CHAPTER 8

Almac: An Industrial Perspective of Ene Reductase (ERED) Biocatalysis

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8.1 Introduction

8.1.1 Almac Group

For more than 50 years, Almac has supported the global biopharmaceutical industry with their drug development activities and bringing new drugs to market to advance human health. Its comprehensive solutions range from R&D, biomarker discovery and commercialisation, API manufacture, formulation development, clinical trial supply, IRT (IVRS/IWRS) through to commercial-scale manufacture. The privately owned, global organisation has grown organically over 45 years, now employing in excess of 4500 highly skilled personnel and has a strong reputation for innovation, quality and exceptional customer service. The Group is headquartered in Craigavon, Northern Ireland with additional operations based throughout Europe, USA and Asia.

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Almac Group focuses on five core values; outstanding quality, sustainable financial performance, inspirational people, superlative customer focus and exceptional innovation. We continually strive to identify, design and implement novel and exciting methods of delivering industry-leading solutions which ultimately accelerate the drug development process.

Almac combines the expertise from its range of specialised services to provide bespoke solutions to develop and commercialise drugs through its five complementary business units: Almac Diagnostics (diagnostics and biomarker development); Almac Sciences (API services, chemical development & analytical services); Almac Pharma Services (pharmaceutical development); Almac Clinical Services (clinical supply chain); and Almac Clinical Technologies (trial supply management technology).

8.1.2 Biocatalysis at Almac

Within Almac Sciences, the biocatalysis team consists of computational, molecular and microbiologists, enzymologists, organic chemists and analysts. The group has demonstrated proficiency in gene identification, expression, enzyme evolution, fermentation, enzyme production and biotransformation scale-up. Expertise ranges from enzyme production to the synthesis of complex chiral APIs from mg–tonne scale.

The application of biocatalysts in the pharmaceutical, fragrance and fine chemical industries continues to grow as it moves from the domain of specialised technology to mainstream methodology. This movement has been facilitated by the increasing commercial 'off-the-shelf' availability of biocatalysts. Almac has heavily invested in this area to secure the supply of selectAZyme™ biocatalysts for clients' non-GMP and GMP manufacturing projects. Our exclusive technology platform consists of a number of recombinant enzyme panels that can be used in chemical processing across multiple functional group interchanges with the timelines required to develop these bioprocesses comparable with chemistry scale-up.

Our team is closely linked to the process chemistry and manufacturing chemistry groups, where enzyme discovery and development is integrated with screening and route definition. Each member of the team brings expertise to complex processes procedures and can rapidly implement an enzymatic process to significantly improve the yield and timelines of a multistep synthesis.

8.1.3 The Rise of Biocatalysis

Biocatalysis has risen to become a prominent and mainstream technology in pharmaceutical chemistry.[1–9] Several reasons have been responsible for this including the ability of enzymes to deliver shorter more expedient synthetic routes to sophisticated molecules, with considerably lower environmental burden than traditional chemical approaches. Furthermore, it is often the case now that in addition to lowering toxic solvent and reagent inventories

the introduction of enzyme catalysis can provide cost competitive alternatives to chemical synthesis alone with resultant lower final product cost.

A number of requirements can be identified for enzymes to achieve industrial application. Firstly, a suitable collection of enzymes needs to be readily available so that a screening process can be conducted to identify candidate enzymes to be carried forward for development. Such enzymes need to be available at the required scale and at a suitable cost. Process intensity for biotransformations need to be comparable to chemical processes, with an achievable product concentration in the range of 50–100 g L^{-1} and a biocatalyst loading with respect to substrate input that enables a reasonable enzyme cost contribution to the process. Finally, processes and enzymes need to be unencumbered by intellectual property constraints and demands.

The properties of ene reductases and their applications have been extensively reviewed in the literature.[10–17] This chapter discusses the impact of biocatalysis on a specialised reaction type, namely the reduction of the alkene carbon–carbon double bond, from an industrial perspective. It focuses on the ene reductase enzyme class, which is making rapid progress towards routine industrial application for alkene hydrogenation. In future it is also foreseeable that ene reductases may be used for the reverse reaction, namely desaturation and the formation of an alkene.

8.2 Introduction to Alkene Reduction

The reduction of unsaturated alkenes to their saturated products is of fundamental importance as a chemical reaction type across the chemical and pharmaceutical industries. Many products, especially pharmaceuticals, feature this chemical transformation in their synthesis, and a particular focus has been on reactions that provide chiral products through high yielding asymmetric chemistry.

One approach for saturation of carbon–carbon double bonds is by asymmetric hydrogenation technology, which involves the use of precious metals such as rhodium, ruthenium and iridium in conjunction with chiral phosphines, and where the reducing source is hydrogen gas.[18] This approach requires specialist catalysis expertise, and of course access to the appropriate pressurised hydrogenation equipment.

An alternative approach to alkene reduction is by organocatalysis. The organocatalytic hydrogenation of cyclic enones has been reported, using amino acids to build the chiral organic catalysts, and the Hantzsch ester (a mimic of a nicotinamide enzyme cofactor) as the hydrogen source.[19] Whilst conversions and enantioselectivities were good for the substrates tested, the methodology does require a relatively expensive hydrogen source and catalyst. Furthermore, the catalyst loading was high (20 mol%), and these factors serve to reduce, or perhaps preclude, the industrial viability of this approach.

Both approaches described above have limitations that have stimulated the search for alternative ways in which to reduce the carbon–carbon double bond. Precious metal-based catalysis in particular has been vulnerable to

reliability of source and price volatility of the metals used, and in the pharmaceutical field the presence of residual metal in drug substances has been an on-going concern. Enzymatic approaches have recently emerged as viable alternatives for alkene reduction and these are starting to challenge established chemical methodology. The enzyme class involved is the ene reductases and associated enzymes of the Old Yellow Enzyme family.

8.3 An Introduction to Ene Reductases and How They Work

Ene reductases and the associated Old Yellow Enzyme class have been identified as a class of enzymes that catalyse reactions that are very relevant to current pharmaceutical intermediate synthesis. They perform the asymmetric reduction of the alkene double bond in certain substrate types where an electron withdrawing group is appended to the alkene. The natural function of ene reductases is varied and includes for example response to oxidative stress.[20] In other cases they are involved in the biosynthesis of secondary metabolites such as ergot alkaloids and plant hormones like jasmonic acid.[21,22]

The overall reaction catalysed by the ene reductase family is the NAD(P)H-dependent reduction of activated alkenes (Figure 8.1). The reaction proceeds in two stages: the first stage involves NAD(P)H oxidation by hydride transfer from the nicotinamide cofactor to the ene reductase FMN flavin cofactor (this is the reductive half reaction). In the second stage, hydride transfer from the reduced flavin to the substrate effects the reduction of activated alkenes (this is the oxidative half reaction).

Only carbon–carbon bonds that are electronically activated by a conjugated electron-withdrawing group are reduced and non-activated (isolated)

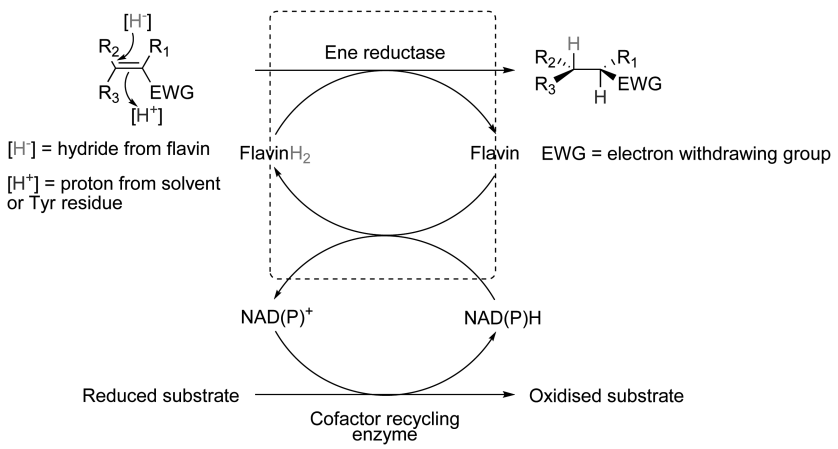


Figure 8.1 General ene reductase reaction.

alkenes are unreactive. Various functional groups may serve as 'activators' including unsaturated carboxaldehydes (enals), ketones (enones), and conjugated nitroalkenes, all making good substrates. Alkenes with appended unsaturated carboxylic acids or esters tend to behave as 'borderline' substrates, as is the case for unsaturated nitriles.

