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REVIEW
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Alcohol Dehydrogenases as Catalysts in Organic Synthesis

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Alcohol dehydrogenases (ADHs) have become important catalysts for stereoselective oxidation and reduction reactions of alcohols, aldehydes and ketones. The aim of this contribution is to provide the reader with a timely update on the state-of-the-art of ADH-catalysis. Mechanistic basics are presented together with practical information about the use of ADHs. Current concepts of ADH engineering and ADH reactions are critically discussed. Finally, this contribution highlights some prominent examples and future-pointing concepts.

Keywords: alcohol dehydrogenases (ADH), oxidation reactions, reduction reactions, enantioselective synthesis, alcohols, ketones, aldehydes, biocatalysis

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1 INTRODUCTION—ALCOHOL DEHYDROGENASES AS CATALYSTS FOR ORGANIC CHEMISTRY

Redox reactions constitute a central theme of organic synthesis; particularly, the conversion of alcohols into aldehydes or ketones as well as the reverse reaction (i.e., reduction of carbonyl compounds into alcohols) play an important role also in industrial practice (Sheldon et al., 2002; Tojo and Fernández, 2006; Magano and Dunetz, 2012). Decades of intensive research have yielded a vast portfolio of efficient oxidation- and reduction procedures and –catalysts. Interestingly, chemical catalysts applicable for both, oxidation and reduction reactions are scarce. In contrast, alcohol dehydrogenases (ADHs, sometimes also termed carbonyl reductases, KREDs) are natural redox catalysts capable of catalysing the oxidation of alcohols and the reduction of carbonyl compounds. This versatility together with their often high selectivity makes ADHs powerful catalysts for the redox transformation of alcohols and carbonyl compounds. The past decades have seen enormous research efforts dedicated to the applicability of ADHs in organic synthesis. Earlier issues such as poor availability, narrow substrate scope or low economic attractiveness of ADH-catalyses reactions have been solved and ADHs are increasingly used on industrial scale (Hauer, 2020; Wu S. et al., 2021).

The aim of this contribution is to present the current state-of-the-art of ADHs in organic synthesis. It builds on and extends previous review articles covering various aspects of ADH catalysis (Li et al., 2021c; de Gonzalo and Paul, 2021; Hollmann et al., 2021; Musa et al., 2021; Musa, 2022).

2 BASIC CONSIDERATIONS

ADHs catalyse the reversible oxidation of alcohols to the corresponding carbonyl products. Being a redox reaction by nature, the oxidation or reduction reaction has to be accompanied by a reduction or oxidation reaction of a stoichiometric cosubstrate. ADHs utilise nicotinamide cofactors for this (**Figure 1**). The nicotinamide cofactors exist in ribose-phosphorylated [NAD(P)] and ribose-non-phosphorylated (NAD) form; their basic physicochemical properties (e.g., redox potentials) are

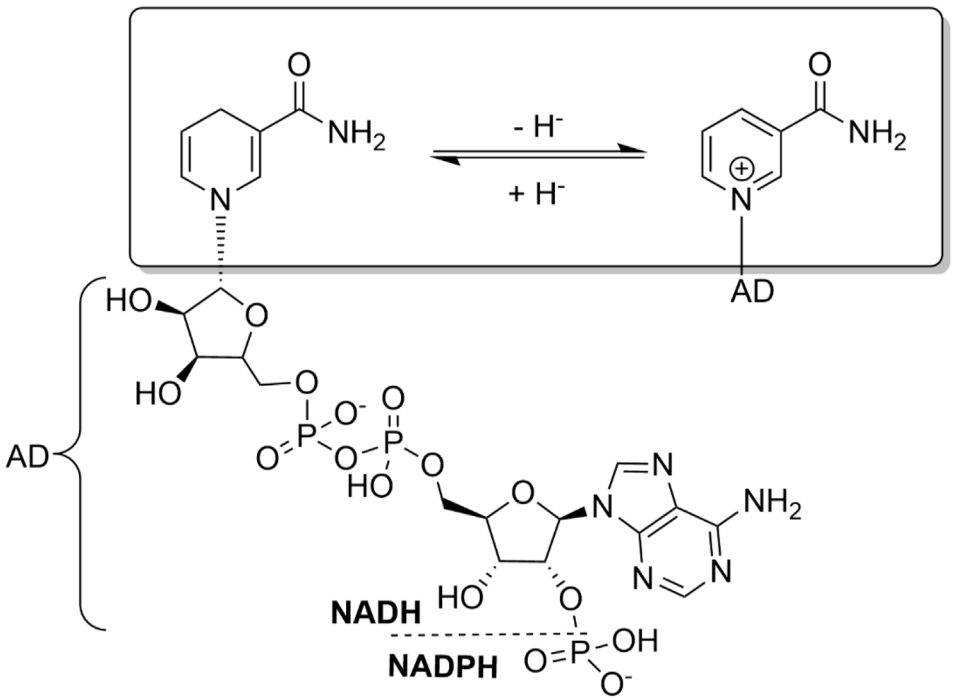


FIGURE 1 | Structure and basic redox chemistry of the nicotinamide cofactors. The catalytically relevant nicotinamide moiety is highlighted. The ribose-phosphorylated (NADP) and ribose-non-phosphorylated (NAD) forms differ in their adenine dinucleotide (AD) moiety.

identical but, frequently, ADHs, depending on their role in the host organism, exhibit high selectivity for one of the two forms. As a rule of thumb, ADHs involved in anabolic pathways generally prefer NADP while metabolically relevant ADHs utilize NAD. As redox-cofactors, NAD(P) obviously exist in an oxidised [$NAD(P)^+$] and a reduced [NAD(P)H] form. In essence, NAD(P)H and $NAD(P)^+$ function as hydride ion donors or–acceptors, respectively.

