REVIEW ARTICLE

Cofactor regeneration – an important aspect of biocatalysis

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Biocatalysis has made tremendous advances in the field of synthesis of industrially important products and intermediates. Cofactors are an important part of many enzymes which are involved in biocatalysis. These cofactors are expensive and stoichiometric additions are not economically feasible. This necessitates the *in situ* cofactor regeneration in biocatalytic processes. Various methods of regeneration of NAD(H)/ NADP(H), an important class of cofactors, have been reviewed in this article. We discuss their salient features, suitability for current bioprocesses, drawbacks and scope of improvement.

Keywords: Biocatalysis, biotransformation, cofactor regeneration, enzymes.

BIOCATALYSIS, the use of microbial cells or isolated enzymes, has made significant progress from the time enzymes were discovered to the current scenario where it is used in major industrial processes[1–10]. Many classes of enzymes such as acylases, amidases, hydrolases, cellulases, etc. are used to carry out reactions involved in the production of antibiotics, herbicides, fuel alcohols and pharmaceutical intermediates[11].

The advance of biocatalysis has been possible because of three specific advantages of enzymes over the traditional chemical catalysts. First, enzymes have naturally evolved to carry out reactions at low temperature and pressure conditions hence subverting the need for high temperature and pressure, which are essential for most of the other catalysts. A classic example is the conversion of nitrogen to ammonia in biological systems, in contrast to the well-known Haber–Bosch process that involves high pressure and temperature. From an industrial perspective, mild reaction conditions imply significant savings in energy cost.

The second and probably the most important advantage of biocatalysts over chemical catalysts is that they are fully chemo-, regio- and stereoselective. Therefore where side products or multiple isomers of a single product are formed in chemical catalysts-based reactions, biocatalysts have the potential of producing only the desired product with very high selectivity[12,13]. Such high selectivity is of obvious interest to the pharmaceutical sector, from the point of view of synthesis of chiral intermediates or end-products. As shown in Figure 1, using the enzyme pyruvate decarboxylase (PDC), (R)-phenyl acetyl carbinol (a pharmaceutical intermediate) could be obtained with an enantiomeric excess (ee) of 98% (ref. 14).

Another example is the industrial production of L-tertiary leucine, from trimethyl pyruvate and ammonium formate (Figure 2). L-leucine and its derivatives are important chiral building blocks for the pharmaceutical industry. It acts as a precursor for the production of antiviral drugs (Sandoz, Biomega Abbott), antitumour agents (Zeneca), anti-inflammatory, anti-HIV (Atazanavir), etc. The biocatalytic production of these compounds has been achieved both by isolated enzymes and genetically designed microbial cells[15,16].

The third advantage of biocatalytic processes is their environment-friendliness. In chemical processes based on soluble metal complexes (i.e. homogeneous catalysts), heavy metal toxicity and the use of volatile organic solvents are often issues of concern. Most enzymes utilized in biocatalytic processes use water as the reaction medium. The negative impact on the environment because of accumulation of heavy metal residues and the use of organic solvents is therefore completely eliminated.

Further, the ability to produce large amounts of enzymes such as cellulases, proteases and lipases through engineered organisms has made the economics of use of these enzymes for many industrial processes practically feasible. Biological route is being integrated into processes which were earlier completely dependent on the chemical route for synthesis of many important compounds. For example, Lonza has developed a biological route to produce 6-hydroxynicotinic acid from niacin which is used in the synthesis of insecticides. This method is more cost-effective than the chemical synthesis route used earlier[8,17].

Many of the reactions catalysed by these industrially important enzymes are redox reactions. Gain or loss of electrons with or without the accompaniment of proton transfer, i.e. redox reactions, is ubiquitous in biological systems. Many of the enzymes involved in these reactions have cofactors which are the actual active sites for electron transfer. In cofactor-dependent redox reactions, the enzymes themselves are not modified; the cofactors are

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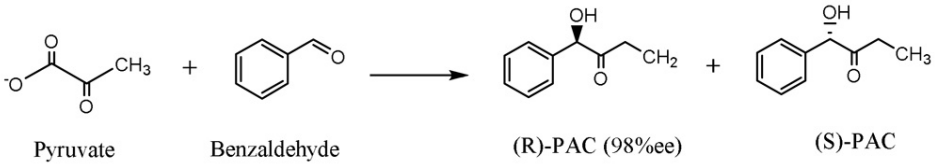

Figure 1. Production of (R)-phenyl acetyl carbinol (PAC) in enatiomeric excess.

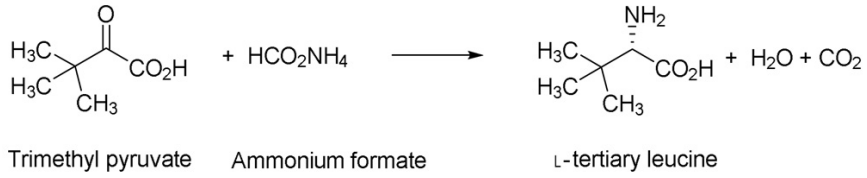

Figure 2. L-tertiary leucine production.

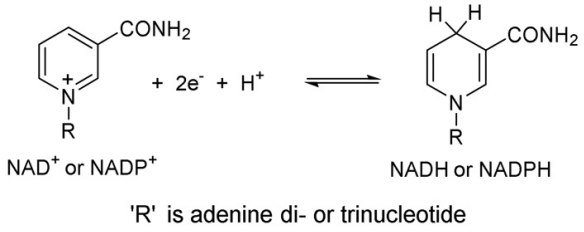

Figure 3. Interconversion of NAD/NADP and NADH/NADPH.