8.4 Examples of Ene Reductase Reactions Reported in the Literature

To demonstrate that ene reductases are receiving serious attention from industrial pharmaceutical process chemistry groups one can cite the work that Pfizer Inc. have published, pertinent to the synthesis of pregabalin.[23,24] This is a γ-aminobutyric acid mimic used in the treatment of CNS disorders such as epilepsy. The key bioreduction utilises an ene reductase cloned into *E. coli*, from *Lycopersicon esculentum*, that can reduce both geometric isomers in high enantiomeric excess as shown in Figure 8.2. The enzyme was considerably faster for the (*E*)-isomer. Both acid and ester were substrates. Preparative scale reduction of the (*E*)-isomer was achieved (>99% ee in 69% yield) using NADPH as the cofactor, with recycling of the cofactor enabled by *Lactobacillus brevis* alcohol dehydrogenase and isopropanol as the hydrogen source. Whilst the authors noted that issues such as enzyme activity and stability still need to be addressed before an effective industrial reduction of the geometric isomer mixture can be realised, reaction and enzyme engineering offers the potential to achieve this.

Porto *et al.*[25] reported for the first time the ene-reduction of aromatic malononitriles with electron withdrawing substituents (Figure 8.3a) by biotransformation using whole cells marine-derived *Penicillium citrinum* CBMAI 1186, with the products obtained in good yields (>93%). With electron donating substituents (Figure 8.3b) the yield of desired products was much lower. It is likely that the OH substituent, which results in a 12% yield, disfavours biohydrogenation. The methoxy substituent resulted in an 84% yield and the OH and OMe functionalised substrate returned a 66% yield. The reduction

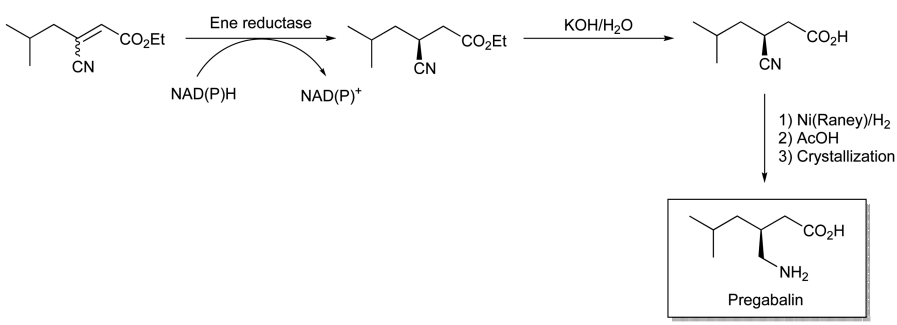


Figure 8.2 Synthesis of pregabalin using an ene reductase.

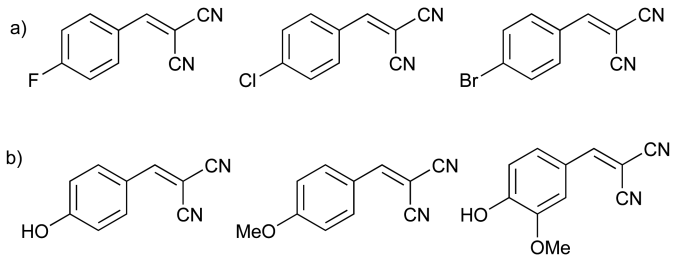


Figure 8.3 Substrates with (a) electron withdrawing and (b) electron donating substituents for bioreduction with CBMAI 1186.

of the C=C bond by CBMAI is shown to depend on the electronic effects promoted by the attached substituents.

Utilising biocatalysts in the synthesis of menthol is highly desirable, as the monoterpenoids are of high commercial value, and mint crop prices can be volatile due to reliance on harvest yields with the process including expensive steam distillation and filtration processes.[26–28] The optimisation strategies for biocatalytic terpenoid synthesis focus on an expression host and the metabolic engineering of a biosynthesis pathway. Scrutton *et al.*[29] have demonstrated a one-pot biotransformation of (1*R*,2*S*,5*R*)-(−)-menthol and (1*S*,2*S*,5*R*)-(+)-neomenthol from pulegone, a commonly found flavour in perfume products. They developed a strategy using recombinant *E. coli* extracts containing the biosynthetic genes for an ene reductase (NtDBR from *Nicotiana piperita*) and two menthone dehydrogenases (MMR and MNMR from *Mentha piperita*) with moderately pure menthol (79.1%) and neomenthol (89.9%) obtained. The advantages of the one-pot strategy are the ability to optimise each enzymatic step and the ability to generate libraries of pure compounds for use in high-throughput screening. Further to this work the authors also pinpointed a mechanistic switch between ketoreduction and ene-reductase activity in the short-chain dehydrogenases/reductases family which could potentially afford ene-reductases from the transformation of SDR ketoreductases.[30]

Characterisation of two OYEs from the genome of *Chryseobacterium* sp. CA49 for their ene reductase activities showed that *Chr*-OYE1 had broad substrate scope (reduced 18 out of 19 substrates studied) and excellent stereoselectivity, while *Chr*-OYE2 had limited activity towards activated alkenes. The mutant *Chr*-OYE2-M183Y displayed improved activity and stereoselectivity (>99%) when compared with the wildtype catalyst.[31]

8.4.1 Ene Reductases as Part of a Reaction Sequence

Whilst still at an early stage, a greater level of sophistication is emerging in the operation of biocatalysis, where more than one biotransformation is performed in sequence. This in effect creates an artificial metabolic pathway, and can take a net conversion of substrate to product well beyond what might be

achieved simply through chemical methods alone. The phrase 'reaction cascade' has been coined to describe such sequences. Ene reductases have been demonstrated as useful players in this emerging technology. As an example, the biotransformation of allylic alcohols into lactones was reported.[32,33] Figure 8.4 shows the scheme for the biotransformation of carveol into a related lactone. The sequence consisted of an oxidation by alcohol dehydrogenase to form an enone, the stereoselective hydrogenation of the alkene by an ene reductase, and finally the lactone formation by a Baeyer–Villiger monooxygenase. Overall, a 60% yield was obtained with an excellent >99% diastereomeric excess. This approach was shown to work for a range of unsaturated alcohols. Furthermore, the three biocatalysts were expressed simultaneously in *E. coli*, so that a single whole biocatalyst was able to perform the illustrated sequence, representing an operationally simple procedure, with the full redox balance of cofactors effected by internal metabolism of the *E. coli* cells.

A second example reported the one-pot conversion of (*R*)- and (*S*)-carvone into carveol.[34] This synthesis utilised the two-enzyme sequence shown in Figure 8.5. In the first step carbon–carbon double bond reduction, ene reductase LacER from *Lactobacillus casei* was used. For the second step, a carbonyl reductase from *Sporobolomyces salmonicolor* (SSCR) or *Candida magnolia* (CMCR) was employed to perform the asymmetric ketone reduction. The aim was to develop a more effective process for the conversion of carvones into

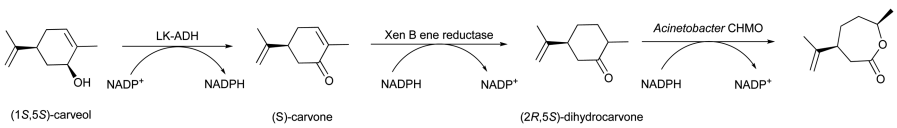


Figure 8.4 Transformation of allylic alcohol into lactone.