2.1 Catalytic Mechanism

The catalytic mechanism of ADH-catalysed transformations comprises the binding of the starting material (alcohol or carbonyl compound) together with the nicotinamide cofactors to the enzyme active site followed by a hydride transfer between cofactor and substrate (**Figure 2**). Some ADHs contain a metal ion in their active site (also Fe ions), which, however, predominantly participates in coordination of the starting materials and does not fulfil redox activities. There are also metal-free ADHs, does not which principally follow the same hydride transfer mechanism (Zhou et al., 2020).

One key-feature of ADH-catalysis is the reversibility of the reaction. Any given ADH catalyses both the NAD(P)H-driven reduction of carbonyl compounds to the corresponding alcohol and the $NAD(P)^+$-driven oxidation of alcohols to the corresponding carbonyl products.

As both the carbonyl starting material and the nicotinamide moiety are chiral, four different stereochemical hydride transfer pathways are possible (**Figure 3**) (De Wildeman et al., 2007) Hydride transfers from the *si*-face of the prochiral ketone result in (*R*)-configured alcohols whereas hydride attacks from the *re*-face yield (*S*)-alcohols. In both cases the hydride transferred can stem from either the *re*- or *si*-face of the nicotinamide ring.

ADHs catalysing hydride addition from the *re*-face of the ketone (or abstraction of a hydride from (*S*)-alcohols) are termed Prelog-selective ADHs whereas those ADHs attacking from the *si*-face (or abstracting a hydride from the (*R*)-alcohol) are termed *anti*-Prelog ADHs. Hence, for any given enantiopreference a set of suitable ADHs is available (**Table 1**, *vide infra*) (Prelog, 1964)

2.2 Factors Influencing Activity/Selectivity of ADHs.

2.2.1 Influence of the Reaction Conditions on the Selectivity of ADH-Catalysed Reactions

The selectivity, particularly the enantioselectivity of ADH-catalysis is predominantly determined by the geometrical composition of the active site and the structure of the starting material that control the binding of the substrate relative to the nicotinamide cofactor (**Figure 3**). Controlling this binding and the resulting selectivity is nowadays predominantly achieved through enzyme engineering (*vide infra*). There are, however, a range of other factors that may have a significant influence on the stereoselectivity of ADH-catalysed reactions.

As early as 1986, Keinan, Lamed and coworkers reported an influence of the reaction pH (and buffer) on the enantioselectivity of an ADH-catalysed reduction reaction (Keinan et al., 1986). Using the ADH from *Thermus ethanolicus* (*Te*ADH) as model enzyme, Philips and coworkers identified the so-called racemic temperatures (T_R) above and below which *Te*ADH exhibits

