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Recent developments in pyridine nucleotide regeneration

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NAD(P)-dependent oxidoreductases are valuable tools for the synthesis of chiral compounds. Due to the high cost of the pyridine cofactors, *in situ* cofactor regeneration is required for preparative applications. In recent years, existing regeneration methodologies have been improved and new approaches have been devised. These include the use of newly discovered dehydrogenases that are stable in high contents of organic solvent and novel enzymes that can regenerate either the reduced or oxidized forms of the cofactor. The use of electrochemical methods has allowed cofactor regeneration for monooxygenases and natural or engineered whole-cell systems provide alternatives to approaches relying on purified enzymes.

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Abbreviations

ADH	alcohol dehydrogenase
CLEC	cross-linked enzyme crystal
DH	dehydrogenase
FDH	formate dehydrogenase
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
STH	soluble pyridine nucleotide transhydrolyase
TTN	total turnover number
UF	ultrafiltration

Introduction

The interest in using enzymatic transformations in industrial processes continues to grow very rapidly [1,2,3,4]. Among the enzymes for potential application towards synthetic chemistry are the oxidoreductases that catalyze the asymmetric reduction of carbonyl groups to alcohols and amines or promote the oxygenation of C–H bonds [5,6]. Most of the enzymes in this class require pyridine nucleotide cofactors for catalysis. Given the cost of NAD(P)⁺ and NAD(P)H, their stoichiometric use is economically not feasible and hence various *in situ* regenera-

tion methods have been devised that allow the use of catalytic quantities [7,8]. In addition to reducing the cost of stereoselective enzymatic transformations, cofactor regeneration simplifies product isolation, prevents problems of product inhibition by the cofactor, and can drive thermodynamically unfavorable transformations by coupling to favorable regeneration reactions.

The effectiveness of an *in situ* cofactor regeneration process is typically measured in terms of turnover numbers (TN, defined as the number of moles of product formed per mole of cofactor per unit time) and total turnover numbers (TTN, defined as the number of moles of product formed per mole of cofactor during the course of a complete reaction) of the cofactor [9]. TTNs of 10³ to 10⁵ may be sufficient to make a process economically viable depending on the value of the reaction product. This review focuses primarily on developments of new or improved technologies since 2000. The numerous reports in which traditional means of cofactor regeneration were used are not covered here.

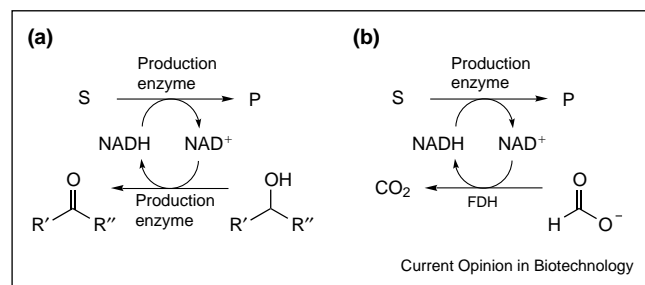
Enzymatic regeneration of NADH

The reduced forms of the pyridine nucleotide cofactors are more expensive and less stable than the oxidized forms. Regeneration of NADH has been extensively investigated for the preparative use of alcohol dehydrogenases (ADHs), amino acid dehydrogenases, and hydroxy acid dehydrogenases [10,11]. Conceptually, the most advantageous approach would involve regeneration of NADH by the production enzyme itself using a cheap sacrificial cosubstrate, typically 2-propanol or ethanol (Figure 1a). In this scheme, the oxidation of the auxiliary alcohol is coupled to reduction of a carbonyl group, a process that has similarities to the Meerwein-Ponndorf-Verley reduction in organic chemistry. To overcome thermodynamic limitations, typically high concentrations of the auxiliary alcohol are required to drive the equilibrium towards the desired product, which unfortunately tends to inactivate the production enzyme.

In recent years, several dehydrogenases (DHs) have been reported that can operate at reasonably high alcohol concentrations. In one study, halogenated propargylic ketones were reduced with high enantioselectivities in the presence (25%) of a short-chain alcohol by horse liver ADH (HLADH) and *Lactobacillus brevis* ADH [12]. The enzymes were still >80% active after 15 h in ethanol and 2-propanol, respectively, and TTNs for the cofactor reached up to 20 000.

