)H (reductive quenching mechanism), or

on the light-induced excitation of the reduced mediator, thus allowing its re-oxidation (oxidative quenching mechanism).

This methodology is very advisable since it is homogeneous making possible a correct mass and redox transfer, does not differentiate between NADH and NADPH cofactors, and can be effective in a broad range of medium conditions. In addition, it can be considered as an environmentally friendly methodology, and does not need very special equipment. On the contrary, nowadays it is still being developed in order to improve its robustness and scalability.

A very recent example was described by Hollmann and co-workers,[87] where in several ADH-catalyzed oxidations, $NAD(P)^+$ was regenerated by visible light irradiation of the FMN present in the reaction medium as photosensitizer (Figure 7), since it is known that flavin photoexcitation is generally induced by wavelengths in the visible range ($\lambda_{max}$≈360-450 nm). Moreover, in this system molecular oxygen was the terminal electron acceptor, very desirable from an environmental point of view, and the high thermodynamic driving force obtained from the oxygen reduction was also beneficial to shift the ADH-catalyzed redox equilibrium. Catalase was also used to dismutate the $H_2O_2$ obtained as by-product. With this setup, the light-driven regeneration was coupled to the oxidation of cyclohexanol catalyzed by ADH from *Thermus* sp. ATN1, as well as the oxidation-lactonization of 1,4-butanediol and 1,5-pentanediol catalyzed by horse liver alcohol dehydrogenase (HLADH). Acceptable TTNs were achieved under non-optimized conditions (between 170-340).

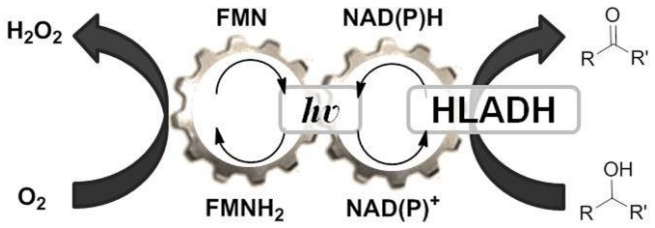



**Figure 7.** Photochemical regeneration of $NAD(P)^+$ by means of light-induced photoexcitation of FMN.

### 3. Nicotinamide-free cofactor regeneration

As pointed out in the Introduction, in most cases, the nicotinamide coenzyme merely acts as an electron shuttle transferring redox equivalents to and from the desired biocatalytic redox reaction in order to, *e.g.* regenerate the active form of the prosthetic group implicated in the enzymatic oxidation process. Therefore, it is obvious that the methodologies described in the previous section can also be applied to perform the direct regeneration of prosthetic groups avoiding the expensive nicotinamide cofactors. For instance, when employing monooxygenases, electron supply to the biocatalyst will be mandatory (Figure 8).

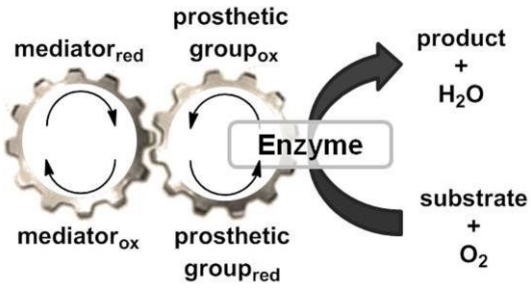



**Figure 8.** Reductive nicotinamide-free cofactor regeneration for enzyme-catalyzed oxidations.

### 3.1. Electrochemical regeneration

Due to their high synthetic potential, there are many examples about electrochemical recycling in monooxygenase-catalyzed oxidations. In these cases, a reductive electrochemical regeneration of these enzymes must be achieved since they reductively activate molecular oxygen incorporating one oxygen atom into the substrate, while the second one is reduced by the cofactor affording water. This cofactor is recycled by providing reducing equivalents like NAD(P)H.

Although there are some examples related to the indirect flavin regeneration employing, *e.g.* 1,1'-dicarboxy-cobaltocene [88] as mediator, in the last few years most of the examples appeared in the literature make use of the direct cathodic $FADH_2$ regeneration. One of the first examples on this topic was demonstrated by Schmid and co-workers,[89] who performed the (*S*)-epoxidation of several styrene derivatives with styrene monooxygenase (StyA) from *Pseudomonas* sp. VLB120 in the presence of a carbon cathode as working electrode (Figure 9). Anyway, the rate of the electroenzymatic reaction did not meet the values obtained with the native cycle using NADH plus the reductase enzyme due to the low *in situ* concentration of $FADH_2$. Furthermore, the high uncoupling rate producing $H_2O_2$ most likely caused the fast StyA inactivation. Interestingly, the ratio of the cathode surface and the reaction volume had a significant influence on the rate of the electroenzymatic epoxidation.