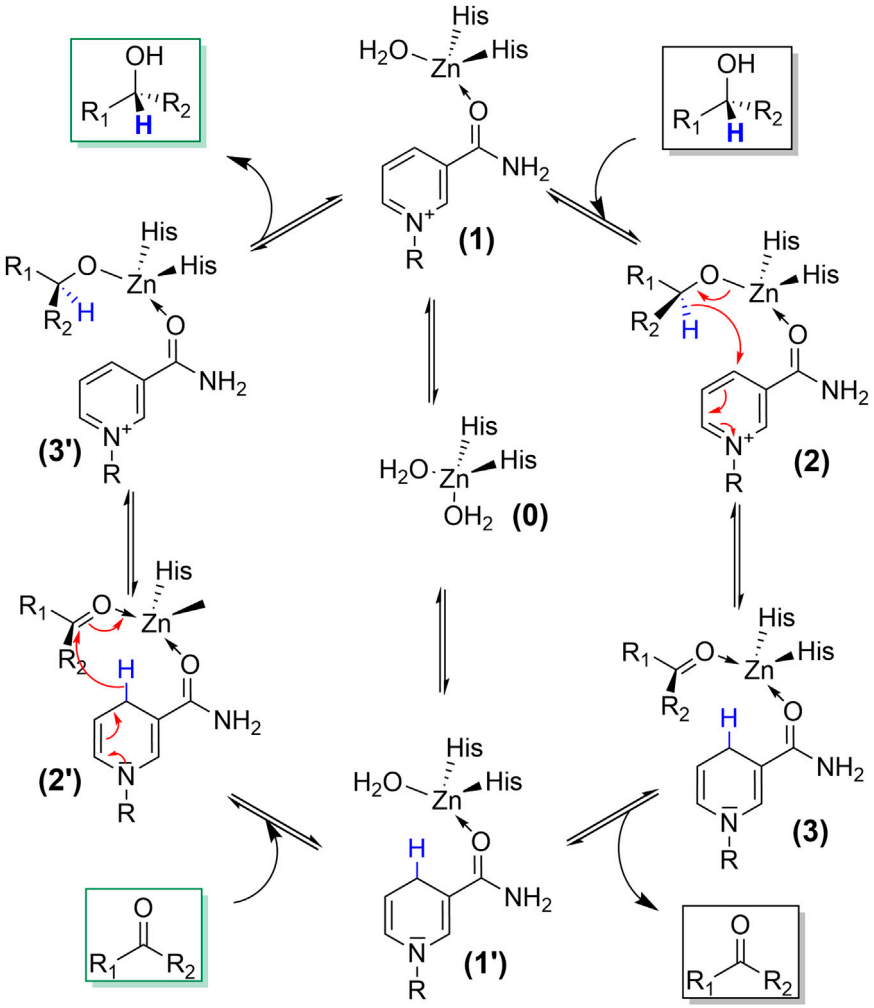


FIGURE 2 | Simplified catalytic mechanism of ADH reactions. Upon binding of both substrates (e.g., NAD(P)$^+$ and alcohol; 0->1->2) a hydride transfer occurs from the alcohol-carbon atom to the oxidised nicotinamide moiety yielding the Zn-coordinated carbonyl product and NAD(P)H (3). Both can dissociate from the active site yielding apo-ADH (0). Alternatively, only NAD(P)H stays bound and the reduced ADH can undergo a reductive conversion (1'->2'-> 3'-> 1).

opposing stereoselectivity for a given substrate (Pham et al., 1989; Pham and Phillips, 1990). For the reduction of 2-butanone and 2-pentanone, for example, T_R was determined to be 26°C and 77°C, respectively. Below these temperatures, (*S*)-alcohols were the predominant enantiomer whereas above T_R the (*R*)-alcohol was formed. The enantioselectivity increased the further away the reaction temperature was from T_R (above and below). A positive correlation between increasing temperature and (enantio)selectivity may appear counterintuitive as generally lower reaction temperatures are evaluated to increase selectivity. On condition the Curtin–Hammett principle applies to ADH-catalysed reactions, the enantioselectivity of a reaction (E) is the difference of the two Gibbs free energies of the transition states leading to the (*R*)- and (*S*)-product ($\Delta\Delta G^{\ddagger}$) and taking into account that ΔG is composed of an enthalpic (ΔH) and an entropic term ($T\Delta S$): $\Delta\Delta G^{\ddagger} = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger}$ (Eq. 1), a racemic temperature can be defined at which the reaction occurs non-enantioselectively ($\Delta\Delta G^{\ddagger} = 0$): $T_R = (\Delta\Delta H^{\ddagger})/(\Delta\Delta S^{\ddagger})$. Crossing T_R, the sign of $\Delta\Delta G^{\ddagger}$ changes corresponding to a switch in enantioselectivity (Phillips, 1992).

Also pressure has been demonstrated to exhibit a measurable influence on the stereodiscrimination of enzymes (Patel and Phillips, 2014). Water soluble cosolvents can also exhibit some influence on the stereochemical outcome of ADH-catalysed redox reactions as exemplified by various authors (Fitzpatrick and Klibanov, 1991; Schumacher et al., 2006; Musa et al., 2007; Li et al., 2009; Nealon et al., 2015).

It should, however, be emphasised that the influence of these parameters generally is not pronounced enough to serve as convenient measure to control the stereoselectivity of ADH-reactions. Also, the molecular effect of, e.g., cosolvents today is by far not understood yet.

2.2.2 Factors Influencing Activity/Stability

pH: ADHs generally exhibit two pH optima, one for the reductive direction and one for the oxidation reaction. As a rule of thumb,