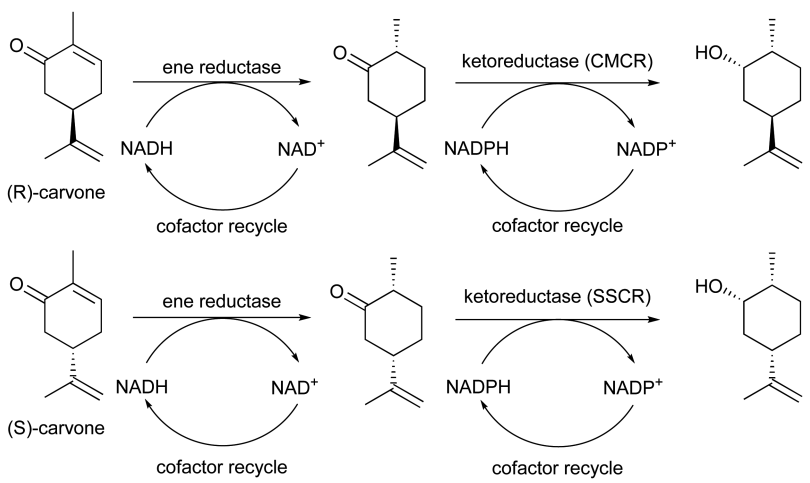


Figure 8.5 Biotransformation of carvone enantiomers.

enantiomerically pure dihydrocarveols, compounds of high interest due to their application as fragrance ingredients. A one-pot process was demonstrated at 0.1 M substrate concentration, yielding in the case of (*R*)-carvone a product with 99% diastereomeric excess, and in the case of (*S*)-carvone 86% de.

A similar approach using an ene reductase followed by alcohol dehydrogenase was described for the conversion of a range of enals and enones into primary and secondary alcohols, respectively.[35] The target alcohols are intermediates for subsequent amine formation in CNS drugs. In the system developed here, for which one example is shown in Figure 8.6, the ene reductase (Old Yellow Enzyme 2) from *Saccharomyces cerevisiae* was cloned into *E. coli* and used in tandem with horse liver alcohol dehydrogenase, giving the product in 89% isolated yield with 99% ee.

The system encompasses an ene-reductase (from the Old Yellow Enzyme family) with an alcohol dehydrogenase (ADH), for the reduction of either α,β-unsaturated aldehyde or ketone to give the alcohol in both high yields and optical purity. Although ene-reductases significantly improve chemoselectivity and conversions, optically pure α-substituted aldehydes can spontaneously racemise under typical biotransformation conditions even at neutral pH (Figure 8.7).[36] Two factors have been shown to minimise the loss of optical purity – using a biphasic system[37,38] or applying the *in situ* substrate feeding product removal (SFPR) technology,[2,39–41] ensuring that the unstable saturated aldehyde is immediately reduced to the more stable alcohol.

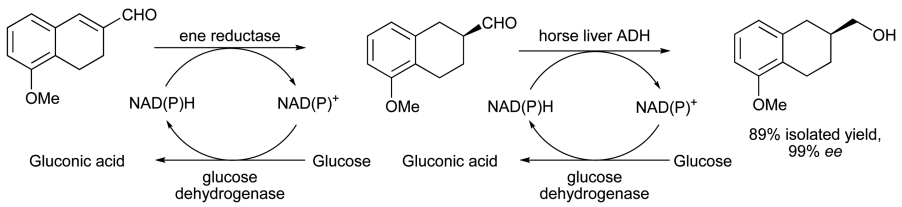


Figure 8.6 Conversion from enal into primary alcohol dual enzyme sequence.

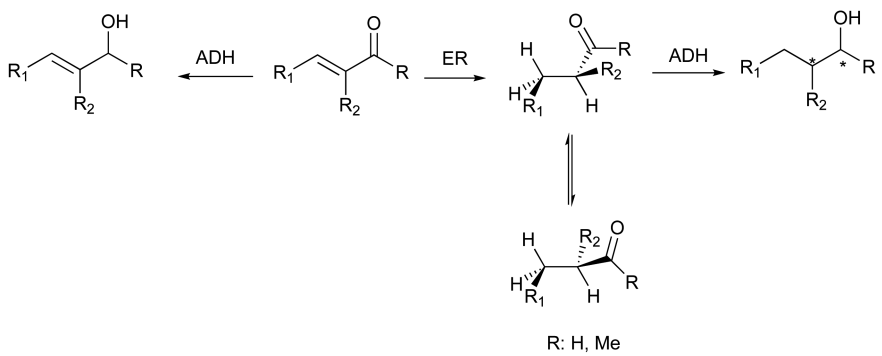


Figure 8.7 Typical chemical path of α,β-unsaturated aldehyde/ketone reductions with isolated enzymes or microorganisms.

Coupled use of ene reductases and ω-transaminases have been shown to result in diastereomerically enriched (*R*)- and (*S*)-amine derivatives in one-pot sequential and cascade processes (Figure 8.8) as demonstrated on a β-substituted cyclic enone.[42] The one-pot synthesis required no modifications to reaction conditions, and a high chemoselectivity of the ω-TAs was shown in the cascade reaction. Ene reductases from the Old Yellow Enzyme family coupled with commercially available ω-TAs gave >99% conversion and de for a variety of α- or β-substituted unsaturated ketones as substrates.

A further example from Hauer *et al.*[43] reports the enzymatic reduction of allylic alcohols, which are normally not directly reduced by enzymes such as Old Yellow Enzymes as they do not contain an electron withdrawing group to activate the C=C bond. It was found that the ene reductase nicotinamide-dependent cyclohex-2-en-1-one reductase (NCR) did not catalyse the reaction but the morphinone reductase (MR) from *Pseudomonas putida* M10 and OYE1 from *Saccharomyces pastorianus* gave good activity towards allylic substrates. The grafting of loop A and loop B regions from OYE1 and MR into the inactive NCR scaffold resulted in active variants for the cascade reduction (Figure 8.9).

Moran *et al.*[44] reported that several α-acetoxymethyl enones participated in bioreduction cascades. The substrate is reduced by an OYE with the loss of the acetoxy group, forming a new enone that is subsequently reduced by an OYE to form an enantiomerically enriched ketone (Figure 8.10). The acetoxy group is an adequate leaving group for the enones investigated containing electron-rich aromatic rings, thus avoiding the hydrolysis that occurs with the α-halomethyl enones. The main biocatalytic cascade pathway for α-acetoxymethyl enones with EREDs is most likely an allylic substitution followed by a hydrogenation.

More recently, Zhao *et al.*[45] report a one-pot sequential chemoenzymatic system for the formation of 2-aryl-succinate derivatives. The sequence consisted of Rh-catalysed diazo-coupling, giving more than 9:1 selectivity for

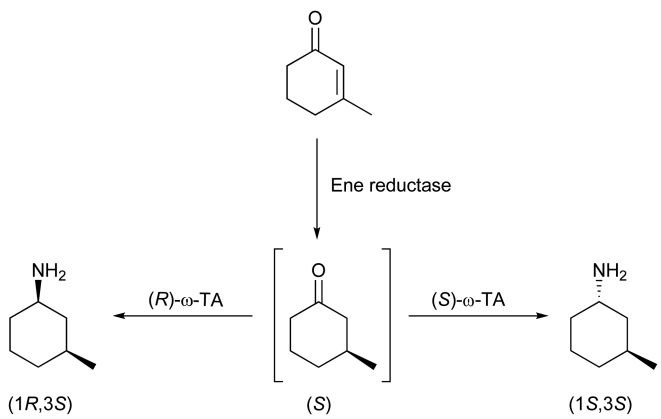


Figure 8.8 Examples of ER/ω-TA cascade reactions.

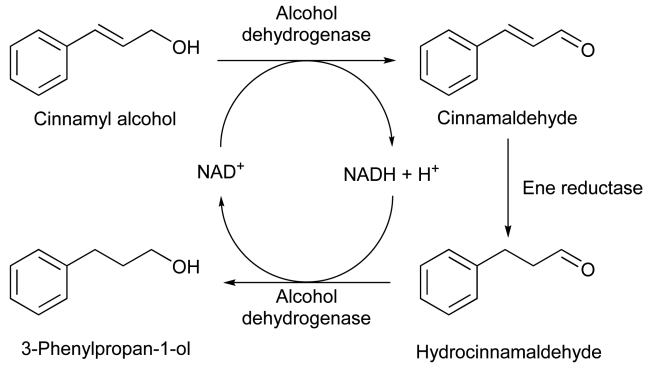


Figure 8.9 Bienzymatic three-step cascade reaction for the reduction of cinnamyl alcohol through the coupling of an alcohol dehydrogenase (ADH) with ene reductase wild-type enzymes and loop-grafted variants.