An intriguing recent technological advance features the use of cross-linked enzyme crystals (CLECs) in which the

Figure 1



Enzymatic methods for regeneration of NADH. **(a)** Coupled-substrate regeneration of NADH uses the same enzyme that converts substrate (S) to product (P) for cofactor recycling. **(b)** Coupled-enzyme regeneration utilizes an auxiliary enzyme such as FDH to recycle the reduced cofactor.

cofactor was present during crystallization. HLADH-NADH-CLEC preparations achieved about 90% of the activity of the soluble enzyme, and remarkably retained full activity after three months [13[•]]. The half-life of the two-component CLEC in 25% 2-propanol at 40°C was determined to be more than 4 days, and a TTN of >12 000 was obtained in a 15 mmol scale reduction of cinnamaldehyde. Another recently reported two-component CLEC combined lactate dehydrogenase and lipoamide dehydrogenase for electrochemical cofactor regeneration [14]. These preliminary findings are very encouraging and undoubtedly this area will be a fertile ground for further improvements and scale-up.

The successful use of the production enzyme for cofactor regeneration discussed above is still relatively rare, and by far the most common solution involves utilization of a second DH with high specificity for a sacrificial substrate that is not used by the production enzyme (Figure 1b). Formate dehydrogenase (FDH) from *Candida boidinii* is still the gold standard in this area and is the only method discussed in this review that is used on an industrial scale (in the production of *L-tert*-leucine at Degussa [15,16]). The large-scale use of enzymatic regeneration systems typically involves continuous ultrafiltration (UF) membrane reactors in which the cofactor is tethered to a large macromolecule, usually polyethylene glycol; both the enzymes and cofactors are retained because their sizes are larger than the molecular weight cut-off of the UF membrane. Other types of reactors have been reviewed elsewhere [8,10]. The advantages of FDH involve the use of formate as an innocuous substrate and the production of CO₂, which renders the process essentially irreversible providing a strong driving force for NADH regeneration. Disadvantages of FDH are its relatively low specific activity (~6 U mg⁻¹) [17], its cost of production, and its general sensitivity to organic solvents preventing the use of substrates with low aqueous solubility (for a review of regeneration methods in organic media, see

[18]). The latter impediment was recently overcome by the use of biphasic mixtures containing 10–20% (v/v) of *n*-hexane in the asymmetric reduction of ketones by ADH from *Rhodococcus erythropolis* [19]. The stability of FDH under these conditions was still high (>90%) after 65+ h, and substrate concentrations up to 100 mM allowed a much improved volumetric productivity. A similar high activity of FDH in hexane was reported in cell-free biotransformations with 2-hydroxybiphenyl 3-monooxygenase (HbpA) [20]. The use of biphasic emulsions not only allows use of hydrophobic substrates and products but can also alleviate product inhibition and facilitate product isolation. In a separate study, the mutation of cysteine residues that were implicated in deactivation processes occurring under biotransformation conditions have improved the stability of FDH [17].

A recently discovered new enzyme, phosphite dehydrogenase [21,22], shows much promise for NADH regeneration. The enzyme catalyzes the NAD-dependent oxidation of phosphite to phosphate, a reaction that is strongly thermodynamically driven ($\Delta G^0 = -15$ kcal mol⁻¹ at pH 7). The specific activity of the enzyme is ~6 U mg⁻¹ with a *K_m* of ~50 μM for both NAD⁺ and phosphite [22]. A preliminary study shows its use with several DHs [23^{••}], but its scope and utility under large-scale production conditions is still undetermined. Efforts to optimize the enzyme and its use are ongoing in the laboratories of the authors. An interesting application of this phosphite dehydrogenase features the production of isotopically labeled products. Deuterium- or tritium-labeled phosphite is economically accessible from labeled water sources, and it has been shown that the label is stereospecifically transferred to NAD⁺ [21]. Use of isotopically labeled phosphite during NADH regeneration has been shown to efficiently generate labeled products in high isotopic purity [23^{••}].