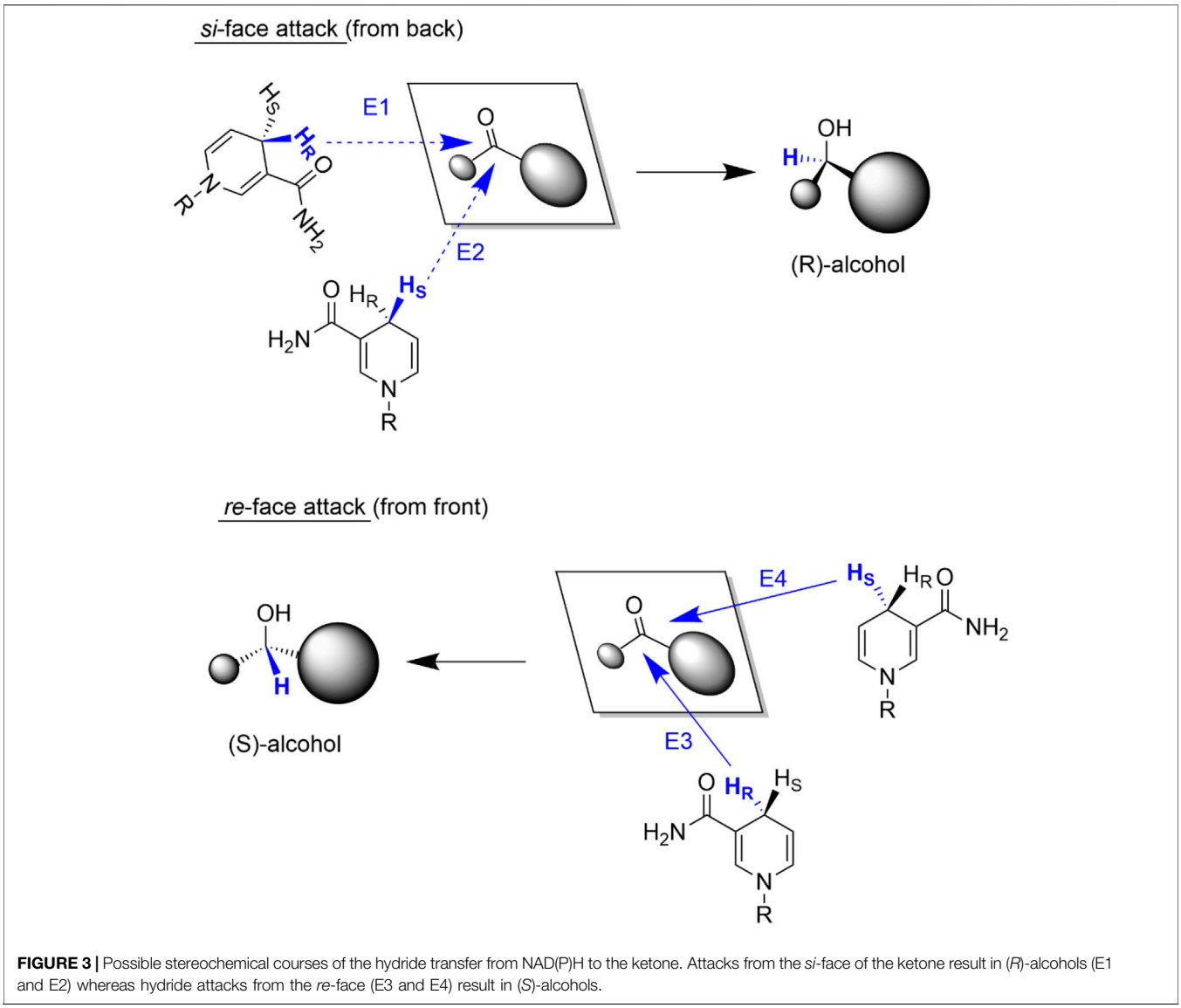


FIGURE 3 | Possible stereochemical courses of the hydride transfer from NAD(P)H to the ketone. Attacks from the *si*-face of the ketone result in (*R*)-alcohols (E1 and E2) whereas hydride attacks from the *re*-face (E3 and E4) result in (*S*)-alcohols.

TABLE 1 | Selected examples for ADHs with (*anti*-) Prelog selectivity (de Gonzalo and Lavandera, 2021).

Prelog ADHs		Anti-Prelog ADHs	
Source	**Cofactor**	**Source**	**Cofactor**
Horse liver (HLADH)	$NADH/NAD^+$	*Lactobacillus kefir* (*Lk*ADH)	$NADPH/NADP^+$
Rhodococcus ruber (ADH-A)	$NADH/NAD^+$	*Lactobacillus brevis* (*Lb*ADH)	$NADPH/NADP^+$
Thermoanaerobacter brokii (*Tb*ADH)	$NADPH/NADP^+$	*Candida parapsilosis* (*Cp*ADH)	$NADPH/NADP^+$

aldehyde/ketone reduction reactions are favoured in (slightly) acidic media whereas for the oxidation of alcohols alkaline media are favourable (Chang et al., 2009). Nevertheless, operating ADH reactions at neutral pH usually represents an acceptable compromise, especially if a substrate-coupled nicotinamide regeneration approach is used (*vide infra*). The pH of the reaction mixture also can have a significant influence on the stability of the nicotinamide cofactor: the reduced forms (NAD(P)H) are more stable in alkaline media whereas the oxidised forms ($NAD(P)^+$) are more stable in acidic media (Chenault and Whitesides, 1987).

Temperature: The rate of ADH-catalysed reactions increases with the temperature of the reaction medium. A good approximation for the influence of the temperature on the rate of a chemical reaction is the $Q_{10} = 2$ value stating that the rate doubles if the reaction temperature is increased by 10°C. As a

TABLE 2 | Selection of commercial ADH suppliers.

Supplier	Examples
Almac[a]	ADH screening KITs
Amano[b]	Several ADHs
Biocatalysts[c]	Phenylalanine Dehydrogenase, Mannitol Dehydrogenase
c-LEcta[d]	ADH screening KITs
Codexis[e]	ADH screening KITs
EnzymeWorks[f]	ADH screening KITs
Evoxx[g]	Several R- or S-selective ADHs covering aliphatic and aromatoic ketones, ketoesters and aldehydes
Gecco[h]	ADHA (*Pyrococcus furiosus*) *Tb*ADH (*Thermoanaerobacter brockii*) ADH*Mi* (*Mesotoga infera*) *Lb*ADH (*Lactobacillus brevis*) *Eh*ADH (*Entamoeba histolytica*) *Gt*ADH (*Geobacillus thermodenitrificans*) *Pp*ADH (*Pseudonomas putida*) *Rj*ADH (*Rhodococcus jostii*)
Johnson Matthey[i]	17 alcohol dehydrogenase (ADH) enzymes for the reduction of ketones and aldehydes to the corresponding alcohols. The enzymes in this kit belong to different protein folds (i.e., short-chain ADHs, zinc-containing ADHs, aldo-ketoreductases), both from prokaryotes and eukaryotes. Also included are enzymes identified from genomic and metagenomic samples and engineered enzymes
Prozomix[j]	Several ADHs and screening KITs
Sigma-Aldrich[k]	Several ADHs including HLADH, *Sc*ADH, *Ec*ADH