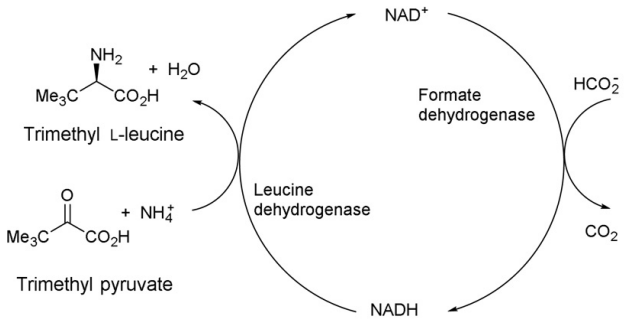

Figure 4. Industrial manufacture of trimethyl L-leucine using two enzymes.

reduced or oxidized. Thus for the enzyme to be practically useful by catalysing multiple rounds of the same reaction, the correct form of the cofactor must be available. In living systems the correct balance between the oxidized and reduced cofactor is maintained by an intricate network of coupled redox reactions.

A large number of redox active enzymes use two nicotinamide-containing cofactor-based redox couples. These are NAD^+/NADH and $NADP^+$/NADPH (collectively abbreviated hereafter as $NAD(P)^+$/NAD(P)H). Both of these have the same nicotinamide fragment and an adenine di- or trinucleotide tail. As shown in Figure 3 for NAD(P)/NADP(H), electron transfers accompanied by proton transfer take place selectively at the 4-position of the nicotinamide ring.

In this article we deal with only those redox reactions that involve the nicotinamide cofactors. This is because $NAD(P)^+$/NAD(P)H redox couples are numerous in biology, and most of the cofactor regeneration reports are based on these two cofactors[18,19].

In NAD^+/NADH or $NADP^+$/NADPH-dependent biological reactions, many turnovers of the enzymes are possible only if an efficient cofactor regeneration pathway is available. As NADH and NADPH are expensive chemicals (cost 10^6 and 10^5 USD/mol respectively)[20] providing them in stoichiometric quantities in the reaction medium is not economically feasible. Hence the need for the cofactor regeneration methods.

As an example, the enzyme-based industrial process for the manufacture of trimethyl L-leucine may be considered. As shown by the left-hand side reaction of Figure 4, leucine dehydrogenase catalyses the reaction between trimethyl pyruvic acid, ammonium ion and NADH. In this reaction trimethyl L-leucine is produced and NADH is oxidized to NAD^+. As shown by the right-hand side reaction of Figure 4, a second enzyme, formate dehydrogenase is also used to catalyse the reduction of NAD^+ by formate ions to regenerate NADH necessary for further round of catalysis.

Syntheses of many other pharmaceutical intermediates or final products have been successfully achieved by this strategy. Antiviral drugs such as Atazanavir[21] and antimalarial drug component amorphadiene[22] (Figure 5) are two important examples where cofactor regeneration has been shown to lead to enhancement of their production.

A good cofactor regeneration system should have a large total turnover number (TTN). In the context of cofactor regeneration-based catalysis, the definition of turnover is the total number of moles of product formed per mole of cofactor[23]. To be of practical use, the cofactor regeneration system must be compatible with the reaction

conditions under which biocatalysis are carried out. The product isolation should not be complicated by the regeneration system. It should be economical and practically feasible.

As shown in Figure 6, the strategies reported for cofactor regeneration are many. Over the last three decades, attention has shifted away from electrochemical, chemical or isolated enzyme-based methods, to the use of genetically modified designer microbes. We explore the advantages and disadvantages of these systems in the following sections.

Direct electrochemical method

In direct electrochemical methods for the reduction or oxidation of $NAD(P)^+$ or NAD(P)H, the electrodes provide or accept the electrons directly. In case of reduction, the proton(s) are supplied by the aqueous environment, whereas in direct oxidation, NADH or NADPH donates electrons at the anode and is converted to NAD^+ or $NADP^+$. As high potentials are involved, this method can be used only for substrates that give oxidation products that are stable towards further oxidation[24].

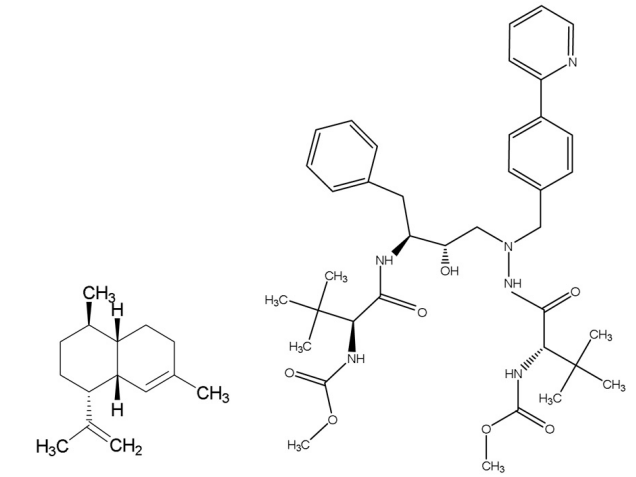

Figure 5. Two pharmaceuticals syntheses which involve cofactor regeneration.