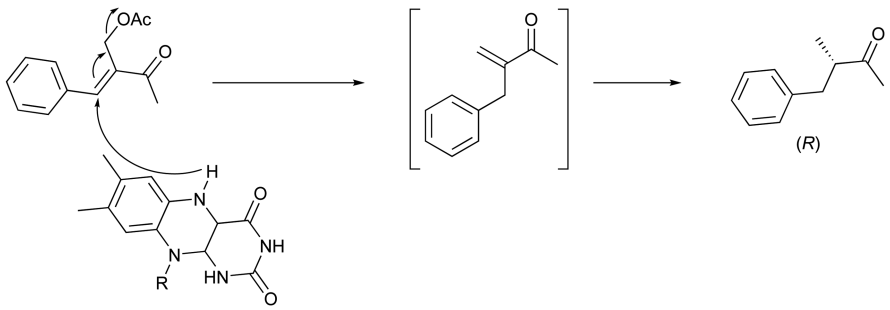


Figure 8.10 S_N2' type mechanism proposed for ERED catalysed reaction of α-acetoxymethyl enones.

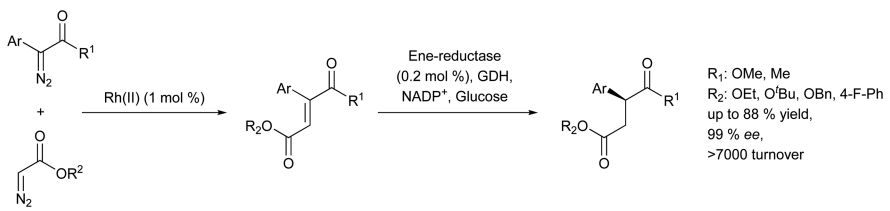


Figure 8.11 One-pot sequential chemoenzymatic system.

heterocoupling of the two diazo-esters, and a reduction mediated by an ene reductase with up to 99% ee (Figure 8.11). There was a preferential generation of the (*E*)-alkene from the diazo-coupling reaction giving high yield and enantioselectivity. The ene-reductase selectively reduced the (*E*)-alkene in a mixture of (*E*) and (*Z*) isomers. The combination of organometallic and enzymatic catalysis allows unusual transformations without the need to purify and isolate intermediates.

To encourage the uptake of this type of approach by industry more literature examples are urgently needed, and it is important to emphasise the need for multidisciplinary collaboration between practitioners of synthetic biology to produce and characterise new enzymes and chemists to apply these in enzyme cascades, with shared understanding of specific challenges such as cofactor and equilibrium demands. The academic sector has a key role to play in this process of enzyme discovery, development and demonstration, not just for ene reductases but many other enzyme types also.

8.4.2 Ene Reductases and Solvents

The use of organic solvents is an integral feature of biocatalysis, where they serve a number of purposes. These include solubilisation of substrates, *in situ* removal of products and modulation of rate or selectivity. Process development of biotransformations will frequently involve screening for optimisation of an appropriate co-solvent. In some cases the solvent may even serve as the substrate. Ene reductases have been shown to demonstrate good solvent tolerance.[38] The ene reductases KYE1 from *Kluyveromyces lactis* and YersER from *Yersinia bercovieri* (both derived from the Old Yellow Enzyme family) were shown to be active against a broad range of substrates including cyclic enones, cyclic enol-ethers, alkene esters/diesters and nitrostyrenes. In this work, glucose dehydrogenase/glucose was used as the reducing source and recycling system for nicotinamide cofactor. When the enzymes were tested with a range of co-solvents the rate and selectivity was maintained surprisingly well, with little drop in performance in 20% v/v ethylene glycol, DMSO, hexane and toluene, representing both water miscible and immiscible solvents. In biphasic systems with toluene or hexane, the reaction was not impaired even at 70% v/v solvent.

(2*R*,5*R*)-Dihydrocarvone is a key intermediate in the production of natural products, antimalarial drugs and valuable chiral building blocks.[46] However, its synthesis by whole-cell biotransformation often results in by-products (Figure 8.12). It has been reported that the ene-reductase NostocER1 from the cyanobacterium *Nostoc* sp. PCC 7120 and a $NADP^+$ accepting mutant of the formate dehydrogenase (FDH) from *Mycobacterium vaccae* can be applied to the whole-cell batch bioreduction of (*R*)-carvone.[47] In aqueous medium, the biotransformation resulted in a low conversion of 27.2% and 81.7% de of (2*R*,5*R*)-dihydrocarvone. The introduction of a second phase was found to have an impact on the biotransformation. Three key observations were made:

1. The second phase served as a substrate reservoir for the poorly water soluble (*R*)-carvone.[48]
2. Ionic liquids or adsorbent resins were found to improve substrate conversion at concentrations ≥50 mM. Carvones are known for their antimicrobial activity and toxic effects on the biocatalyst are reduced by lowering the substrate concentration present in aqueous phase.
3. The stereoselectivity improves due to efficient product extraction from the aqueous phase and the concomitant protection from isomerisation by the *E. coli* cells.

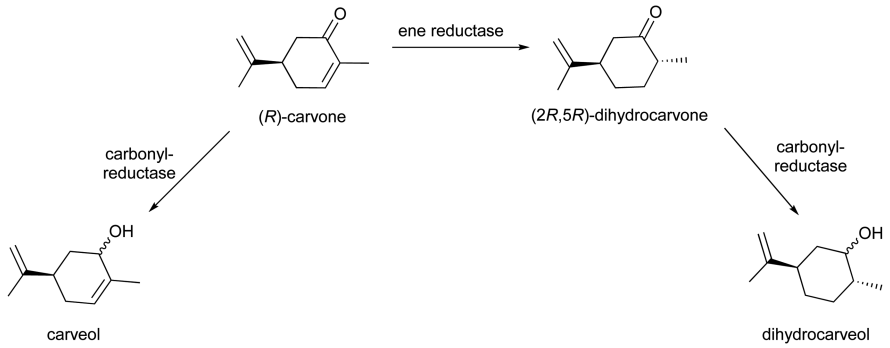


Figure 8.12 Asymmetric reduction of (*R*)-carvone to (2*R*,5*R*)-dihydrocarvone.

With the use of a biphasic system the conversion increased to >85% and high stereoselectivities were observed (96.0–99.2% de). Notably the undesired formation of carveols or dihydrocarveols as side products was not observed. The batch biotransformation was demonstrated at litre scale and shows potential for production of (2*R*,5*R*)-dihydrocarvone at larger scales.

Thus, the use of co-solvents during ene reductase process development should routinely be considered where appropriate, if they can bring a benefit to the reaction.

8.4.3 Challenges of Co-factor Recycle

The catalysis of ene reductases utilises nicotinamide cofactors (NADH, NADPH) in a manner similar to carbonyl reductases. Stoichiometric use of these natural coenzymes is not viable economically, and their instability can hinder catalytic processes that employ coenzyme recycling. Whilst this presents additional challenges for operation and cost, these challenges should in no way be viewed as insurmountable by pharmaceutical chemists. This reasoning is derived from the extensive success of carbonyl reductases now being realised for ketone into alcohol biotransformations in pharmaceutical intermediate manufacture, where cofactor requirements are routinely addressed. Whilst ene reductases as an enzyme class can utilise both NADH and NADPH as reducing hydride equivalents, they tend to have a preference for NADPH over NADH.

Various cofactor recycling systems may be used, utilising methods and enzyme sources developed for carbonyl reductases. The most commonly used approaches are in Figure 8.13.

Of these approaches, the first using glucose/glucose dehydrogenase is the most frequently used. In carbonyl reductase biocatalysis cofactor recycle is often achieved using isopropanol as the hydrogen donor for cofactor recycle. Furthermore, the same enzyme may be used both for ketone reduction and isopropanol oxidation, with equilibrium driven by using an excess of the alcohol solvent. The same methodology, where a solvent stable alcohol dehydrogenase is used to oxidise isopropanol has been established for ene reductase

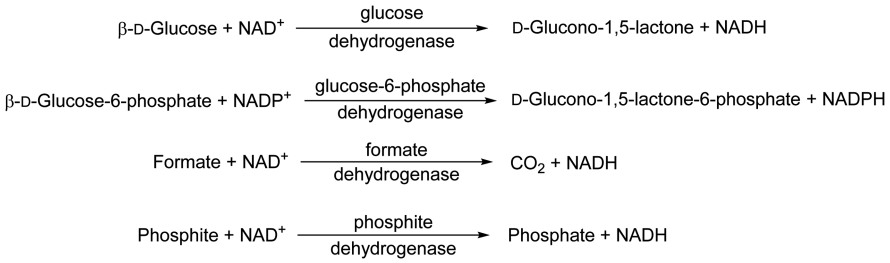


Figure 8.13 Commonly used cofactor recycling systems.