[a]*https://www.almacgroup.com/api-chemical-development/enzyme_kits/carbonyl-alcohol-interconversion/*
[b]*https://www.amano-enzyme.com*
[c]*https://www.biocatalysts.com/enzyme-products/*
[d]*https://www.c-lecta.com/products-services/products/customized-enzyme-c-lections/*
[e]*https://www.codexis.com/biocatalytic-enzymes/#Ketoreductases151026204116415103461166l7f911-9218*
[f]*http://www.enzymeworking.com/page98.html?_l=en*
[g]*https://evoxx.com/products/biocatalysis/alcohol-dehydrogenase/*
[h]*https://www.gecco-biotech.com/product/alcohol_dehydrogenases/*
[i]*https://matthey.com/en/products-and-services/pharmaceutical-and-medical/catalysts/co-reduction-kit*
[j]*http://www.prozomix.com/products/listing?searchby=application&searchby_application=456&category=21&x=29&y=9*
[k]*http://sigmaaldrich.com*

result, exponential increase of the rate of ADH-catalysed reactions with temperature is to be expected. However, also the thermal degradation of enzymes (such as ADHs) represents a chemical process accelerating with temperature. Hence, the optimal temperature for an ADH should be at the intersection of the (increasing) activity curve and the decreasing stability curve of an enzyme (Almeida and Marana, 2019).

Solvents: The effect of solvents on the activity/stability and selectivity of ADHs is difficult to predict. Different enzymes are differently affected by a given solvent whereas a given enzyme usually displays variable tolerance to different solvents. One study on the effect of water-immiscible organic solvents on three ADHs showed a higher or even increased stability in the presence of ethers whereas detrimental effects were observed for aromatic and aliphatic hydrocarbons and halogenated solvents (Villela Filho et al., 2003). Many ADHs exhibit decreased activity and stability in the presence of water-miscible organic solvents (Miroliaei and Nemat-Gorgani, 2002; Gröger et al., 2003; Schumacher et al., 2006; Li Y. et al., 2021). For instance, 1,4-dioxane and acetonitrile have been found to negatively affect stability and activity of *Lb*ADH but at the same time to also reduce substrate inhibition (Schumacher et al., 2006). Especially ADHs from thermophilic host organisms such as the ADH from *Pyrococcus furiosus* tend to exhibit higher solvent-stability compared to their mesophilic counterparts (Zhu et al., 2006b) but also ADHs from mesophilic origins can exhibit considerable robustness against water soluble cosolvents(Yan et al., 2021).

2.3 Sources of ADHs

ADHs are ubiquitous in all three domains of life—Archea, Bacteria and Eukarya (Machielsen et al., 2006; Gaona-López et al., 2016; Thompson et al., 2018). In the early days of biocatalysis, naturally available enzymes have been used; the ADH from horse liver (HLADH) (Batelli and Stern, 1910; Lutwak-Mann, 1938) or the ADH from yeast (YADH) (de Smidt et al., 2008) being some prominent examples. With the advent of molecular biology and the possibility to recombinantly express enzymes of interest in well-characterised expression hosts, this situation has changed dramatically in the past 30 years (Bornscheuer et al., 2012). Today, recombinant expression of almost any gene of interest in robust and efficient expression hosts such as *E. coli*, *S. cerevisiae* or *P. pastoris* is possible and has become a standard technique. This has also made ADH production independent from seasonal changes as faced with plant sources, ethical issues as in case of animal-derived enzymes or fundamental issues as in case of (yet) unculturable microorganisms (Singh et al., 2016; Bodor et al., 2020). As a result, today, an enormous versatility of wild-type ADHs is available, which is also reflected by the variety of enzymes mentioned throughout this contribution. Various commercial suppliers also offer ADHs in their portfolios (**Table 2**).

The seemingly high costs of enzymes compared to "classical" chemical catalysts remains to be a persistent myth. Indeed, the prices found for small amounts of ADHs (and enzymes in general) often significantly exceed those of chemical components. It should, however, be kept in mind that enzyme production is subject to economy of scale (Tufvesson et al., 2010). Enzymes produced at small scale, where labour and equipment costs dominate the cost structure of enzyme production, tend to range at 10.000 € kg^{-1} and higher. However, if produced at scale, enzyme costs can go as low as 250 € kg^{-1}. Furthermore, the cost contribution of a (bio)catalyst to the final product significantly depends on the catalyst's performance in the chemical transformation of interest. For example, an ADH produced at large scale will be economically feasible to produce pharma products if approx. 20–30 kg product can be obtained per kg of ADH. An enzyme produced at smaller scale will have to generate one ton of product per kg of enzyme to meet the maximal cost contribution allowable for pharma products.

The formulation of the ADH catalyst is directly linked to its cost. Especially in the academic literature, often highly purified ADHs are reported. Enzyme purification, however, also in times of affinity tags still represents additional efforts and costs that can easily add up to more than five times of the enzyme fermentation costs (Tufvesson et al., 2010). Therefore, the majority of commercialised ADH preparations are crude cell extracts rather than purified ADHs. Provided that none of the "contaminating" metabolites and enzymes negatively influence the reaction of interest, crude extracts are usually more an advantage rather than a disadvantage as these preparations contain the nicotinamide cofactor thereby often making additional supplementation with $NAD(P)^+$/NAD(P)H superfluous. Nevertheless, a commonly observed obstacle with crude enzyme preparations is the presence of endogeneous ADHs from the expression host, which may exhibit activity on the target starting material but not necessarily the desired selectivity. In such cases (partial) purification is almost inevitable.