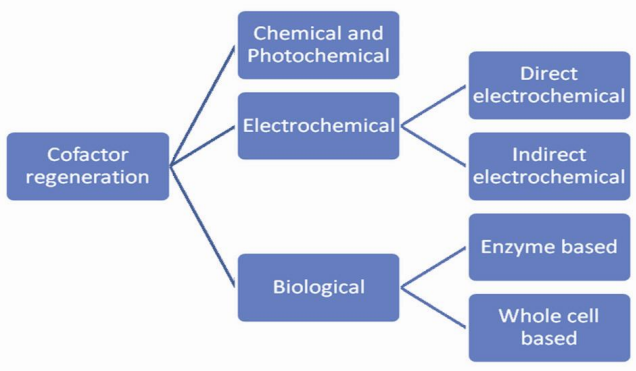

Figure 6. Various methods of cofactor regeneration.

Direct electrochemical oxidation has been used for the conversion of glucose to gluconate by the enzyme glucose dehydrogenase (GDH). In the stoichiometric oxidation of glucose to gluconate by GDH, NAD^+ was reduced to NADH. By direct electrochemical oxidation, NAD^+ was regenerated, and a turnover number greater than 10^4 was obtained[25]. Metal salts of gluconate are used in various industrial processes and the process of conversion of glucose to gluconate has been patented[26]. Some other examples of direct oxidation have been listed in Table 1.

Over the last several decades, the electron transfer processes of $NAD(P)^+$/NAD(P)H have been much studied[19,27]. It is generally accepted that the direct electrochemical reduction of $NAD(P)^+$ is a two-step process in which the first electron is transferred from the cathode to form a free radical, which then accepts a proton. The cation then accepts a second electron to give NAD(P)H[28]. Examples and references of the work involving direct electrochemical reduction are listed in Table 1.

Direct electrochemical reduction suffers from certain disadvantages. Due to the formation of NAD(P) free radical, NADP dimers (kinetics of this inactive product is faster than the active product formation kinetics) and 1,6 $NADP^+$ are formed instead of the required form, i.e. 1,4 $NAD(P)^+$ (ref. 29), which leads to the wastage of usable cofactor. This necessitates keeping the cofactor concentration low so as to avoid the formation of radicals and dimers.

Other drawbacks of direct electrochemical regeneration include the need for high over potentials and electrode fouling[30]. It has been observed that for effective cofactor regeneration, the enzyme molecules need to be in the vicinity of the electrode[31]. In spite of these drawbacks direct electrochemical reduction has been successfully used in certain cases such as the conversion of α-ketoglutarate to L-glutamate using glutamate dehydrogenase from bovine liver with 100% conversion and 3300 turnovers[32].

Indirect electrochemical method

As shown in Figure 7, in indirect electrochemical methods a redox active organic or organometallic molecule is used as a mediator. The mediator molecules act as electron carriers and facilitate the kinetics of the electron transfer process. In other words, the use of such mediators reduces the kinetic barrier, i.e. high over potentials.

The use of the organometallic mediator $[Cp^*Rh(bpy) \times (H_2O)]^{2+}$ (Cp^* = pentamethyl cyclopentadienyl, bp = 2, 2′-bipyridine) for the regeneration of NADH is well established. Some examples of use of such mediators are listed in Table 2.

Apart from the mediators described above, a second enzyme in combination with viologens has also been used for cofactor regeneration[33,34]. Bayer Corporation has

Table 1. Examples of direct electrochemical methods for cofactor regeneration

Reaction	Electrode/potential	Turnover number	Comments	Reference
Glucose to gluconate (GDH) NADH to NAD	Reticulated non-microporous vitreous carbon/800 mV	>10,000	99.99% efficient	24
2-Propanol to acetone (ADH) NADH to NAD	Tin oxide/–500 mV	ND	–	82
Pyruvate to lactate (LDH) NADH to NAD	Cholesterol-modified gold amalgam electrode/–1400 mV	1400	–	83
NAD to NADH	Ruthenium-modified glassy carbon electrode/–576 mV	ND	96% yield	84
DL-lipoamide to dihydrolipoamide (lipoamide dehydrogenase) NAD to NADH	Platinum and nickel nano-patterned carbon nanofibres/–1500 mV	ND	99.3% yield	85
α-Ketoglutarate to glutamate (glutamate dehydrogenase) NAD to NADH	Platinum electrodes in presence of vanadia–silica xerogels	3300	Conversion rate increased from 30% to 100%	32

ND, Not determined.

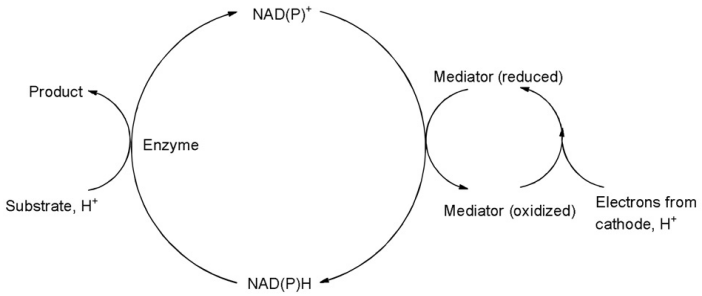

Figure 7. Indirect electrochemical method for NAD(P)H regeneration.

patented 9H-acridin-2-one and 11H-dibenz-[b,f][1,4]-oxazepin-8-one as efficient mediators for the electrochemical regeneration of cofactors[35].

Prevention of electrode fouling and reduction of high over potentials have also been attempted by the use of modified electrodes. Examples are: meldola blue modified graphite electrode, hexacyanoferrate modified nickel electrodes and 3,4-dihydroxy benzaldehyde modified glassy carbon electrode[36].