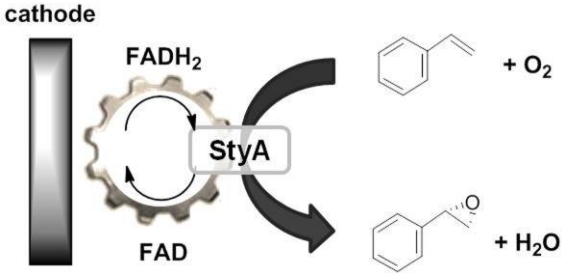



**Figure 9.** StyA-Catalyzed epoxidation of styrenes following a direct electroenzymatic flavin regeneration approach.

In a subsequent work from the same group,[90] this methodology was improved with the same biocatalyst for the asymmetric epoxidation of styrene with high enantiomeric excess. In this case, a highly porous reticulated vitreous carbon electrode, where the volumetric surface area is maximized, was employed in a flow-through mode to regenerate the consumed $FADH_2$ cofactor. Increasing FAD concentrations

negatively influenced product space-time yields and efficiencies due to the enhanced accumulation of acetophenone and phenylacetaldehyde side-products. The hydrogen peroxide accumulation was minimized by the presence of a catalase. In comparison to the previously described electroenzymatic batch reaction system, productivity in terms of specific enzyme activity and synthesis time was 150-fold higher regarding to (*S*)-styrene oxide, and 215-fold higher with respect to the overall electroenzymatic performance. Thus, using a flow rate of 150 mL $min^{-1}$, an average space-time yield of 0.35 g $L^{-1}$ $h^{-1}$ could be achieved during 2 h with a final (*S*)-styrene oxide yield of 75.2%. In a more recent contribution by Lim *et al.*,[91] the low electroenzymatic efficiency arising from fast $FADH_2$ reoxidation could be overcome employing a zinc oxide/carbon black composite electrode. Again, StyA was successfully used for the epoxidation of styrene. By correctly adjusting the oxygen concentration, high electroenzymatic efficiency was obtained.

This approach has also been applied to regenerate highly oxidized Fe–heme species in CYP450s. Udit and co-workers reported the anodic electrocatalytic generation of Fe(IV)–heme active species of cytochrome P450cam from *Pseudomonas putida* with a graphite electrode, achieving the oxidation of thioanisole into methyl phenyl sulfoxide.[92] Concerning the indirect electrochemical regeneration of heme prosthetic groups, a recent contribution using an electrochemical cell with a platinum electrode and several mediators, showed that P450cin from *Citrobacter braakii* could stereoselectively hydroxylate its natural substrate 1,8-cineole to form 2β-hydroxy-1,8-cineole. Phenosafranine and safranine T were able to transfer the electrons to the heme active center of P450cin, although productivity must still be highly improved.[93]

Very recently, the employment of nicotinamide cofactor analogs has been shown for electrochemical indirect regeneration. It has been described that NAD analogs, 3-acetylpyridine adenine dinucleotide (APAD) and 3-pyridinealdehyde adenine dinucleotide (PAAD) could be electrochemically reduced more efficiently than the original nicotinamide coenzyme by means of $[Cp^*Rh(bpy)H_2O]^{2+}$ as electrochemical mediator.[94] Furthermore, the temperature stability of their reduced forms was also much higher than the one found for NADH. This work has demonstrated that NAD analogs can be excellent cofactors to be reduced via electrochemical regeneration to be applied to redox enzymatic reactions.