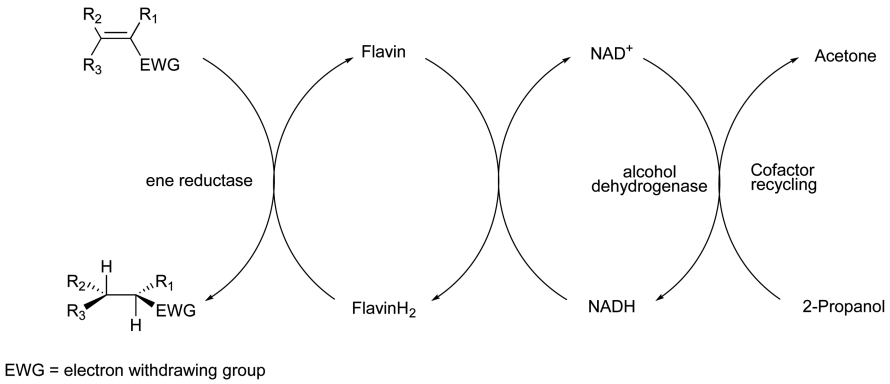


Figure 8.14 Co-factor recycling with isopropanol.

systems.[49] In this system, the oxidation of the sacrificial 2-propanol to acetone by the solvent-stable alcohol dehydrogenase (ADHA) from *Rhodococcus ruber* has been coupled to the bioreduction of activated alkenes using ene reductases as shown in Figure 8.14.

A various typical ene reductase substrates were reduced by this method, and performance was generally similar to other recycling methods based on glucose/glucose dehydrogenase or formate/formate dehydrogenase.

The key message is that for a given ene reductase biotransformation cofactor regeneration may be achieved by different enzymes and substrates. Crucially though, the literature reports that different systems may give different results in terms of yield and selectivity for a specific reaction, and choice of the cofactor recycling system should be an integral part of biotransformation development for large scale application.

8.4.4 Avoiding the Use of Nicotinamide Co-factors

In some cases it may be desirable to avoid the requirement for regeneration of the nicotinamide cofactor by a second enzyme-catalysed redox cycle. To address this desire a coupled substrate approach was developed.[50,51]

This demonstrates a simpler concept whereby a low cost substrate such as a 2-enone or 1,4-dione serves as the hydrogen donor for the direct recycling of the flavin cofactor. The system uses only a single enzyme and is independent of nicotinamide cofactor. During this recycle process the co-substrate is desaturated, and the reaction is driven thermodynamically by the rapid aromatisation of the desaturated product. This is illustrated in Figure 8.15, where cyclohexenone is used as the co-substrate for reduction of 4-ketoisophorone. A wide range of enones were demonstrated as suitable co-substrates for the reduction of 4-ketoisophorone to form (*R*)-levodione. This appears to be a promising approach, and from an industrial perspective has an appealing degree of simplicity over dual enzyme systems requiring nicotinamide cofactors. It is anticipated that more examples based on this approach will emerge in due course.

Industrial interest in this approach is evident through a patent issued to BASF.[52] This patent is based on the bioreduction of various enones by the YqjM gene product of *Bacillus subtilis* and the FCC248 gene product (an estrogen binding protein).

An alternative approach to address the cofactor issue is by the use of synthetic mimics of NAD(P)H.[53] Several synthesised analogues are shown in Figure 8.16.

These were tested against a range of ene reductases for the reduction of ketoisophorone to levodione. Good activity was found, and in some cases performance exceeded that of the natural cofactor. Recycling of the cofactor

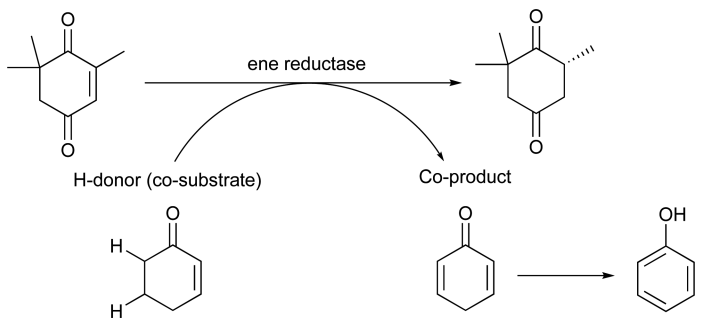


Figure 8.15 Bypassing cofactor requirement by use of a co-substrate.

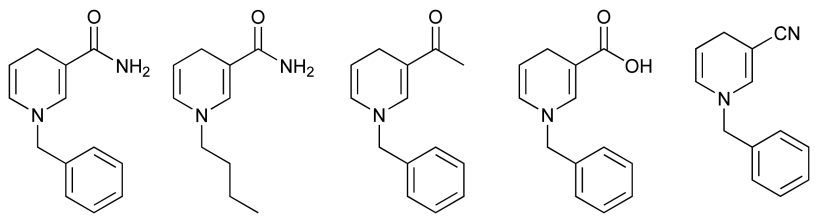


Figure 8.16 Synthetic mimics of nicotinamide cofactor.

mimics was also demonstrated using formate as the reductant and a rhodium-based catalyst. It is claimed that these mimics are inexpensive to synthesise and have greater stability than their natural counterparts, making them an intriguing potential alternative to use of the natural cofactors.

Until more recently synthetic mimics were not reported to significantly enhance enzyme activity; however, kinetic investigations with the ene-reductase Old Yellow Enzyme found the flavin-reducing step to be slower than the substrate reduction. Hauer *et al.*[54] reasoned that varying the electrochemical potential of the utilized cofactor could improve the rate of flavin-reduction. The introduction of aromatic residues could serve as electron donating residues when attached to the nitrogen atom thus altering electrochemical properties (Figure 8.17). When compared with NADH, cyclic voltammetry identified that the lowered oxidation potential of HPNAH represented a higher ability for hydride donation. The enzyme 2-cyclohexen-1-one reductase from *Zymomonas mobilis* (NCR)[55] was a highly active cofactor enzyme pair with HPNAH, with a sixfold higher v_{max} relative to NADH most likely due to the ability of HPNAH to reduce the flavin faster.

There are further reports of biomimetics (**1**–**5**) that can outperform natural coenzymes in biotransformations (Figure 8.18).[53] The synthetic coenzymes are stable relative to biological equivalents, inexpensive to manufacture and have been proven to work with a wide range of ene-reductases. The biomimetics can be used in only catalytic amounts at the expense of formate.

Scrutton *et al.*[56] further demonstrated an approach using photosensitive transition metal complexes of Ru(II) or Ir(II) as electron donors for OYE-catalysed α,β-unsaturated alkene reduction. The light driven biocatalytic systems are a cheaper alternative to costly redox coenzymes and potentially avoid the need for enzyme-based cofactor regeneration systems. As an alternative to NADPH as the hydride donor, the yields and enantioselectivity for C-terminally histidine-tagged enzymes PETNR-His_8 and TOYE-His_6 were comparable for a broad range of substrates. With cyclohexen-2-one there were high levels of product accumulation with the main electron transfer pathway *via* photoexcitation of the photosensitiser. This is followed by successive reductive

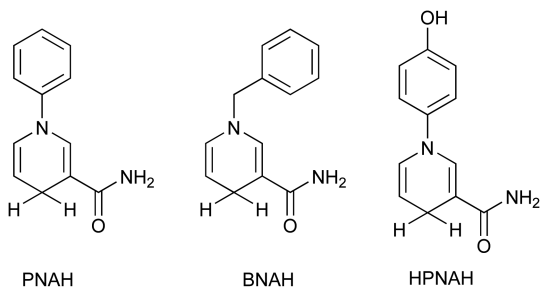


Figure 8.17 Synthetic cofactors, PNAH (1-phenyl-1,4-dihydronicotinamide), BNAH (1-benzyl-1,4-dihydronicotinamide) and HPNAH (1-(4-hydroxyphenyl)1,4-dihydronicotinamide).