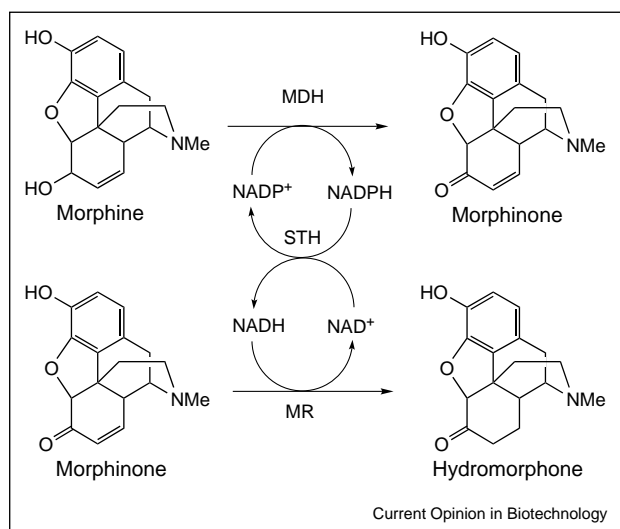
Enzymatic regeneration of NADPH

NADP-dependent enzymes are less common than their NAD-dependent counterparts by a factor of about four [1]. Cofactor regeneration for these enzymes has focused on two general approaches. In the first strategy, NADPH is regenerated using ADH from *Thermoanaerobium brockii*, glucose-6-phosphate dehydrogenase or an engineered FDH that can accept NADP⁺ instead of its natural substrate NAD⁺ [24]. The second general approach involves engineering of the production enzyme such that it can utilize NAD⁺. The latter approach has the advantage that the less costly and more stable NAD⁺ cofactor can be used.

An interesting recent development involves the use of soluble pyridine nucleotide transhydrogenase (STH), which catalyzes the transfer of reducing equivalents between NAD(P)⁺ and NAD(P)H (Equation 1).



Figure 2



Recycling of both pyridine nucleotide cofactors in the production of hydromorphone from morphine. MDH, morphine dehydrogenase; MR, morphinone reductase; STH, soluble pyridine nucleotide transhydroxylase. (Figure adapted from [25**] with permission.)

Biocatalytic production of the potent analgesic hydromorphone from morphine requires both an NADP^+ -dependent morphine dehydrogenase (MDH) and an NADH -dependent morphinone reductase (MR). In a carefully fine-tuned cell-free system, the reducing equivalents produced in the first step were efficiently transferred to NAD^+ by using STH as cycling catalyst to produce hydromorphone in up to 84% yield (Figure 2) [25**]. This strategy is a variant of substrate-coupled regeneration [26]. The general utility of STH for NADPH regeneration by coupling its activity with, for instance, FDH has yet to be explored. As described in a later section, many applications that rely on NADPH are conducted with whole-cell preparations instead of purified enzymes.

Enzymatic regeneration of NAD(P)^+

Whereas regeneration of the reduced forms of the pyridine nucleotide cofactors has been extensively investigated, recycling of NAD(P)^+ has been less well developed. The motivation for NAD(P)^+ regeneration stems from the potential to either use DHs to resolve racemic mixtures of chiral alcohols and amines by oxidizing one of the two enantiomers or to synthesize ketones that are difficult to prepare chemically. This strategy would be particularly useful to access isomers having non-physiological configurations (e.g. for the production of D-amino acids with L-amino acid dehydrogenases). Regeneration of NADP^+ from NADPH can be achieved using the glutamate dehydrogenase catalyzed reduction of α -ketoglutarate to L-glutamate, and NAD can be recycled from NADH by using NADH oxidases. The

latter method has the distinct disadvantage, however, of producing H_2O_2 which damages most proteins, hence catalase must be added.

Two recent studies have provided a potentially attractive alternative [27*,28*]. Certain NADH oxidases generate water as the reduced product according to Equation 2, a process that involves an enzyme-bound flavine adenine dinucleotide (FAD) and a redox-active cysteine residue in the enzyme's active site [29].