3 ENGINEERING OF ADHS AND ADH REACTIONS

Despite the vast natural diversity of ADHs accessible nowadays from commercial sources, strain collections or databases a given wild-type ADH may not meet the requirements for cost-efficient synthesis of the desired target product. Enzymes have evolved to meet the needs of their host organism for survival and not the needs of an organic chemist. Fortunately, protein engineering has made tremendous advances in the past 20 years (Fasan et al., 2019; Acevedo-Rocha et al., 2020). Adapted enzymes that fulfil the needs of organic chemists can be built by directed evolution or semi-rational design using diverse standard molecular biology techniques, computational modelling and bioinformatics. In directed evolution experiments, random mutations are introduced into the parent gene; the resulting enzyme variants are investigated and selected for improvements in the desired property, finally serving as parents for following evolutionary rounds. The random nature of this approach yields many variants with mutations in irrelevant positions for the desired property which is why huge mutant libraries have to be screened. This makes pure random directed evolutionary approaches very time- and resource-intensive. Smaller libraries of higher quality can be achieved by restricting the mutation sites based on structural or mechanistic information. This semi-rational approach needs prior knowledge (mostly crystal structures or homology models of the enzyme) to determine the locations for randomisation. In turn, the library sizes (as well as the screening efforts) are greatly reduced. Fully rational design of improved variants using *in silico* methods are yet in their infancy (Bornscheuer et al., 2012; Hammer et al., 2017; Li et al., 2018; Fasan et al., 2019; Yang et al., 2019; Acevedo-Rocha et al., 2020; Bell et al., 2021).

Next to engineering the biocatalyst itself, also engineering the reaction conditions, e.g., by adjusting the solvent composition or immobilising the biocatalysts can have a decisive effect on the practicability of an ADH-catalysed redox reaction.

Some selected examples of enzyme- and reaction engineering will be discussed in the following paragraphs.

3.1 Protein Engineering

Substrate scope enlargement. A broad substrate scope is a key requirement for an enzyme to be an effective biocatalyst to foster their application in organic synthesis. For instance, the secondary alcohol dehydrogenase from the thermophilic bacterium *Thermoanaerobacter pseudoethanolicus* ATCC 33223 (*Te*ADH) is a very robust ADH exhibiting a rather narrow substrate scope (small substrates such as aliphatic ketones, **Figure 4**) (Musa et al., 2021). By analysing the active site architecture of wt-*Te*ADH Phillips and co-workers identified Trp110 to interfere with the binding of sterically more demanding starting materials. Indeed, exchanging this amino acid residue for a smaller alanine residue resulted in a *Te*ADH mutant accepting much larger substrates than the wild type while not being impaired in its thermal stability. A further mutant of this enzyme (W110A/I86A) accommodated even bulkier starting materials and also influenced the stereoselectivity of the enzyme (**Figure 4**) (Musa et al., 2018).

Improving/reversing enantioselectivity. Similar to the substrate scope, the enantioselectivity of an ADH is a property intimately linked to the geometry of the active site. The company Codexis has for long excelled in improving enzymes for various properties using the protein sequence activity relationship (ProSAR) algorithm (Fox et al., 2007). Using ProSAR, they, for example, improved the enantioselectivity of *Lk*ADH for tetrahydrothiophene-3-one from 63% to 99.3% (Liang et al., 2010b). By simultaneously challenging the mutant libraries with increasing thermal- and solvent-stress, the final mutant enzyme also showed significantly improved properties here. Starting from wt-*Thermoethanolicus brokii* ADH, Reetz and coworkers applied triple code saturation mutagenesis to engineer it into an *S*- or *R*-selective enzyme for tetrahydrotfuran-3-one and similar starting materials (Sun et al., 2016). Using this enzyme Reetz and his team also demonstrated that enantiodiscrimination of sites further away from the carbonyl group can be achieved (Agudo et al., 2013).