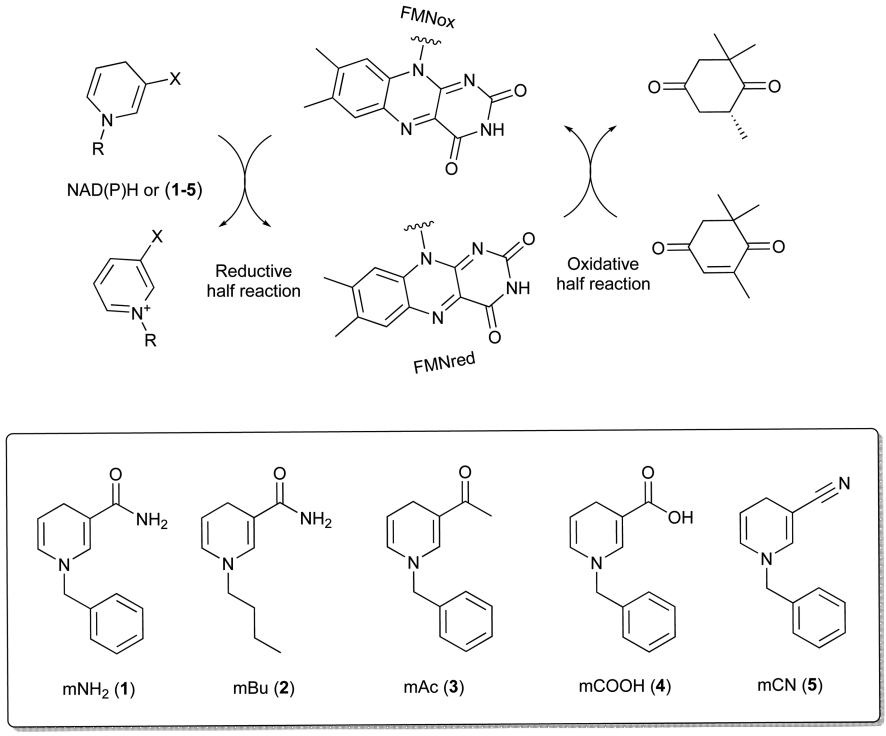


Figure 8.18 Synthetic nicotinamide biomimetic mNADHs (**1**–**5**) and the catalytic cycle of ER-catalysed reactions.

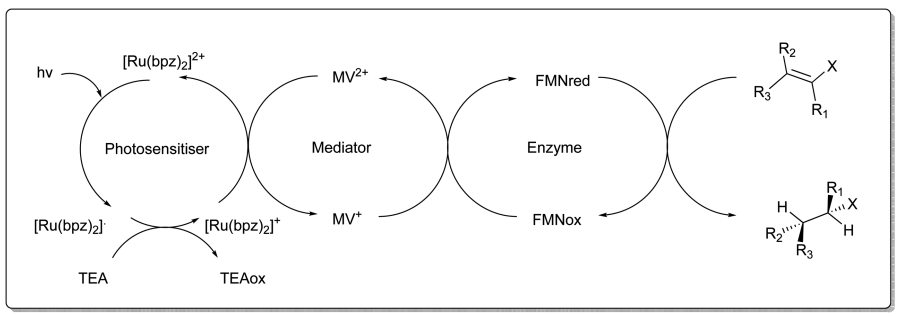


Figure 8.19 Electron-transfer processes that contribute to enzyme reduction in light-driven biocatalytic cycles of PETNR and TOYE.

and oxidative steps between TEA, methyl viologen and enzyme bound flavin (FMN) to the alkene substrate (Figure 8.19).

Finally, artificial electron donors, such as reduced methyl viologen, can be used as an alternative to NAD(P)H in ene reductase biotransformations. However, this donor, perhaps better known as the herbicide paraquat, is

highly toxic and therefore not a desirable reagent for utilisation in biocatalytic products such as foods and pharmaceuticals.[57]

Since the cost contribution of enzymes for $NAD(P)^+$ reduction and recycle can be demanding from an industrial perspective it is essential that cofactor recycle or methods that avoid cofactor altogether are evaluated carefully during the process of scale up and development. It is apparent that there are numerous approaches that can be evaluated, and for industrial ene reductase biocatalysis this is a crucial factor for consideration in the development of a viable process.

8.4.5 Impact of Synthetic Biology

The recent impact of synthetic biology on multiple aspects of biocatalysis is striking, and is manifested by greatly decreasing the cost, time and risk associated with cloning enzymes. Furthermore, the ability to utilise published data relating to enzyme amino acid sequences and three-dimensional structure to semi-rationally alter enzyme performance is unprecedented. These techniques have filtered through to ene reductases, with good examples in the literature where enzyme performance has been improved and new enzymes have been discovered.

Gene shuffling, where the gene sequences of a small number of similar enzymes are cut into fragments then recombined together to give new chimeric 'shuffled' proteins is a methodology practised by companies such as Codexis Inc. The technique is one method of several for altering the properties of enzymes. Codexis demonstrated this approach by creating new ene reductases from three Old Yellow Enzymes derived from *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*.[58] Some of the mutants showed dramatic improvements in the level of activity against tested substrates. Two examples are shown in Figure 8.20. In the reduction of (*Z*)-ethyl 2-cyano-3-phenylbut-2-enoate several mutants were found where the activity was improved more

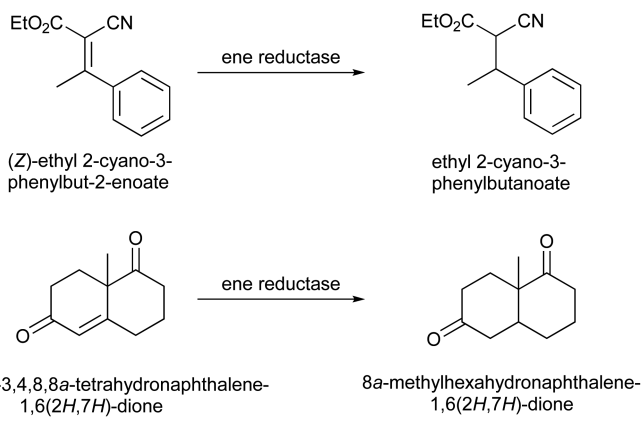


Figure 8.20 Substrates for engineered ene reductases.

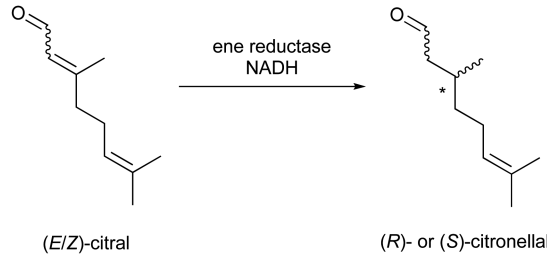


Figure 8.21 Hydrogenation of (*E*/*Z*)-citral to (*R*/*S*)-citronellal catalysed by ene reductase.

than 20-fold relative to the parent enzyme. With 8a-methyl-3,4,8,8a-tetrahydronaphthalene-1,6(2*H*,7*H*)-dione several enzymes demonstrated over ten-fold increase in activity relative to the parent enzyme.

These results show that the activity levels of ene reductases can be altered substantially by synthetic biology and the application of routine random mutagenesis methods. Recently, Hauer and coworkers[59] achieved a deeper understanding of the >99% (*S*)-selective reduction of both isomers of citral, where the reaction is catalysed by NADH-dependent cyclohexenone ene reductase (NCR) from *Zymomonas mobilis*, using active-site mutational studies and docking simulation. The selectivity demonstrated, by the structurally similar (*E*/*Z*)-isomers, was shown to be dependent on the introduced mutations. It was possible to invert (*E*)-citral reduction enantioselectivity from >99% (*S*) to an ee of 46% (*R*) with the introduction of mutation W66A. For (*Z*)-citral the ee remained as >88% for all single residue variants. The studies concluded that W66 offers a leverage position which can induce (*R*) selectivity in NCR-catalysed citral reduction with reversed citral binding modes (Figure 8.21).