The enzymes from *Lactobacillus sanfranciscensis* and *Borrelia burgdorferi* were recently cloned and heterologously expressed in *Escherichia coli* [27*]. The former enzyme accepts NADPH with about 30% of the activity detected with NADH ($V_{max} = 39 \text{ U mg}^{-1}$), and hence may be used for regeneration of both cofactors. The first example of using an NADH oxidase in NAD^+ regeneration was recently demonstrated with the protein from *Lactobacillus brevis* in the resolution of *rac*-1-phenylethanol with ADH from *L. brevis* resulting in the isolation of enantiomerically pure *S*-isomer [28*]. Unlike methods that use ADH, lactate dehydrogenase or glutamate dehydrogenase for NAD(P)^+ regeneration, this method does not generate by-products, but the oxygen sensitivity of the protein is currently still a disadvantage.

Electrochemical regeneration of pyridine nucleotide cofactors

Electrochemical recycling of either the reduced or oxidized forms of pyridine nucleotide cofactors has long been acknowledged as a potentially powerful technology as it would not require a second enzyme and cosubstrate, and hence not lead to the production of by-products. Obstacles for the effective use of this approach include the necessity of high overpotentials with direct oxidation or reduction of the cofactor, electrode fouling, dimerization of the cofactor, and the fact that only enzyme in the immediate vicinity of the electrode will be productive [10]. These problems have been successfully addressed for the development of electrochemical biosensors, but extension to preparative electrochemical reactors has been challenging because of costs related to scale-up and the necessity of larger electrode surface areas. Attempts to prevent electrode fouling and the use of high overpotentials have focused on surface modifications [30], and several methods have been explored to retain the enzyme and/or cofactor close to the working electrode including electrode deposition [8,14,31,32] and membranes surrounding the electrode [33]. In the area of so-called membrane electrochemical reactors, dialysis and UF membranes have been compared with the latter proving more efficient [33]. The overpotentials required were significantly reduced in this and other studies by the use of low molecular weight mediators. A large number of

mediators have been examined with viologens, anthraquinones and $[\text{Cp}^*\text{Rh}(\text{bpy})\text{Cl}]\text{Cl}$ receiving much attention. Viologens require the enzyme diaphorase (lipoamide dehydrogenase) to reduce NAD(P)^+ , obviating some of the advantages of using electrocatalysis presented above. Furthermore, molecular oxygen is incompatible with this system precluding its use in combination with monooxygenases. The rhodium complex originally developed by Steckhan and coworkers [34] was recently used in the first example of electrochemical NADH regeneration for a monooxygenase reaction, the *ortho*-hydroxylation of several α -substituted phenols by HbpA [35]. Despite some inhibition of the NADH regeneration by molecular oxygen, the substrate for the enzyme, the overall electrochemical process had a productivity rate about half of that with FDH as regeneration system [36]. The mechanism of reduction of NAD^+ by the reduced rhodium complex has been studied in detail. A hydridorhodium species is believed to carry out selective 1,4-hydride transfer to the nicotinamide in a process that shows saturation kinetics [37,38]. The K_m for NAD(P)^+ was determined to be around $9\ \mu\text{M}$, allowing comparable concentrations to those used for enzymatic NAD(P)H regeneration. A current drawback in comparison with, for instance, FDH lies in the low turnover rate of $36\ \text{h}^{-1}$ compared with $\sim 8000\ \text{h}^{-1}$. Nevertheless, the methodology has some attractive features, including the capability to recycle either NADH or NADPH and the use of chemical reductants such as formate to generate the hydridorhodium species [34].

Regeneration of NAD(P)H with whole-cell catalysts

Monooxygenases have great potential for asymmetric hydroxylations and epoxidations as well as hydroxylations at non-activated positions for which currently no synthetic organic catalysts exist [1,39]. Although several notable examples of the use of monooxygenases in cell-free systems using enzymatic cofactor regeneration have been reported in recent years [36,40–44], most uses thus far involve either metabolically active or non-growing cells of bacterial or yeast origin [1,45,46]. Similarly, many applications of dehydrogenases use whole cells, which either have intrinsically high activities and stereoselectivities or have been genetically engineered to contain the desired enzymes [5]. These applications circumvent laborious protein purification steps and, when growing cells are used, take advantage of the cellular machinery for NAD(P)H recycling from cheap feed stocks like glucose. The natural NAD(P)H regeneration rate is sufficient for oxygenase/dehydrogenase activities of about $100\ \text{U g}^{-1}$ dry weight, but may become limiting at higher activities [46]. In the latter scenario, or if resting cells are used, simultaneous overexpression of production and regeneration enzymes has been applied or purified cofactor regeneration enzymes have been added to whole-cell preparations [47].