A related approach is the immobilization of the cofactor-containing enzyme on the electrode. The enzyme molecules on the surface of the electrodes have a higher probability of electron transfer than the ones that are far away. Consequently, better kinetics of cofactor regeneration is expected[37]. However, industrial feasibility of any of the approaches based on modified electrodes remains to be proven.

Chemical and photochemical methods for cofactor regeneration

Chemical and photochemical methods are similar in approach and for this reason they are discussed together. Chemical methods have so far been limited to regeneration of NAD(P)H. As shown in Figure 8, dihydrogen (H_2) rather than an electrode (cathode) is the final electron donor. The electrons are carried to the cofactors by one or more mediators. A few organometallic complexes are known to activate dihydrogen to give protons. Not surprisingly, these are the same ones that have been investigated as mediators for indirect electrochemical regeneration so far. Due to the insolubility and instability of many enzymes and cofactors in organic solvents, the overall reaction must be carried out in water. In other words either the organometallic mediator must be soluble in water, or some other method must be found for electron transport in a biphasic system.

Thus, water-soluble $[RuCl_2(TPPTS)_2]_2$ (TPPTS = triphenyl phosphine trisulfonated) has been used to catalyse the transfer of electron from H_2 gas to $NAD(P)^+$. In conjugation with the $NADP^+$-dependent alcohol dehydrogenase from *Thermoanaerobium brockii*[38], this catalytic system has been shown to be effective for stereoselective reduction of ketones. In the same study, rhodium complexes such as $[Cp^*Rh(bpy)(H_2O)]^{2+}$ have also been used for similar purposes.

A biphasic system involving water and a water-immiscible organic solvent such as dichloromethane was used for the catalysis of pyruvate to L-lactate by lactate dehydrogenase. For dihydrogen oxidation, a platinum carbonyl cluster was used. A redox active dye, 3,7-diamino-2,8-dimethyl-5-phenylphenazinium was the electron carrier across the phase boundary[39].

Various photosensitive compounds such as Sn(II)-meso-tetramethylpyridinium porphyrin, Sn-$TMPyP^{4+}$, $Ru(bpy)_3^{2+}$, acridine dyes, methylene blue and *N*-methyl phenazonium methyl sulphate, P-doped TiO_2 nanoparticles, etc. undergo facile electron transfer reactions in

Table 2. Examples of indirect electrochemical methods for cofactor regeneration

Reaction	Electrode potential (mV)	Mediators	Turnover number	Comments	Reference
Acetophenone to (R)-phenyl ethanol (ADH) NADP to NADPH	688	$[Cp^*Rh(bpy)(H_2O)]_2^+$	35	ee >99.9%	86
Monohydroxyphenyl compounds to 2,3-dihydroxyphenyl derivatives (monooxygenase) NAD to NADH	650–800	[CpRh(I)(bpy)H]Cl	ND	–	87
Meso-diol – chiral lactones (ADH) NADH to NAD	585	2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate)	30	Yield 93.5%, ee >99.5%	88

ND, Not determined.

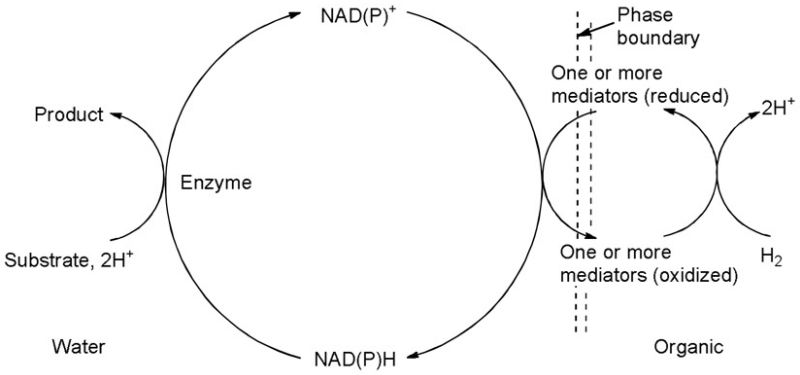

Figure 8. Regeneration of NAD(P)H by chemical methods. For a biphasic system more than one mediator is used.

their excited states[40–43]. They have been investigated for cofactor regeneration, but to the best of our knowledge none of them has been coupled with industrial processes.

Enzymatic cofactor regeneration

As already seen, biological method of cofactor regeneration includes the use of a second enzyme (see Figure 4). In theory, due to the law of microscopic reversibility the production enzyme itself can regenerate the cofactor with the use of cheap substrates such as ethanol and 2-propanol. However, thermodynamically for such a process to be feasible, large amounts of these substrates are needed which can inhibit the production of enzyme[31]. In spite of this, there are examples where this approach has been successful. Halogenated propargylic ketones were reduced with high enantioselectivity by horse liver alcohol dehydrogenase (HLADH) using ethanol with a turnover number of 20,000 (ref. 44). Another example of such an approach is the enantioselective synthesis of (2S)-2-phenylpropanol and (2S)-2-(4-iso-butylphenyl) propanol ((S)-Ibuprofenol) using (HLADH) and ethanol[45].

The use of a second enzyme is still the more prevalent option. Enzymatic cofactor regeneration could be *in vitro* or *in vivo*, i.e. in genetically modified microbial cells (Figure 9). In the following sections we describe both the methods.