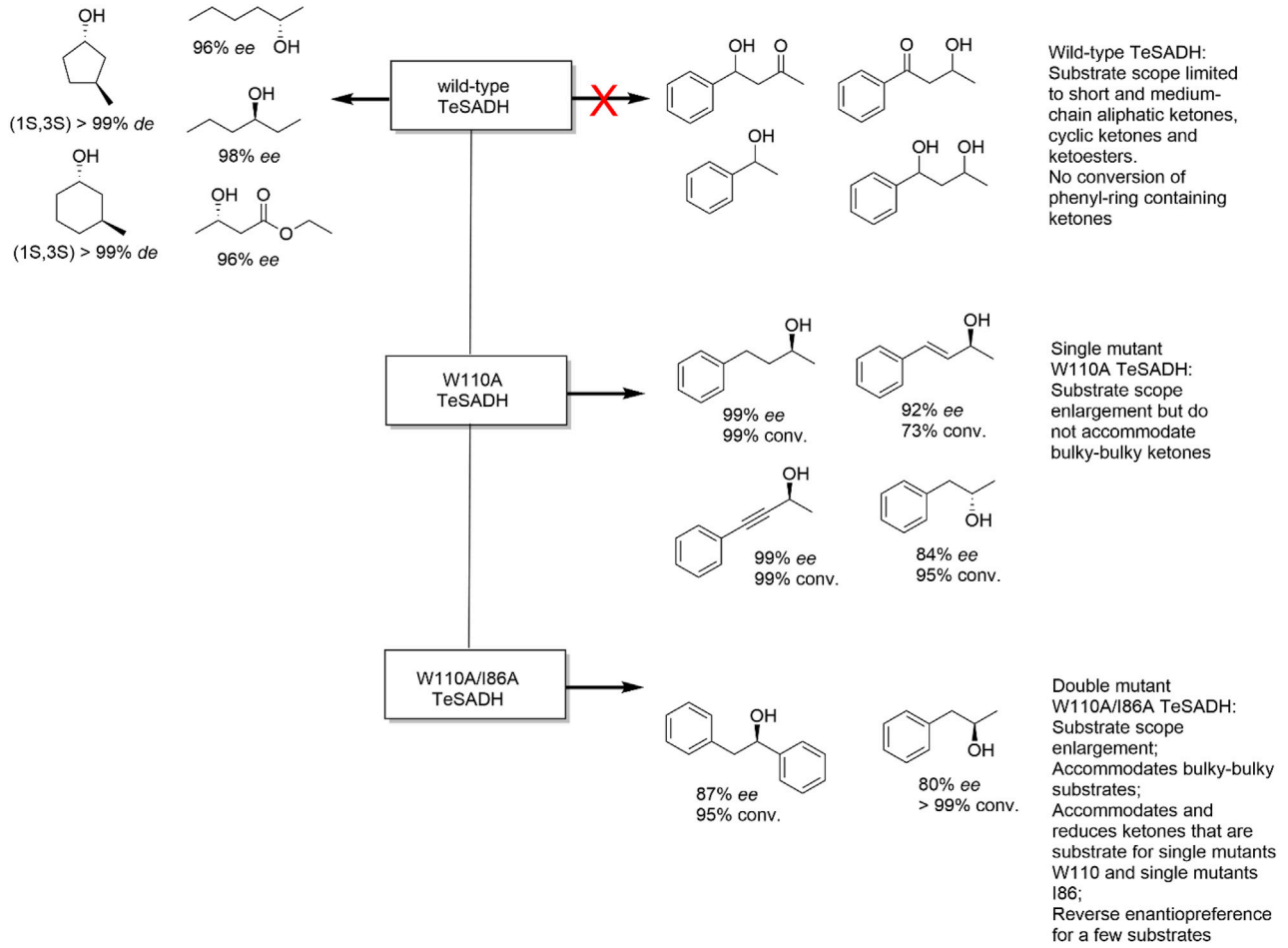


FIGURE 4 | Substrate scope enlargement and reverse enantiopreference achieved with single and dual mutants from *Te*SADH (*Thermoanaerobacter pseudoethanolicus* secondary alcohol dehydrogenase).

Axially chiral 4-alkylidene cyclohexanone represent an almost impossible target for enantioselective reduction chemistry. Using combinatorial active-site saturation test (CAST)-based directed evolution, highly *R*- and *S*-selective mutants were obtained.

Another impressive example of engineering the selectivity of an ADH was reported recently by Ni and coworkers (Xu et al., 2018). (4-Chlorophenyl) (pyridin-2-yl)methanone (CPMK) is widely recognised as "difficult" substrate due to the similar steric demand of both substituents of the carbonyl group impeding differentiation of the prochiral substrate faces. The authors chose the ADH from *Klyveromyces polysporus* (*Kp*ADH) exhibiting modest enantioselectivity in the reduction of CPMK (84% ee). In a first step they identified possible amino acid residues in the active site (using a homology model of the enzyme) that may be involved in substrate binding. From those, potential hotspots were identified via a combined alanine- and tyrosine-scanning. The six hotspots were submitted to combinatorial mutagenesis using reduced codon alphabets. Overall, several *Kp*ADH mutants with significantly improved enantioselectivity (99.4% ee) and increased catalytic activity compared to the wt-enzyme were identified.

Catalytic activity improvement. Recently, Zheng and co-workers reported engineering the ADH from *Lactobacillus kefir* (*Lk*ADH) for improved activity for the synthesis of (*S*)-2-chloro-1-(2,4-dichlorophenyl) ethanol [(*S*)-TCPE] from the corresponding ketone starting material (Zheng et al., 2021). wt-*Lk*ADH showed high enantioselectivity but rather poor catalytic activity. A rational design approach was applied based on the structural characteristics of the *Lk*ADH substrate binding complex. Molecular docking calculations were used to generate the binding structure of ADH and 2,2′,4′-trichloroacetophenone (TCAP) and to identify the key residues responsible for *Lk*ADH activity. Additionally, molecular dynamic simulations of the *Lk*ADH-substrate binding complex demonstrated that the substrate binding pocket in wt-*Lk*ADH should be reconfigured to allow TCAP to be well accommodated in the correct orientation with the 2,4-dichlorophenyl group being located at the large binding pocket and the chloromethyl group at the small one. Based on these studies, three sites, specifically A94 (located at the large binding pocket), E145 (located at the small binding pocket) and S96 (located at the loop responsible for modulating the open and

closed state of the binding pocket), were sequentially selected for site mutation and subsequently combined. The enantioselective triple mutant A94S/S96E/E145A displayed a 117- fold increase in relative activity compared to the wild-type enzyme.

Thermal and solvent stability improvement. Next to selectivity, robustness of a biocatalyst against hostile reaction conditions is one of the most desired properties in biocatalysis. Particularly, resistance against thermal inactivation and inactivation by organic solvents is of interest for the practical applicability of ADHs in organic synthesis.