Disadvantages of using whole cells include high biomass waste, lower enzymatic activity per unit dry weight, inhibition of cell growth by substrate or product, and degradation of the product such as overoxidation. Specifics of some of these limitations have been reviewed [46]. Discussion of all reports in which NAD(P)H was regenerated by the central metabolism of whole cells is beyond the scope of this review. We instead focus on examples where exogenous enzymes or cosubstrates were added to whole-cell processes.

A *sec*-ADH from *Rhodococcus ruber* DSM 44541 was shown to be relatively resistant to inactivation by 2-propanol and acetone [48]. In this report lyophilized whole cells were used for the asymmetric reduction of sulcatone to (*S*)-sulcatonol in 30% (v/v) 2-propanol with the enzymatic activity displaying a half-life of 35 h. Interestingly, the system could also be used for the oxidation of racemic secondary alcohols. Several alcohols were resolved with high selectivities using acetone (20%) as the sacrificial oxidant to regenerate the oxidized form of the cofactor [49]. These results are remarkable given the typical problems of cosubstrate and product inhibition that are usually encountered in these type of processes and the reactivity of acetone which tends to inactivate enzymes. A similar approach was used for the asymmetric reduction of various arylketones and 2-alkanones by phenylacetaldehyde reductase from *Corynebacterium* strain ST-10 [50]. NADH regeneration was achieved by the addition of up to 7% 2-propanol.

Conclusions

Many different avenues for recycling pyridine nucleotide cofactors have been devised, each with its own advantages and disadvantages. The strategy of choice will much depend on the stability of the production enzymes towards organic solvents, the level of enzymatic activity in whole cells, and the effect of substrate and product on enzyme activity and cell survival. At present, the most profitable methods are the use of FDH for NADH regeneration, for cases in which purified enzymes are used, and the use of whole cells when conditions are favorable. Yeast ADH has proven more suitable than FDH in some applications. NADPH regeneration is best carried out in whole cells; when purified enzymes are used for medium- to large-scale processes, engineered FDH that can accept NADP^+ is preferred over glucose-6-phosphate dehydrogenase given the expense of its substrate glucose-6-phosphate. Electrochemical methods are continuing to be explored and new developments described in this review hold much promise. Most of these methods will require investigation of scale-up and performance in reactor settings. At present, no general solution for the regeneration of the oxidized forms of pyridine nucleotides has been developed, but the water-producing NAD(P)H oxidases will probably see much attention in the near future as will the use of CLECs.