In vitro cofactor regeneration

Formate dehydrogenase is one of the first enzymes used for NADH regeneration. Shaked and Whitesides[46] reported the use of formate dehydrogenase from *Candida boidinii* for the conversion of NAD^+ to NADH using formate as substrate. The product in this case is CO_2, which is removed from the system easily. Also, the reaction has a large positive entropy change which is thermodynamically favourable. Formate dehydrogenase has the disadvantage of low specific activity (4–6 U/mg protein)[47]. Nevertheless, this enzyme has been patented[48], and is used in the industrial-scale production of L-trimethyl leucine at Degussa[2,15,48].

There are several other reports on the use of a second enzyme for cofactor regeneration in a variety of enzyme-catalysed reactions. Thus glucose dehydrogenase[49], glucose-6-phosphate dehydrogenase[50], alcohol dehydrogenase[51], hydrogenase[52] and lactate dehydrogenase[53] have all been used for NADH regeneration. These cofactor regeneration methods have certain drawbacks. Glucose dehydrogenase and glucose-6-phosphate dehydrogenase consume the glucose for cofactor regeneration, thus limiting the available glucose for the main reactions which use glucose or glucose-derived substrates.

Many new alternative enzymes such as glutamate dehydrogenase and NADH oxidase are now being explored. Glutamate dehydrogenase catalyses the reaction

Table 3. Summary of the *in vivo* approaches for cofactor regeneration

Target and starting material(s)	*In vivo* modification	Effect on production	Reference
Production of 12-ketochenodeoxycholic acid from cholic acid	Over-expression of ADH to convert NADPH-NADP	NA	89
1,3 PD from glycerol	Over-expression of FDH to convert NAD to NADH	Initially even after the expression of 1,3 PD operon with no enhanced 1.3 PD, which improved after NADH formation	59
Vaniallic acid to vanillin	Over-expression of GDH to NADP-NADPH	Reduction of reaction time from 8 to 6 h	60
Accumulation of pyruvate	Over-expression of NADH oxidase NADH-NAD	Accumulation of pyruvate increased from 0.52 to 0.67 g of pyruvate/g glucose	63
Production of (R)-1-phenylethanol	Single-point mutation in LDH to accept both NAD and NADP	Reaction rate decreased to 5 min in comparison to 210 min	90
Isobutanol production in anaerobic conditions	Affinity of the enzymes was altered for NADH than NADPH	Yield increased from 86% to 100%	63
Hydromorphone production	Over-expression of pyridine nucleotide transhydrogenase PntAB, which converts NADPH to NADH	Yield in consecutive biotransformation increased from negligible to 60%	68
Amorphadiene production	NADH-dependent HMG-CoA reductase from *Delftia acidovorans* instead of NADPH-dependent native enzyme	120% increase in production of amorphadiene	22
Lycopene production	NADP-dependent GAPDH from *Clostridium acetobutylicum* replacing native NAD-dependent enzyme	2.5-fold increase in lycopene production	91
Acetoin production	Expression of NADH oxidase which decreases the availability of NADH for lactate production, thereby directing pyruvate towards acetoin production	Increase in acetoin from 57% to 74% of glucose fermented	70
Ethanol production	Over-expression of nicotinic acid phosphoribosyl transferase for synthesis of NAD	Two-fold increase in ethanol production	72 72
Catechin production	Deletion of glucose-6-phosphate isomerase diverts the flow towards pentose phosphate pathway generating NADPH	Increase in production of catechin from 18.6 to 38.8 mg/l	73

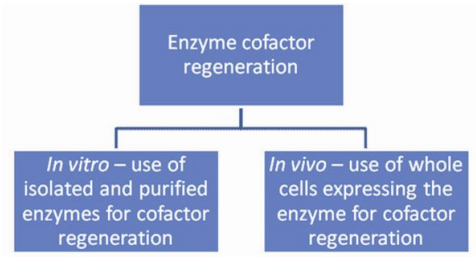

Figure 9. Types of enzyme cofactor regeneration methods.

between glutamate, NAD^+ and water to give α-keto glutarate, NADH and ammonia. NADH oxidase from *Lactobacillus sanfranciscensis* regenerates NAD^+ by oxidizing NADH with a turnover number of >5000 (ref. 54).

The demonstrated industrial viability of cofactor regeneration with a second enzyme has spurred the filing of many patents; examples of these include ADH from *Rhodococcus erythropolis* for the production of chiral organic compounds using formate dehydrogenase/NADH oxidase[55], malate dehydrogenase for cofactor regeneration in a similar process[56], glucose dehydrogenase and malate dehydrogenase for primary alcohol production from aldehydes[57], and the use of formate dehydrogenase for cofactor regeneration in the production of ethyl (3R, 5S)-dihydroxy-6-benzyloxy hexanoate[58]. There is scope for discovery of more enzymes of similar nature.

In vivo cofactor regeneration

The recycling of cofactors to the preferred form is the only method to solve the problem of cofactor availability in *in vitro* processes. Alternative approaches are possible to solve the cofactor availability for *in vivo* processes. Since organisms are naturally adapted to regenerate only the amount of cofactor required for their own cellular processes, various genetic modifications are typically required to ensure sufficient cofactor availability for an *in vivo* biotransformation involving exogenous substrates. These methods could include production of more cofactor, use of alternative cofactor systems and other strategies as will be discussed in detail below.