### 3.2. Chemical regeneration

As for the case of nicotinamide coenzymes, $[Cp^*Rh(bpy)(H_2O)]^{2+}$ is able to regenerate FAD by reducing it into $FADH_2$. This fact has allowed the substitution of the nicotinamide cofactor directly employing the metallic complex plus formate as final electron acceptor. The first example of a direct flavin regeneration with this system was demonstrated by the group of Schmid,[95] using styrene monooxygenase (StyA) from *Pseudomonas* sp. VLB120 to achieve the chemoenzymatic epoxidation reaction of different vinyl aromatic derivatives to synthesize enantiopure (*S*)-epoxides. In addition, StyA was also able to perform sulfoxidation reactions under these conditions. The productivity of this approach was significantly lower (70%) to the natural system, which was ascribed to the mutual inactivation of StyA and the regeneration catalyst. The same approach could be carried out for the regioselective tryptophan-7-halogenase-

catalyzed chlorination and bromination reactions [96] and to obtain chiral sulfoxides employing different Baeyer–Villiger monooxygenases.[85]

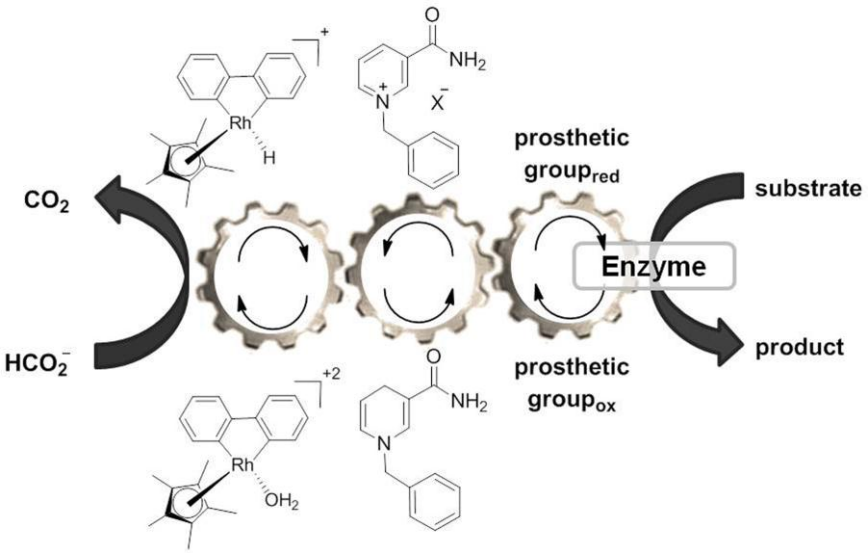



**Figure 10.** *In situ* cofactor regeneration using $[Cp^*Rh(bpy)(H_2O)]^{2+}$ with formate and a biomimetic nicotinamide cofactor.

In recent contributions, $[Cp^*Rh(bpy)(H_2O)]^{2+}$ with formate has been applied to some FAD- or heme-dependent oxidative transformations using biomimetic nicotinamide cofactors (Figure 10). Thus, *N*-benzyl-1,4-dihydronicotinamide was applied as NADH mimic for the hydrogen transfer in the FAD-catalyzed hydroxylation of 2-hydroxybiphenyl to the corresponding catechol accomplished by 2-hydroxybiphenyl 3-monooxygenase (HbpA) from *Pseudomonas azelaica* HBP1 together with $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and formate.[97] However, productive coupling of this method to achieve the enzymatic hydroxylation reaction was not totally successful due to a deactivation process by reaction of the peripheral -$NH_2$ or -SH groups of HbpA with the precatalyst $[Cp^*Rh(bpy)(H_2O)]Cl_2$, thus inhibiting the cofactor regeneration process. To successfully circumvent this fact, the reacting amino acids were treated with a polymer containing epoxides. This system was also applied with CYP450s.[98] In this case, *N*-benzyl- or *N*-(4-methoxybenzyl)-1,4-dihydronicotinamide were used with P450 BM3, P450cam and several mutants of both biocatalysts to perform the hydroxylation of *p*-nitrophenoxydecanoic acid and camphor, respectively. While wild-type P450cam oxidation reactions could be supported with both biomimetic cofactor analogues, wild-type P450 BM3 was not active with them. Mutations of a key residue in the reductase unit afforded only a small increase in the initial rate for P450cam, while mutations in the P450 BM3 reductase domain enabled the use of NADH analogues at comparable rates to that of the natural coenzyme.