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
 - of outstanding interest
1. Faber K: *Biotransformations in Organic Chemistry - a Textbook*, edn 4. Berlin: Springer-Verlag; 2000.
 2. Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, Witholt B:
 - **Industrial biocatalysis today and tomorrow.** *Nature* 2001, **409**:258-268.
 This review presents an excellent overview of the current status and the emerging frontiers in biocatalysis from an industrial perspective.
 3. Koeller KM, Wong CH: **Enzymes for chemical synthesis.** *Nature* 2001, **409**:232-240.
 4. Zhao H, Chockalingam K, Chen Z: **Directed evolution of enzymes and pathways for industrial biocatalysis.** *Curr Opin Biotechnol* 2002, **13**:104-110.
 5. Stewart JD: **Dehydrogenases and transaminases in asymmetric synthesis.** *Curr Opin Chem Biol* 2001, **5**:120-129.
 6. Li Z, van Beilen JB, Duetz WA, Schmid A, de Raadt A, Griengl H, Witholt B: **Oxidative biotransformations using oxygenases.** *Curr Opin Chem Biol* 2002, **6**:136-144.
 7. Chenault HK, Simon ES, Whitesides GM: **Cofactor regeneration for enzyme-catalyzed synthesis.** *Biotechnol Genet Eng Rev* 1988, **6**:221-270.
 8. Leonida MD: **Redox enzymes used in chiral syntheses coupled to coenzyme regeneration.** *Curr Med Chem* 2001, **8**:345-369.
 9. Chenault HK, Whitesides GM: **Regeneration of nicotinamide cofactors for use in organic synthesis.** *Appl Biochem Biotechnol* 1987, **14**:147-197.
 10. Devaux-Basséguy R, Bergel A, Comtat M: **Potential applications of NAD(P)-dependent oxidoreductases in synthesis — a survey.** *Enzyme Microb Technol* 1997, **20**:248-258.
 11. Hummel W: **Large-scale applications of NAD(P)-dependent oxidoreductases: recent developments.** *Trends Biotechnol* 1999, **17**:487-492.
 12. Schubert T, Hummel W, Muller M: **Highly enantioselective preparation of multifunctionalized propargylic building blocks.** *Angew Chem Int Ed Engl* 2002, **41**:634-637.
 13. St Clair N, Wang YF, Margolin AL: **Cofactor-bound cross-linked enzyme crystals (CLEC) of alcohol dehydrogenase.**
 - *Angew Chem Int Ed Engl* 2000, **39**:380-383.
 This paper describes the use of CLECs to stabilize both the dehydrogenase enzyme and the cofactor in a nicotinamide cofactor regeneration system.
 14. Leonida MD, Fry AJ, Sobolov SB, Bartoszko-Malik A: **Two-enzyme cross-linked crystals for chiral synthesis coupled with electroenzymatic regeneration of the cofactor.** *Intl J Biochromat* 2001, **6**:207-217.
 15. Bommarius AS, Drauz K, Hummel W, Kula MR, Wandrey C: **Some new developments in reductive amination with cofactor regeneration.** *Biocatalysis* 1994, **10**:37-47.
 16. Bommarius AS, Schwarm M, Drauz K: **Biocatalysis to amino acid-based chiral pharmaceuticals — examples and perspectives.** *J Mol Catal B Enzym* 1998, **5**:1-11.
 17. Slusarczyk H, Felber S, Kula M-R, Pohl M: **Stabilization of NAD-dependent formate dehydrogenase from *Candida boidinii* by site-directed mutagenesis of cysteine residues.** *Eur J Biochem* 2000, **267**:1280-1289.
 18. Adlercreutz P: **Cofactor regeneration in biocatalysis in organic media.** *Biocat Biotransform* 1996, **14**:1-30.
 19. Groeger H, Hummel W, Buchholz S, Drauz K, Nguyen TV, Rollmann C, Huesken H, Abokitse K: **Practical asymmetric enzymatic reduction through discovery of a dehydrogenase-compatible biphasic reaction media.** *Org Lett* 2003, **5**:173-176.
 20. Schmid A, Vereyken I, Held M, Witholt B: **Preparative regio- and chemoselective functionalization of hydrocarbons catalyzed by cell-free preparations of 2-hydroxybiphenyl 3-monoxygenase.** *J Mol Catal B Enzym* 2001, **11**:455-462.