The turnover number used to calculate the efficiency of a cofactor regeneration method in *in vitro* processes cannot be used for balancing of *in vivo* cofactor systems. A process which involves optimization of cofactors is

considered superior if the yields are closer to the theoretical yield (product obtained in the stoichiometric ratio of substrates) and are obtained in lesser time (higher reaction rates) in comparison to the processes which do not include cofactor optimization. Instead of turnover numbers, the effect of cofactor optimization on the total yield and productivity of a process is used to determine the efficiency of the process.

One straightforward approach that is widely used towards cofactor balancing under *in vivo* conditions is over-expression of the enzymes responsible for cofactor regeneration. As a specific example, the conversion of glycerol to 1, 3 propane diol (1, 3 PD) by *Klebsiella pneumonia* may be considered. Increase in the production of 1, 3 PD was not observed even after over-expression of the key enzymes, due to the non-availability of a sufficient amount of NADH. This problem was resolved after over-expressing the *C. boidinii* NAD^+ formate dehydrogenase which regenerated NADH[59].

Similarly, reduction rates of vaniallic acid to vanillin by aldehyde oxidoreductase (Figure 10) were accelerated by NADPH regeneration which was obtained by over-expressing of glucose dehydrogenase (GDH) of *Bacillus subtilis*[60]. The reaction time decreased from 8 to 6 h. Although the final titres were similar, the decrease in reaction time contributed significantly to the favourable economics of the new process.

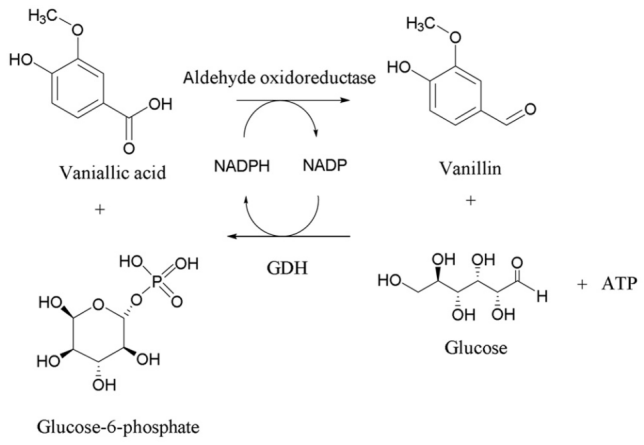

Figure 10. Vanillin production involving NADPH regeneration by glucose dehydrogenase.

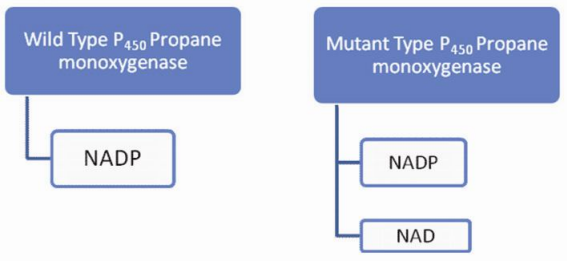

Figure 11. Change in cofactor specification of mutants of P_{450} propane monooxygenase.

Over-expression of water-forming NADH oxidase ($NADH + H^+ + 0.5O_2 = NAD^+ + H_2O$) from *Lactococcus lactis* in a pyruvate decarboxylase (PDC) minus strain of *Saccharomyces cerevisiae* led to accumulation of more pyruvate from glucose (0.67 g pyruvate/g glucose as against 0.52 g pyruvate/g glucose by wild type) by oxidizing the NADH formed during glycolysis[61]. NADH oxidase is one of the promising enzymes for *in vivo* cofactor regeneration as it utilizes oxygen as the substrate and water is the product. One of the ways to increase the availability of cofactors for a process is to engineer enzymes to accept structurally similar coenzymes such as NAD^+ and $NADP^+$ with equal efficiency.

Cytochrome P_{450} monooxygenase is an important class of enzymes which carries out biotransformation involving oxidation of organic compounds. P_{450} propane monooxygenase of *Escherichia coli* is used for the conversion of propane to propanol. A variant of this enzyme was generated by directed evolution so that it could accept NAD and NADP as cofactor with equal efficiency[62], rather than just NADP which is the cofactor for the wild-type enzyme. With this both NAD and NADP metabolic pools could be tapped for this process (Figure 11).

Another method of meeting the cofactor requirement is by changing the dependence of a pathway from one cofactor to another. This is achieved by engineering the enzymes involved in a given pathway. The percentage yield of isobutanol production from glucose increased from 86 to 100 under anaerobic conditions (which is more economical because of lower operational cost and higher theoretical yield) using *E. coli*. This was achieved by the use of engineered (random mutagenesis followed by screening for higher ratios of NADH/NADPH activities) ketol acid reductoisomerase and alcohol dehydrogenase, with higher affinity for NADH over NADPH. The native pathway is dependent on the latter cofactor[63]. It is important to note that *in vivo* NADPH regeneration is an aerobic process. NADPH can be oxidized only by the enzymes of the tricarboxylic acid (TCA) cycle, which operates only in the presence of oxygen. Therefore, an anaerobic process which involves microorganisms expressing the NADPH-dependent enzymes needs alteration of the cofactor specificity of the enzymes to NADH. Similarly, ethanol formation from xylose under anaerobic condition with better yields was obtained by changing the cofactor specificity of the enzymes involved[64,65].