Another chemical approach to regenerate the heme prosthetic group in CYP450s is the employment of cobalt(III)sepulchrate and zinc dust that serves as electron source. Under these conditions, the P450 BM3 F87A mutant performed the hydroxylation of *p*-nitrophenoxydodecanoic acid at a 20% turnover rate in comparison to the usage of NADPH. [99] More recently, Schwaneberg *et al*. obtained several P450 BM3 mutants with enhanced performance for the Zn/Co(III)sep-mediated electron transfer, by directed

evolution experiments. Among them, a variant carrying five mutations (P450 BM3 M5) exhibited for this system a 3-fold improvement in its catalytic efficiency compared with the starting point mutant P450 BM3 F87A.[100] In a more recent study, the same group applied this methodology to achieve continuous hydroxylation of 3-phenoxytoluene in a plug flow reactor for 5 days with TTNs over 2,000, using the immobilized P450 BM3 M9 mutated at nine positions.[101]

### 3.3. Photochemical regeneration

Light-driven regeneration of the flavin prosthetic group appears again as a green and promising alternative to other methods commented before. Thus, Reetz and co-workers showed the Baeyer-Villiger oxidation of different substrates catalyzed by a mutated phenylacetone monooxygenase (PAMO-P3) employing ethylenediaminetetraacetate (EDTA) as photosensitizer.[102] This compound transferred the electrons to the FAD present in the medium acting as mediator in the overall process. Anyway, it was observed that catalytic amounts of $NADP^+$ were necessary to achieve a successful setup due to the known role of the bound nicotinamide coenzyme in sustaining the catalytically active conformation of this type of enzyme.[85] A light bulb was used in order to have a stable system. Although its selectivity remained unaltered, the catalytic performance of PAMO-P3 was significantly lower than using a conventional regeneration system. This fact was ascribed to the slow electron-transfer kinetics under these conditions. In a subsequent work, this system was investigated in more detail and it was discovered that oxidative uncoupling of the flavin-regeneration reaction from enzymatic $O_2$-activation was responsible for the consumption of approximately 95% of the reducing equivalents provided by the sacrificial electron donor (EDTA) that obviously made this process less productive. Furthermore, the electron transfer between the free and enzyme-bound flavins was relatively slow.[103]

In order to avoid the decoupling reaction, the same authors proposed the use of deazaflavins instead of FAD or FMN as mediators, since reduced deazaflavins have been reported to react very slowly with molecular oxygen.[104] In combination with an inexpensive sacrificial electron donor such as EDTA, it was possible to perform the aerobic regioselective hydroxylation of lauric acid using P450 BM3 with a good performance (TTN~ 700).

Very recently, it has been demonstrated the use of a metal-hybrid cytochrome P450 to achieve the light-initiated hydroxylation of lauric acid (Figure 11). In this case, a photosensitizer Ru(II)-diimine complex was covalently attached to the heme domain through the non-native single cysteine residue of the mutated P450 BM3 enzyme. Among several sacrificial reductive quenchers investigated, sodium diethyldithiocarbamate (DTC) was selected. The biocatalytic reaction was irradiated with visible light to successfully achieve this transformation, obtaining comparable TTN values than the ones observed forming *in situ* hydrogen peroxide (see below).[105] This example shows that the development of new hybrid biocatalysts can improve the performance of known biocatalytic reactions, allowing the design of novel transformations.

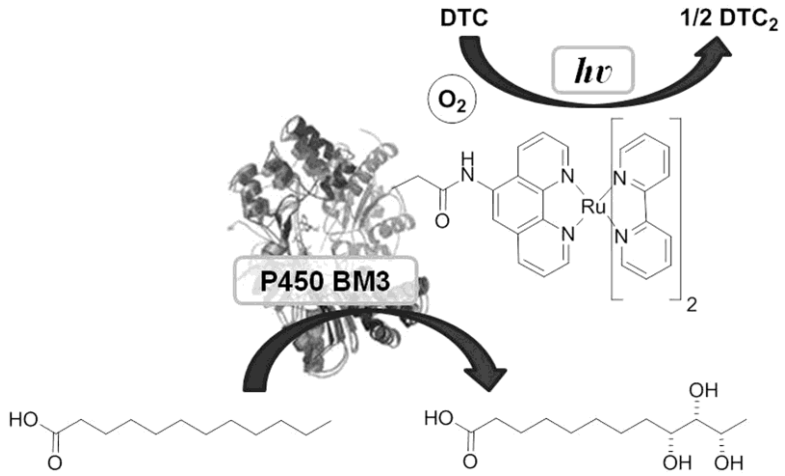



**Figure 11.** Hybrid P450 BM3 enzyme composed by a photosensitizer Ru(II) complex covalently attached to the heme domain of P450 BM3.