 21. Vrtis JM, White A, Metcalf WW, van der Donk WA: **Phosphite dehydrogenase: an unusual phosphoryl transfer reaction.** *J Am Chem Soc* 2001, **123**:2672-2673.
 22. Costas AM, White AK, Metcalf WW: **Purification and characterization of a novel phosphorus-oxidizing enzyme from *Pseudomonas stutzeri* WM88.** *J Biol Chem* 2001, **276**:17429-17436.
 23. Vrtis JM, White A, Metcalf WW, van der Donk WA: **Phosphite dehydrogenase, a new versatile cofactor regeneration enzyme.**
 - *Angew Chem Int Ed Engl* 2002, **41**:3257-3259.
 This study presents a new method for regenerating reduced nicotinamide cofactors based on a recently discovered enzyme, phosphite dehydrogenase. Because of the intrinsic large thermodynamic driving force, the innocuous nature of phosphite and phosphate to the enzymes, and the low cost of phosphite, this phosphite–phosphite dehydrogenase system may prove complementary to the most widely used formate–formate dehydrogenase system.
 24. Tishkov VI, Galkin AG, Fedorchuk VV, Savitsky PA, Rojkova AM, Gieren H, Kula MR: **Pilot scale production and isolation of recombinant NAD⁺- and NADP⁺-specific formate dehydrogenases.** *Biotechnol Bioeng* 1999, **64**:187-193.
 25. Boonstra B, Rathbone DA, French CE, Walker EH, Bruce NC:
 - **Cofactor regeneration by a soluble pyridine nucleotide transhydrogenase for biological production of hydromorphone.** *Appl Environ Microbiol* 2000, **66**:5161-5166.
 This article describes the use of an STH from *Pseudomonas fluorescens* for the regeneration of the nicotinamide cofactors NAD and NADP in the synthesis of hydromorphone in both cell-free and whole-cell systems. STH can transfer reducing equivalents between NAD(H) and NADP(H).
 26. Hummel W, Kula MR: **Dehydrogenases for the synthesis of chiral compounds.** *Eur J Biochem* 1989, **184**:1-13.
 27. Riebel BR, Gibbs PR, Wellborn WB, Bommarius AS: **Cofactor regeneration of NAD⁺ from NADH: novel water-forming NADH oxidases.** *Adv Synth Catal* 2002, **344**:1156-1168.
 - Two novel water-forming NADH oxidase genes were cloned and over-expressed in *E. coli*. These two enzymes can regenerate NAD⁺ from NADH by reducing oxygen directly to water. One of them can also regenerate NADP⁺ from NADPH with slightly reduced activity.
 28. Geueke B, Riebel B, Hummel W: **NADH oxidase from *Lactobacillus brevis*: a new catalyst for the regeneration of NAD.** *Enzyme Microb Technol* 2003, **32**:205-211.
 - This study presents the first example of using an NADH oxidase to regenerate NAD⁺ from NADH in which the NADH oxidase was coupled with an NAD-dependent ADH to produce enantiomerically pure alcohol from a racemic mixture.
 29. Claiborne A, Mallett TC, Yeh JI, Luba J, Parsonage D: **Structural, redox, and mechanistic parameters for cysteine-sulfenic acid function in catalysis and regulation.** *Adv Protein Chem* 2001, **58**:215-276.
 30. Délécouls-Servat K, Bergel A, Basséguy R: **Surface-modified electrodes for NADH oxidation in oxidoreductase-catalysed synthesis.** *J Appl Electrochem* 2001, **31**:1095-1101.
 31. Manjón A, Obón JM, Casanova P, Fernández VM, Ilborra JL: **Increased activity of glucose dehydrogenase co-immobilized with a redox mediator in a bioreactor with electrochemical NAD⁺ regeneration.** *Biotechnol Lett* 2002, **24**:1227-1232.
 32. Suye S, Aramoto Y, Nakamura M, Tabata I, Sakakibara M: **Electrochemical reduction of immobilized NAD⁺ on a polymer modified electrode with a co-polymerized.** *Enzyme Microb Technol* 2002, **30**:139-144.
 33. Délécouls-Servat K, Basséguy R, Bergel A: **Membrane electrochemical reactor (MER): application to NADH regeneration for ADH-catalysed synthesis.** *Chem Engin Sci* 2002, **57**:4633-4642.