Enzyme engineering is typically achieved after detailed structural and modelling studies on the enzymes. Identification of relevant amino acids is followed by site-directed mutagenesis for targeted changes in these enzymes. The cofactor specification of the enzymes can also be altered by random mutagenesis (exposure to mutagenic reagents) and then using a screening reaction that tests the effectiveness of the variant for cofactor regeneration. An example in case is the conversion of trimethyl pyruvate to L-tert-leucine using genetically modified microbial cells

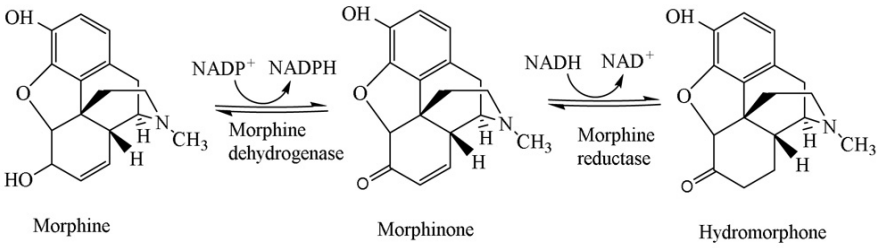

Figure 12. Biotransformation of morphine to hydromorphone.

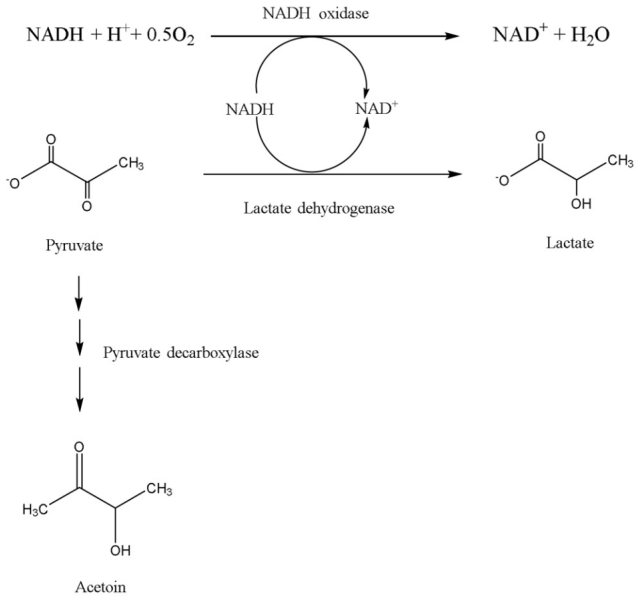

Figure 13. Diversion of flow of pyruvate away from lactate towards acetoin production by over-expression of NADH oxidase.

rather than isolated enzymes. The genetically modified microbial cells were obtained by random mutagenesis followed by screening for phosphite dehydrogenase, which is involved in NADPH generation in *E. coli*[66]. These mutants of phosphate dehydrogenase with relaxed cofactor specificity and increased thermostability have been patented[67].

If the enzyme specificity cannot be changed, then another strategy is over-expression of the enzymes needed for inter-conversion of one cofactor to the other. Over-expression of pyridine nucleotide transhydrogenase PntAB from *Pseudomonas fluorescens* in *E. coli*, which converts NADPH to NADH and vice versa, increased hydromorphone production from morphine. This biotransformation is carried out in consecutive steps involving the same cells by converting excess NADPH generated in one step to NADH required for the following enzymatic step[68] (Figure 12). A patent has been acquired for the use of a strain transformed with PNT[69].

Sometimes changing the cofactor ratio alone (without modulating any other primary enzymatic activity) leads to a desired end-product. Thus instead of an auxiliary step, cofactor ratio alteration in itself is a tool to bring about metabolic changes. This idea has been exemplified by the oxidation of the NADH in *L. lactis* by over-expressing NADH oxidase gene from *Streptococcus mutans* (heterologous expression), which in turn led to the diversion of the flow of pyruvate towards acetoin (Figure 13). In *L. lactis*, lactate dehydrogenase (LDH) converts pyruvate to lactate utilizing NADH in the process. Pyruvate is also converted to acetoin by the action of pyruvate decarboxylase (PDC). If NADH is not available in sufficient quantity, the LDH reaction does not take place and the excess pyruvate is routed through PDC to form acetoin[70].

In another example of changing cofactor levels for altered levels of metabolites, NAD^+ was reduced by *in vivo* over-expression of formate dehydrogenase in *S. cerevisiae*. This resulted in increase in glycerol production from glucose, when this enzyme was over-expressed in cytosol. It also led to an increased production of both glycerol and ethanol when over-expressed in mitochondria. This example demonstrates that compartmentalization of the cofactor also plays a role in the metabolite distribution[71].

Enhancing the quantity of cofactor by over-expressing the enzymes of cofactor biosynthesis has been also explored to overcome the problem of cofactor shortage. Increase in the total levels of NADH pools by over-expression of the enzyme (nicotinic acid phosphoribosyl transferase), required for the synthesis of NAD by salvage pathway led to an increase in ethanol production, particularly during anaerobic cultivation of *E. coli*[72].

Another way to increase the availability of cofactors is to knockout dispensable pathways which utilize a particular cofactor. This makes the cofactor available for the pathway that produces the desired product. Genes for glucose-6-phosphate isomerase (*pgi*), phosphoenolpyruvate carboxylase (*ppc*) and phospholipase A (*pld A*) were deleted to make NADPH available for the production of leucocyanidin and catechin production. The deletion of glucose-6-phosphate isomerase directs the flux through pentose phosphate pathway generating NADPH. These genes were identified by stoichiometry-based modelling studies to optimize the NADPH production without affecting the viability of the strain[73].