Iterative saturation mutagenesis is a more rational approach where, based on known protein structure, key amino acid residues are systematically changed to other amino acids in order to improve key enzyme properties such as selectivity or stability. This approach was exploited for an ene reductase YqjM, cloned from *Bacillus subtilis*.[60] Using the known structure of this enzyme, 20 amino acid sites were subjected to alteration, and the resulting altered enzyme tested for reduction of various cyclopentenone and cyclohexenone substrates. For the biotransformation shown in Figure 8.22, the wild-type enzyme catalyses reduction to the (*R*)-isomer with 99% ee.

Two mutations of this enzyme (cysteine to aspartate at position 26 and isoleucine to threonine at position 69) improved the reaction rate by about one-third, whilst still catalysing a selective reduction of 99% ee. However, a different mutant, where just the cysteine at position 26 was changed to glycine, demonstrated completely reversed enantioselectivity, with the product now 98% ee (*S*)-isomer. This work demonstrated that both reaction rate and selectivity can be altered for ene reductases.

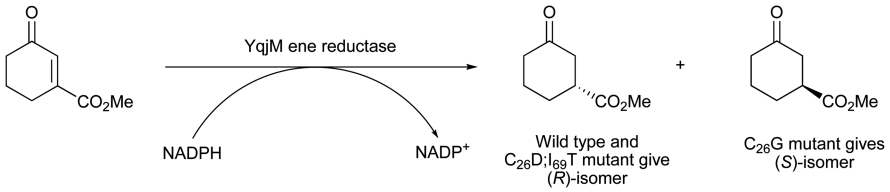


Figure 8.22 Reversal of stereoselectivity in ene reductases by mutation.

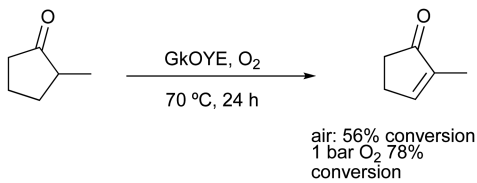


Figure 8.23 Oxidation by ene reductases.

New methods for enzyme discovery continue to emerge, such as the recently reported catalophore method.[61] In this approach the protein structure of known ene reductases was used as a starting point to define the three-dimensional spatial positioning of a few key active site enzyme residues. This 'catalophore' map was then used to interrogate a database of many different enzymes with known structure, and was able to identify new enzymes with ene reductase activity despite having very different amino acid sequences and enzyme topology to established enzymes, and with different stereopreference to known enzymes.

It is important that the academic sector continues to develop enzyme discovery methods, exploiting the full potential of synthetic biology and use these methods to characterise new ene reductase enzymes. Further data on structure, properties and biotransformation performance will allow industrial scientists to increase their ene reductase competencies.

8.4.6 Ene Reductases in Reverse: Oxidation

An interesting and potentially important advance in ene reductase technology was reported where the enzyme functions in the reverse direction.[62] Instead of alkene reduction the authors demonstrated that a range of cyclic ketones could be desaturated by oxidation to the corresponding enones, with an example shown in Figure 8.23.

Unlike the reductive process the reaction did not require a nicotinamide cofactor, and it was demonstrated that oxygen functioned as the oxidant. The enzyme cycle was completed by the aerobic reoxidation of the flavin $FMNH_2$. The reactions were found to work best at higher temperature, around 70 °C. In the case of racemic ketones, the potential for chiral products was evident. The use of pure oxygen was shown to increase the extent of reaction.

The synthesis of substituted naphthols by an efficient enzymatic method from the corresponding tetralones has been reported.[63] This utilises the ability of the ene-reductases of the Old Yellow Enzyme family to work in reverse. Screening of Almac's selectAZyme panel of EREDs resulted in over 60% of the enzymes yielding 2-naphthol when 2-tetralone was used as the substrate. Moderate to excellent conversions (up to >99%) were reported for the selected EREDs for the production of a set of substituted naphthols (Figure 8.24). The robustness of the process was demonstrated with a 2.0 g scale reaction which gave a 91% isolated yield.

Whilst the application of ene reductases as oxidative catalysts is very much embryonic, there is good potential for reaction and enzyme engineering to advance this mode of application towards routine viability. The main challenges to be addressed include broadening the range of substrates amenable to oxidation, dealing with limited oxygen solubility at higher temperature and increasing enzyme stability, turnover and selectivity.

8.4.7 Thermophilic Ene-reductases

Ene-reductases that come from extremophiles are gaining importance due to their higher stability. The genome of the acidophilic iron-oxidising bacterium *Ferrovum* sp. JA12 was reported as the first thermophilic-like ene-reductase (*FOYE*-1).[64] It is closely related to three mesophilic ene-reductases, namely *Dr*OYE, *Rm*OYE and OYE*Ro*$_2$. *FOYE*-1 was found to be highly efficient in the transformation of different maleimides creating stereoselective succinimides. High conversions were achieved using *FOYE*-1 for the reduction of *N*-phenylmaleimide (>99% with NADPH) and also on *N*-phenyl-2-methylmaleimide (>98%, using NADPH, Table 8.1). The stability of the enzyme was studied between 20 and 70 °C with the optimum activity of *FOYE*-1 observed at 50 °C (160 U mg^{-1}). At the upper end of the temperature range studied the activities were reduced (39–51 U mg^{-1}).

8.4.8 Alternative Screening Methods

Typically, for screening ene reductase enzymes, either GC or HPLC is used for analysis, which is advantageous as both stereoselectivity and conversion can be assessed. For primary screening, an alternative and potentially quicker

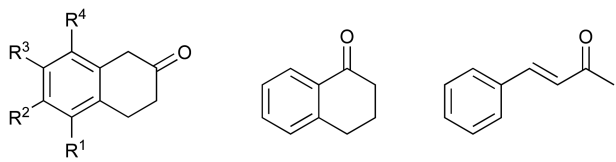
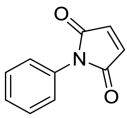
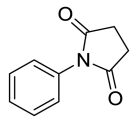
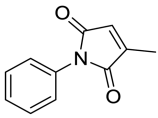
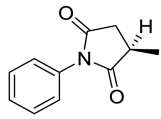


Figure 8.24 Range of tetralones tested for the production of naphthols using EREDs.

Table 8.1 Asymmetric bioreduction of activated maleimides using FOYE-1.

Substrate	Product	Cofactor	Conversion (%)	ee (%)
		NADPH NADH	>99 >89	– –
		NADPH NADH	>98 >97	>94 (*R*) >96 (*R*)

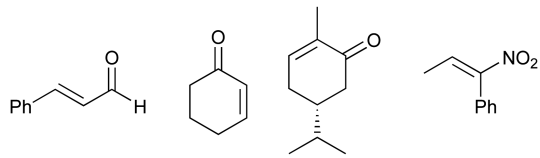


Figure 8.25 Substrate scope for validation of FRED assay.

colorimetric method reported by Monti *et al.*[65] allows for the determination of conversion in a substrate independent way (FRED, fast and reliable ene reductase detection). To validate the FRED assay, the bioreduction of substrates by OYE1 were analysed by both FRED and by GC (Figure 8.25), with a comparable determination of conversion. There is a wide range of possible applications for this technology, not least for quick, reliable primary screening of libraries.

Quertinmont and Lutz have explored the potential alternative methods to evaluate libraries of OYE variants.[66] They developed a protocol – RAPPER (Rapid Parallel Protein EvaluatoR)[67] – that allows for systematic protein engineering studies of OYE1. RAPPER enables fast and more efficient, semi-quantitative evaluation of enzyme variants as an initial screening protocol to discover tailored biocatalysts, but does have limitations such as small sample sizes.

8.5 Example of Utilisation of an ERED at Industrial Scale

A green, renewable alternative to metal-catalysed asymmetric reductions can be achieved by employing EREDs. The first example of a large scale enantiospecific reduction using an ERED catalyst has been reported incorporating a carbonyl reductase (CRED) enzyme in the cofactor recycle.[68] Pairing the ene-reductase step with a hydrolase mediated regioselective ester hydrolysis generated a valuable chiral building block in a one-pot process (Figure 8.26).