**4. Flavin-, vanadate- or heme-regeneration**

In this section we will show some examples about efforts developed during the last years to recycle the prosthetic group of nicotinamide-independent enzymes. In some enzymatic oxidations with flavoenzymes, the flavin cofactor can be regenerated by molecular oxygen. This would be the most convenient method for synthetic applications, but it has a main drawback which is the formation of hydrogen peroxide that in many cases drastically decreases both enzyme activity and stability. An approach that can be used to overcome this problem is the addition of a catalase to remove $H_2O_2$, but this biocatalyst is also unstable in its presence. Therefore, electrochemistry could be considered as a good alternative to perform these transformations under anaerobic conditions (Figure 12).

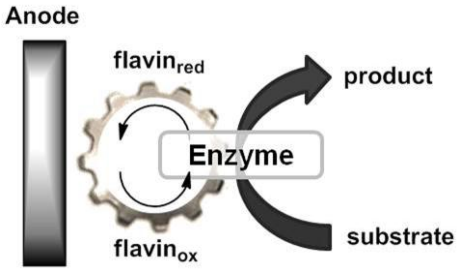



**Figure 12.** Direct electrochemical regeneration of the flavin in enzyme-catalyzed oxidations.

The direct anodic flavin regeneration, that would be the easiest option, has scarcely been employed [106] since the large size of these biomolecules and, in most cases, the tight binding to the active site of the protein hampers a quick electron transfer. Instead, chemical mediators have more often been employed to achieve a more convenient electrochemical process. In this sense, ferrocene derivatives have been successfully used as mediators in several hydroxylase [107] and oxidase-catalyzed [108,109] reactions. Recently, the oxidation of formaldehyde catalyzed by an alcohol oxidase from *Hansenula polymorpha* was mediated by different organic compounds like toluidine blue, methylene blue, 2,6-

dichlorophenolindo-phenol, and *p*-benzoquinone. The last one proved to be the most efficient artificial electron acceptor. For its regeneration a gold electrode was utilized. The poor solubility of benzoquinone in aqueous solutions allowed its immobilization on the surface of the working electrode.[110]

In the last few years laccases have attracted some interest as "coupled enzyme" for flavin cofactor regeneration. These copper-dependent oxidative enzymes [111] reduce one molecule of molecular oxygen into two molecules of water with the concomitant oxidation of four substrate molecules to produce four radicals. Therefore, the application of these enzymes to recycle the flavin prosthetic group of oxidases or dehydrogenases by mediation of a chemical substance (*laccase-mediator system*, LMS) is recently finding some interesting applications. Another advantage of this setup is its homogeneous character, which minimizes diffusion limitations often observed for other systems.

The first examples of LMS-catalyzed oxidations were presented by Haltrich and co-workers,[112,113] where cellobiose dehydrogenase (CDH) from *Athelia rolfsii* or pyranose oxidase (P2O) from *Trametes multicolor* were employed to oxidize lactose to lactobionic acid and glucose into 2-keto-glucose, respectively. Several laccases were used as biocatalysts and different redox mediators were studied such as substituted and unsubstituted *ortho*- and *para*-quinones, benzoquinone imines or ABTS. With this setup, total turnover numbers for the two enzymes utilized were in the range of $10^5$-$10^6$. Due to the formation of hydrogen peroxide in the CDH- or P2O-catalyzed processes, catalase was present in the medium to eliminate it. In a subsequent development of this methodology, various aldoses were oxidized to the corresponding 2-ketosugars by co-immobilization on an acrylic carrier of P2O, laccase and catalase with 1,4-benzoquinone as mediator. Under these conditions, the laccase regeneration system worked efficiently obtaining a higher specific productivity than for the homogeneous system even though oxygen was rate limiting.[114] It has been shown that in the CDH-catalyzed oxidation of lactose to lactobionic acid *via* LMS, the bubble-free oxygenation process was successful to avoid gas/liquid interface inactivation. Furthermore, it was discovered that the oxidized ABTS redox mediator form played a key role in the inactivation mechanism of these biocatalysts.[115] Very recently, in order to demonstrate the applicability of this setup, the oxidation of lactose to lactobionic acid catalyzed by CDH and laccase from *Trametes pubescens* mediated by ABTS, was scaled up in a 20-L membrane-aerated reactor eliminating the high gas/liquid interfacial area harmful for the enzymes, achieving a high space-time yield (74.4 g $L^{-1}$ $d^{-1}$).[116]