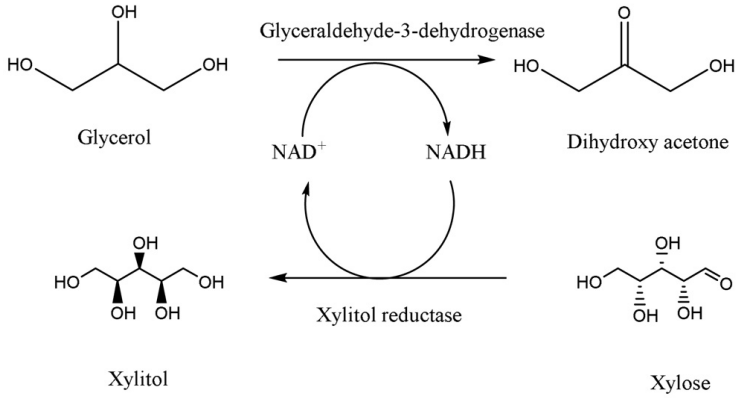

Figure 14. DHA and xylitol being produced from a coupled enzyme system using alternate forms of cofactor.

Finally, it must be noted that strategies based on cofactor regeneration through over-expression of enzymes have an inherent limitation. Living systems maintain a delicate balance of their metabolic aspects. Even small modifications could have deleterious effects on the organism. Hence a proper understanding of the pathways and processes involved with a particular modification is essential for successful changes. This applies to manipulation of cofactor levels too. Most of these cofactors are energy currencies and are involved in maintaining the redox status of the organism; hence changes here have more prominent effect on the metabolism of the organism. For example, when NADH oxidase was over-expressed through high copy number plasmid in a pdc minus strain of *S. cerevisiae* leading to accumulation of pyruvate, it affected the biomass as there was lesser NADH for oxidative phosphorylation and glycerol was produced as a by-product to provide NADH[61]. Therefore, any approach of enzyme and cofactor modification in an organism should take into account the nutrient and energy balance of the organism.

Cofactor regeneration methods in nanotechnology-based applications

Many of the enzymatic reactions are being optimized for operation at nanoscale for biosensors or nanoparticle-based biotransformation[74–78]. In almost all the cases several cycles of enzyme reactions are anticipated from a single batch of enzymes. Hence cofactor regeneration has to be taken into account in these cases.

An example is the use of polymeric nanoparticles-based coupled enzyme systems which utilize the NAD^+/NADH couple. A polymer of methyl methacrylate, ethylene dimethacrylate and methylacrylic acid is used, and two important metabolites, DHA (dihydroxyacetone) from glycerol (catalysed by GDH) and xylitiol from xylose (catalysed by xylitiol reductase) are generated[79] (Figure 14).

A biosensor for the detection of formate was produced using the coupled enzymes formate dehydrogenase and salicylate hydroxylase attached to polyvinyl acetate (PVA) matrix, to detect formate. Formate dehydrogenase converts formate into CO_2 and water-converting NADH to NAD. NADH is regenerated by salicylate hydroxylase using sodium salicylate and oxygen. The oxygen uptake is monitored[80]. A similar method was employed for lactate determination[81]. Several of the enzymes which have been successfully utilized for cofactor regeneration at a large scale are being investigated for deployment as nanoscale sensors and are likely to be commercialized in the near future.

Discussion and conclusion

Biocatalysis is an emerging science which is making rapid inroads into the areas of both bulk and fine chemical synthesis. The specificity and environment-friendly aspects of biocatalysts are responsible for the slow and steady replacement of multistep reagent-based organic synthesis by biocatalysts-based processes. Large investments in terms of money and time are being made to understand the structure and mechanism of enzymes and how they affect the process. The information obtained is used to generate tailor-made enzymes for the process. The recent advances in genomics, proteomics and metabolomics have added to the existing knowledge of biocatalytic processes. Simultaneously, whole-cell biocatalysis is making rapid progress owing to better economics and improvements in fermentation and downstream processing technologies.

Cofactors are an indispensable part of enzymes and cellular processes. Any bioprocess which involves enzymes or whole cells needs to take into account the availability of cofactors. The redox state of a cofactor changes during a reaction and it is essential to bring the cofactor to its original state for the use of enzymes/whole cells for multiple cycles of the reaction. In the absence of cofactor

regeneration to proper extent, the enzyme may not be able to provide the maximum yield. Thus the efficiency of a bioprocess can be increased manifold by addressing the cofactor needs of the process.

Since addition of cofactors is not economically viable, *in situ* regeneration by various chemical, electrochemical and biochemical processes has been applied. As exemplified above electrochemical methods with the involvement of mediators have been successfully applied to many processes and have been patented. The scope of the electrochemical method is however limited by the relatively few electrode and mediator combinations. Also, the assembly and maintenance of apparatus along with high energy requirements have led to the search for alternative methods. Biological cofactor regeneration methods being more compatible with the enzymes/whole-cell process are being increasingly evaluated for cofactor availability. The inexhaustible biological sources give more combinations for cofactor regeneration for a vast range of biological processes. Molecular biology tools can and are being applied to the biological systems to custom-build the enzyme and cofactor required for maximum output from a process.

Metabolic engineering with multi-step and multi-pathway manipulations is rapidly replacing the one-enzyme modification approach. With such large changes in the metabolism of organisms, there is more certainty of cofactor imbalance. Thus any metabolic engineering approach which does not take cofactor balance into account may not deliver the optimum results.

Finally, cofactor availability should be a major target for increasing the efficiency of already established biological processes. It should also be taken into account for designing any new processes to gain the maximum yield of the product and better economics.

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