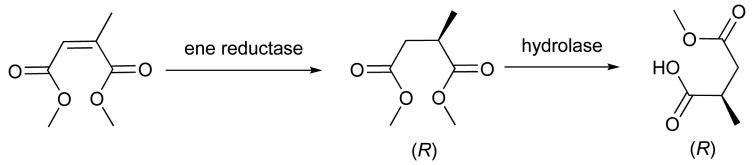


Figure 8.26 Enzymatic route to chiral acid target molecule.

The starting material was screened against the SelectAZyme EESK-1300 kit with ER-104 identified as the preferred enzyme for further optimisation of reaction conditions for scaling up. The traditional glucose–glucose dehydrogenase (GDH) system, which is used to recycle NAD(P)H, was replaced with a CRED enzyme capable of converting isopropanol (IPA) into acetone.[69] This benefits process development since it removes the requirement for pH control, thus allowing large numbers of parallel optimisation reactions. Critically, removing the requirement for pH control is advantageous as it is no longer necessary to adapt large-scale chemical reactors to include circulation loops. After optimisation, A131 was chosen as the recovery enzyme, which proved to be superior to highly purified alcohol dehydrogenase from baker's yeast.

Enzymatic activity is typically influenced by parameters including temperature, pH and organic co-solvent. For this work, optimisation of the process was complicated by the need to suit two enzymes with the reaction parameters. The thermostabilities of ER-104 and A131 were assessed, with optimum activity achieved at 35 °C. There was evidence of denaturing of one or both proteins above this temperature, with a sharp drop in activity. Investigations for optimum pH found that even mildly acidic conditions hindered the desired reaction and subsequent reactions were carried out at pH 8.0, which afforded maximum reaction rate without approaching the alkalinity that potentially would facilitate hydrolysis of the methyl ester moieties present.

The inhibitory effect of the substrate and/or product on an enzyme can be minimised by conducting the desired reaction in a biphasic medium with the addition of a suitable co-solvent. Another alternative is the use of a water miscible co-solvent which increases the reaction rate by improving the availability of the substrate to the enzyme. For the biotransformation discussed here a co-solvent screen of four water-miscible solvents (DMF, DMSO, THF and acetonitrile) was conducted, as well as six water-immiscible solvents (DCM, diisopropyl ether, ethyl acetate, hexane, 2-methyl-THF and toluene) at different solvent : aqueous buffer (10% v/v) ratios. Analysis indicated the most suitable co-solvent was toluene, with an increased conversion (2.5×) over 20 h compared to standard reactions conditions which did not utilise a co-solvent. Optimisation of the toluene to aqueous buffer ratio to 40% v/v resulted in conversions increased by a factor of 4 over the same 20 h timeframe. Conversions of 53% after 20 h and 73% after 44 h were achieved at synthetically viable volume efficiency of 0.2 M (~30 volumes). The optimised conditions included 100% w/w ER-104 cell pellet, 12.5% w/w A131 crude cell-free extract and 5% NADH cofactor.

Attempts to improve conversion further were unsuccessful and it was identified that the acetone produced as a result of the IPA/A131 cofactor recycle system had an inhibitory effect on ER-104. The removal of acetone from the equilibrium can drive the reaction[70–72] and as a result the system shown in Figure 8.27 was employed. The IPA level in the reaction flask was maintained by air sparging through a flask containing 3% v/v solution of IPA in H_2O, with acetone driven off. With this system, 95% conversion was achieved on 5.0 g scale in 40 h.

Following the optimisation, a screen of 48 SelectAZyme hydrolase enzymes (HESK 4800) found AH-33 gave regioselective hydrolysis to the desired product. With an appropriate hydrolase enzyme identified, the reaction was studied to check if it was possible to simultaneously add ER-104, A131 and AH-33 at the beginning of the reaction; however, no conversion was observed. Instead the reaction was conducted stepwise as a one-pot biotransformation.

This study demonstrated that ERED enzymes are applicable to 70 g scale and the use of a CRED/IPA cofactor recycle has simplified optimisation by eliminating the requirement for pH control. The inhibitory effect of the production of acetone was circumnavigated with an ISPR technique with the desired product isolated in 89% yield.

8.6 Transition of Ene Reductases to Mainstream Biocatalytic Use

The field of ene reductases has broadened considerably in recent years, with the impact of synthetic biology becoming more widespread. There is however still some way to go to transition ene reductases into mainstream

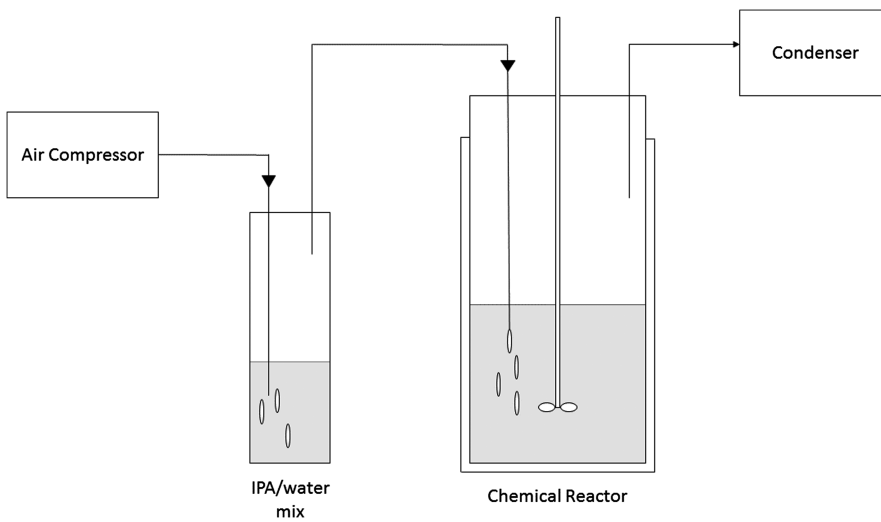


Figure 8.27 Method for IPA/H_2O saturated air sparge to remove acetone formed during the course of the reaction.

biocatalytic use. To advance the field of ene reductase biocatalysis into a state of industrial applicability akin to carbonyl reductases or hydrolases a number of requirements are evident. The first requirement relates to greater availability of enzymes in a format that allows routine screening to be conducted. The bonus here is for academics to continue to discover, characterise and publish high quality data on ene reductases, both at the molecular level and on subsequent performance of the enzymes in real reactions. This will encourage the placement of promising enzymes directly into industrial collections, or the use of published information that can serve as a baseline for synthetic biology-led ene reductase discovery where protein sequences and structures may be exploited.

Ene reductases are no different from other enzyme classes in the general properties that are required to see them utilised in industrial processes. For example, enzyme stability remains a key issue for enabling operation at scale. Total turnover numbers are still somewhat limited at around 10^3–10^4, giving the opportunity for enzyme evolution or immobilisation to help remedy this.[73] Nicotinamide cofactor recycle remains a challenge to be addressed for each ene reductase reaction, and biotransformation process development must scout for the best recycle system or consider the sacrificial co-substrate approach to by-pass their need altogether.

There is every reason to believe that ene reductases will be successful in making the transition from laboratory curiosity to become a mainstream industrial biocatalytic technology for the reduction of the alkene double bond. Furthermore, it is not unreasonable to anticipate that their application may also extend to oxidation and the formation of alkenes.

8.7 Conclusions

Enzyme-enabled syntheses has gained acceptance within the organic toolbox over the last 5–10 years, driven by availability of off-the-shelf enzymes, dramatic advances in speed of gene synthesis (and lower cost) and access to new enzymes *via* molecular and synthetic biology. The decreasing time and cost of the molecular biology in turn decreases the cost associated with redesign and engineering of enzymes whilst increasing the chances of a successful outcome.

As high value products, pharmaceuticals will continue to be the main driving force for enzyme development, but as examples of larger scale syntheses with ERED emerge we will see increasing interest in fields such as flavour and fragrances, specialities and polymer science. These higher volume but lower value products need to be made before commercially viable processes are possible.

Biocatalysis is truly a 21st century technology readily available to all chemists. It brings many benefits, including new route options, process simplification, increased speed of delivery, no heavy metals to control and the potential to generate some new IP to protect your invention. It's time to give it a go!

Acknowledgements

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