34. Westerhausen D, Herrmann S, Hummel W, Steckhan E: **Formate-driven, nonenzymic NAD(P)H regeneration in the alcohol dehydrogenase-catalyzed stereoselective reduction of 4-phenyl-2-butanone.** *Angew Chem Int Ed Engl* 1992, **31**:1529-1531.
35. Hollmann F, Schmid A, Steckhan E: **The first synthetic application of a monooxygenase employing indirect electrochemical NADH regeneration.** *Angew Chem Int Ed Engl* 2001, **40**:169-171.
This paper presents the first example of the use of $[\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})]^{2+}$ for cofactor regeneration with monooxygenases. This Rh complex exhibits high stability and activity over a broad range of pH and temperature. Moreover, both cheap electrical power and chemicals such as formate or alcohols can be used as the source of reducing equivalents. Compared with other *in vitro* enzymatic cofactor regeneration systems, the turnover numbers of this method are somewhat lower.
36. Lutz J, Mozhaev VV, Khmelnitsky YL, Witholt B, Schmid A: **Preparative application of 2-hydroxybiphenyl 3-monooxygenase with enzymatic cofactor regeneration in organic-aqueous reaction media.** *J Mol Catal B Enzym* 2002, **19**:177-187.
37. Lo HC, Leiva C, Buriac O, Kerr JB, Olmstead MM, Fish RH: **Bioorganometallic chemistry. 13. Regioselective reduction of NAD^+ models, 1-benzylnicotinamide triflate and β -nicotinamide ribose-5'-methyl phosphate, with *in situ* generated $[\text{Cp}^*\text{Rh}(\text{Bpy})\text{H}]^+$: structure-activity relationships, kinetics, and mechanistic aspects in the formation of the 1,4-NADH derivatives.** *Inorg Chem* 2001, **40**:6705-6716.
38. Hollmann F, Witholt B, Schmid A: **$[\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})]^{2+}$: a versatile tool for efficient and non-enzymatic regeneration of nicotinamide and flavin coenzymes.** *J Mol Catal B Enzym* 2002, **19**:167-176.
39. Holland HL, Weber HK: **Enzymatic hydroxylation reactions.** *Curr Opin Biotechnol* 2000, **11**:547-553.
40. Rissom S, Schwarz-Linek U, Vogel M, Tishkov VI, Kragl U: **Synthesis of chiral ϵ -lactones in a two-enzyme system of cyclohexanone mono-oxygenase and formate dehydrogenase with integrated bubble-free aeration.** *Tetrahedron Asymmetry* 1997, **8**:2523-2526.
41. Schwarz-Linek U, Krodell A, Ludwig FA, Schulze A, Rissom S, Kragl U, Tishkov VI, Vogel M: **Synthesis of natural product precursors by Baeyer-Villiger oxidation with cyclohexanone monooxygenase from *Acinetobacter*.** *Synthesis* 2001:947-951.
42. Jennewein S, Rithner CD, Williams RM, Croteau RB: **Taxol biosynthesis: Taxane 13 α -hydroxylase is a cytochrome P450-dependent monooxygenase.** *Proc Natl Acad Sci USA* 2001, **98**:13595-13600.
43. Zambianchi F, Pasta P, Carrea G, Colonna S, Gaggero N, Woodley JM: **Use of isolated cyclohexanone monooxygenase from recombinant *Escherichia coli* as a biocatalyst for Baeyer-Villiger and sulfide oxidations.** *Biotechnol Bioeng* 2002, **78**:489-496.
44. Colonna S, Pironti V, Pasta P, Zambianchi F: **Oxidation of amines catalyzed by cyclohexanone monooxygenase.** *Tetrahedron Lett* 2003, **44**:869-871.
45. Endo T, Koizumi S: **Microbial conversion with cofactor regeneration using genetically engineered bacteria.** *Adv Synth Cat* 2001, **343**:521-526.
46. Duetz WA, van Beilen JB, Witholt B: **Using proteins in their natural environment: potential and limitations of microbial whole-cell hydroxylations in applied biocatalysis.** *Curr Opin Biotechnol* 2001, **12**:419-425.
This review gives an excellent overview of the field of whole-cell catalysis for hydroxylation reactions.
47. Kataoka M, Yamamoto K, Kawabata H, Wada M, Kita K, Yanase H, Shimizu S: **Stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by *Escherichia coli* transformant cells coexpressing the aldehyde reductase and glucose dehydrogenase genes.** *Appl Microbiol Biotechnol* 1999, **51**:486-490.
48. Stampfer W, Kosjek B, Moitzi C, Kroutil W, Faber K: **Biocatalytic asymmetric hydrogen transfer.** *Angew Chem Int Ed Engl* 2002, **41**:1014-1017.
This paper reports the first-time use of lyophilized whole cells of *Rhodococcus ruber* DSM 44541 to produce secondary alcohols using acetone as a cosubstrate.
49. Stampfer W, Kosjek B, Kroutil W, Faber K: **On the organic solvent and thermostability of the biocatalytic redox system of *Rhodococcus ruber* DSM 44541.** *Biotechnol Bioeng* 2003, **81**:865-869.
50. Itoh N, Matsuda M, Mabuchi M, Dairi T, Wang J: **Chiral alcohol production by NADH-dependent phenylacetaldehyde reductase coupled with *in situ* regeneration of NADH.** *Eur J Biochem* 2002, **269**:2394-2402.