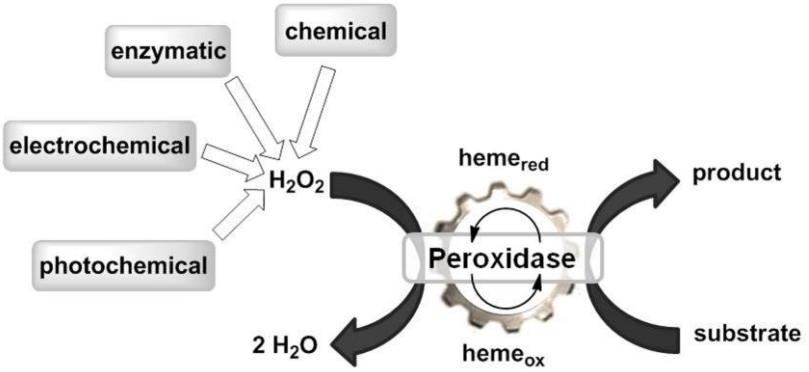



**Figure 13.** Regeneration of the prosthetic group in peroxidase-catalyzed reactions forming *in situ* hydrogen peroxide.

Vanadate- or heme-peroxidases can use hydrogen peroxide instead of molecular oxygen to oxidize their prosthetic group.[117] Unfortunately, many peroxidases are also rather labile towards $H_2O_2$ due to oxidative degradation of the prosthetic group in its presence, especially in the case of heme peroxidases due to the instability of the porphyrin unit. In the last few years, trying to avoid high concentrations of hydrogen peroxide in the reaction medium, a series of enzymatic, electrochemical, chemical, and photochemical approaches have been proposed to achieve the *in situ* catalytic formation of $H_2O_2$ through reduction of dissolved oxygen (Figure 13).

The first methods applied to *in situ* generate catalytic amounts of hydrogen peroxide to carry out peroxidase-catalyzed reactions were enzymatic. Since the first report presented by Therisod and co-workers in 2000,[118] where glucose oxidase from *Aspergillus niger* was used with glucose to generate $H_2O_2$ that was employed by peroxidase from *Coprinus cinereus* to perform stereoselective sulfoxidations, several successful applications have appeared employing this system,[119,120] D-amino acid oxidase [121] or alcohol oxidase from *Pichia pastoris* [122] with peroxidase from *Coprinus cinereus*, glucose oxidase and horseradish peroxidase,[123] and glucose oxidase with chloroperoxidase (CPO) from *Caldariomyces fumago*.[124]

The electrochemical cathodic reduction of $O_2$ to generate $H_2O_2$ has usually been performed using mercury, gold or carbon electrodes. In this sense, CPO has been the most studied biocatalyst due to its high versatility and selectivity to achieve oxidations, although its lability with hydrogen peroxide is also known. Several contributions have appeared studying the generation of $H_2O_2$ with electrodes using this biocatalyst. For instance, it was applied to the stereoselective oxidation of several sulfides to obtain the corresponding chiral sulfoxides such as (*R*)-methyl *p*-tolylsulfoxide or (*R*)-methoxyphenyl methyl sulfoxide in a batch process in the presence of a carbon felt cathode.[125] Later, in a three-dimensional electrolysis cell the productivity for the oxidation of thioanisole could be highly improved (104 g $L^{-1}$ $d^{-1}$).[126] Ionic liquids (ILs) have also been shown to be appropriate solvents to carry out this transformation. These compounds are promising candidates applied to electroenzymatic systems since they entirely consist of ions, have outstanding electrochemical properties, have shown to be biocompatible in some cases and depending on their properties they can solubilize a broad range of substrates. Lütz and co-workers applied different imidazolium and piridinium-based ILs as additives in

CPO-catalyzed sulfoxidation of thioanisole, generating the hydrogen peroxide necessary to complete the reaction by means of a graphite electrode. In the presence of even 10% v $v^{-1}$ of the ionic liquid, the half-life time of CPO improved in many cases, and since thioanisole was more soluble in the reaction medium, the enzymatic conversion was higher.[109]

Regarding chemical approaches, $[Cp^*Rh(bpy)(H_2O)]^{2+}$ with formate was used for controlled *in situ* provision of hydrogen peroxide to obtain an improved cytochrome c-catalyzed sulfoxidation of thioanisole.[127] Another approach was the generation of $H_2O_2$ directly from $H_2$ and $O_2$ using Pd(0)-catalysts for the asymmetric sulfoxidation of thioanisole catalyzed by CPO. In this case, supercritical carbon dioxide ($scCO_2$) was used as medium showing the potential of compartmentalization of catalytic processes in multiphase systems.[128] Recently, a light-driven *in situ* generation of hydrogen peroxide has been described. In this case, FMN was employed as photosensitizer with visible light in a similar system than the previously described in Section 2.4. The flavin derivative was recycled with a simple and cheap electron donor such as EDTA or formate. This setup was applied to several CPO-catalyzed oxidative transformations demonstrating high robustness.[129]

## 5. Summary and Outlook

The wide range of oxidative reactions catalyzed by oxidoreductases comprises the selective oxidation of hydroxyl and carbonyl groups, primary and secondary amines, hydrocarbons, heteroatoms, and double bonds. Despite their unique advantages compared to other non-enzymatic catalysts, their cofactor-dependency has appeared as one of the main challenges to apply these biocatalyzed processes at high scale. Therefore, taking into account the elevated cost of the majority of cofactors, the *in situ* regeneration of a catalytic amount of these derivatives has to be applied in order to design a feasible and scalable process. In this sense, the development of novel systems for cofactor regeneration is in line with the increased applicability of oxidoreductases in preparative biocatalysis. Traditionally, whole-cell systems due to their intrinsic ability to recycle the biological cofactor, or coupled-enzyme approaches employing a second thermodynamically favored biocatalyzed reaction were successfully used to overcome this issue. In the last few years, the recent advances in Molecular Biology and Biochemistry have increased the number of enzymes available to perform a designed transformation and improved the efficiency of these processes by, *e.g.* co-expression of both (oxidative and recycling) biocatalysts in a single host cell or the development of self-sufficient redox enzymes.

More recently, other methodologies have arisen as potential candidates to regenerate the nicotinamide coenzymes or the flavin/heme prosthetic groups in biocatalyzed oxidative processes. The scope of achieving simpler and more robust reaction schemes justifies further developments in this area. Thus, the simplest way to regenerate the oxidized or reduced form of these cofactors would be the employment of electrochemical methods, and although some impressive results have already been obtained improving the mediator performance and the reactor cell design, the productivity of electroenzymatic processes is still low and must be enhanced for biotechnological purposes. Acceleration of the electrochemical reaction

rate is in principle relatively easy by a smart reactor design with a large electrode surface/volume ratio, but still more work must be done in this direction. Furthermore, the use of chemical mediators, photosensitizers, cofactor mimics and hybrid catalysts has also appeared in the last years as potential alternatives to regenerate/replace cofactors. Again, these strategies are very interesting but require more development to achieve the productivity obtained with the enzymatic counterparts.

It is expected that these novel methodologies will be implemented in multistep chemical strategies to avoid the tedious purification and isolation techniques that consume time and decrease the overall process yield, or in the design of novel systems like deracemization of racemic compounds or coupling of two productive reactions. Overall, it can be concluded that although there is still room for improvement concerning the *cofactor challenge*, it is not possible to deny that the great efforts made during the last years by (in)organic and engineering chemists, biochemists, and (micro)biologists are minimizing this issue at levels that were not envisaged some time ago.

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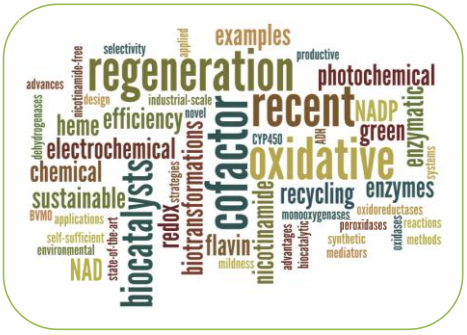
**Graphical Abstract**




**Recent Advances in Cofactor Regeneration Systems Applied to Biocatalyzed Oxidative Processes**

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Recent enzymatic and (electro/photo)chemical strategies to recycle the expensive cofactors in biocatalyzed oxidative processes will be reviewed herein