Organic solvents can influence enzymes (ADHs) in many different ways (Li Y. et al., 2021). Water-immiscible solvents often inactivate enzymes at the solvent interface. The favourable interaction of hydrophobic, inner amino acids with the apolar organic solvent facilitates unfolding and thereby inactivation of the enzyme. Water-miscible solvents can interact in various ways with an enzyme and thereby influence its activity and stability: 1) the changed polarity of the solvent can alternate pKa values and thereby influence internal salt- or H-bridges relevant for catalysis/structural integrity of the enzyme; 2) disrupt hydrophobic interactions in the enzyme and 3) influence the enzyme flexibility by displacing water molecules.

Evolution of solvent-resistance is feasible as, e.g., demonstrated by coworkers of the company Codexis who engineered ADHs for broadened substrate scope and improved solvent stability (Liang et al., 2010a; Ma et al., 2010).

Interestingly, thermal stability and resistance against co-solvents often are correlated, possibly because of the similarities between the two protein unfolding mechanisms (Li S. F. et al., 2021). Nestl and Hauer pointed out the importance of flexible surface regions for stability suggesting to focus on these regions for improving thermal stability of an enzyme (Nestl and Hauer, 2014).

The B value approach developed by Reetz and coworkers addresses such regions by identifying flexible enzyme regions based on atomic displacement parameters of crystallographic data reflecting the smearing of electron densities due to thermal movement (Reetz et al., 2006).

Using an *in silico* method to identify promising residues for thermal stability improvement (FRESCO), Fraaije and coworkers recently reported an impressive stabilisation of ADH-A by 45°C without impairing the catalytic activity at ambient temperature (Aalbers et al., 2020). This is remarkable insofar as often increases in thermostability come with simultaneous decreases in the mutant's enzyme activity at ambient temperature (Machielsen et al., 2006; Willies et al., 2010; Siddiqui, 2017).

In case of the tetrameric ADH from *Leifsonia* (*Ln*ADH) engineering of amino acids involved in the subunit contact areas proofed to be successful (Zhu et al., 2021).

3.2 ADH Immobilisation

Immobilisation has been (and remains) a very active subfield of ADH research. The main motivation to heterogenise enzymes including ADHs is to increase their robustness under hostile, non-natural but industrially relevant reaction conditions such as high reagent concentrations and elevated reaction temperatures (Hanefeld et al., 2009; Sheldon and Pereira, 2017). Further advantages of immobilised enzymes over their soluble counterparts are that they can be applied in continuous processes and/or in repetitive batch reactions, can easily be separated from the reaction mixtures and reused. Immobilised enzymes are also advantageous in multi-step cascade reactions if temporally or spatially separated reaction steps are necessary.

Principally, three immobilisation principles can be distinguished: 1) Entrapment, 2) Immobilisation to a carrier and 3) heterogenisation via crosslinking (**Figure 5**).

Entrapment: Enzymes and also cells can be entrapped in both inorganic and organic polymeric matrices. This is most commonly achieved by preparing the carrier in the presence of the enzymes, so that they become embedded in the matrix lattice. Alternatively, enzymes can also be absorbed in pre-fabricated superabsorbent polymers. As a disadvantage of entrapment, leaching of enzymes is prone to occur, an issue that can be addressed by providing additional covalent binding or by increasing the protein size through cross-linking (Sheldon and Woodley, 2018). However, because of weaker binding forces involved in immobilisation, deactivation of the enzyme by conformational distortions is less likely in comparison with other approaches. Entrapment of ADHs with various gelating materials comprising silica gels (Musa et al., 2007; Nagy-Gyor et al., 2018; Liu et al., 2019; Nagy-Győr et al., 2019) polyvinyl alcohol (Krasnan et al., 2016; Petrovicova et al., 2018; Yildirim et al., 2019) poly ethyleneglycol (Schmieg et al., 2019) superabsorbers (Heidlindemann et al., 2014; Adebar and Groger, 2020), alginates (Milagre et al., 2005, 2006; He et al., 2017; Nasario et al., 2019), metal organic frameworks (MOFs) (Carucci et al., 2018; Ye et al., 2020) or liposomes (Yoshimoto et al., 2008) has been reported.

Immobilisation onto solid carriers. Solid carriers employed for immobilisation include inorganic materials, such as silica-based materials, kaolin or zeolites; organic materials, such as porous acrylic resins, polystyrene polymers and water-soluble natural polymers like cellulose and chitosan; and coordination polymers, particularly metal-organic frameworks (Sheldon and van Pelt, 2013). Inorganic carriers usually display high thermo-, chemo- and mechanical stability. They are also available as nanoparticles, including biocompatible gold nanoparticles and iron-based magnetic nanoparticles, which can offer advantages regarding enzyme loadings and mass transfer due to their high surface-to-volume ratios and controllable sizes (Sheldon and van Pelt, 2013). Some inorganic carriers, like mesoporous silica, present uniform pore diameter, high surface areas as well as high pore volume, so that relatively small enzymes can be immobilised both on the surface and inside the pores. For instance, a study on immobilisation of ADH showed that more enzymes were distributed inside the pores of a mesostructured cellular foam (MCFs) in comparison to ordered mesoporous silica particles such as SBA-15, suggesting MCF as a superior silica-based carrier for immobilisation of ADHs and similarly sized enzymes with higher loading (Zezzi do Valle Gomes and Palmqvist, 2017). Among organic polymers, synthetic acrylic resins are widely employed in enzyme immobilisation, including commercially available hydrophilic acrylic resins with mechanical and thermal stability and

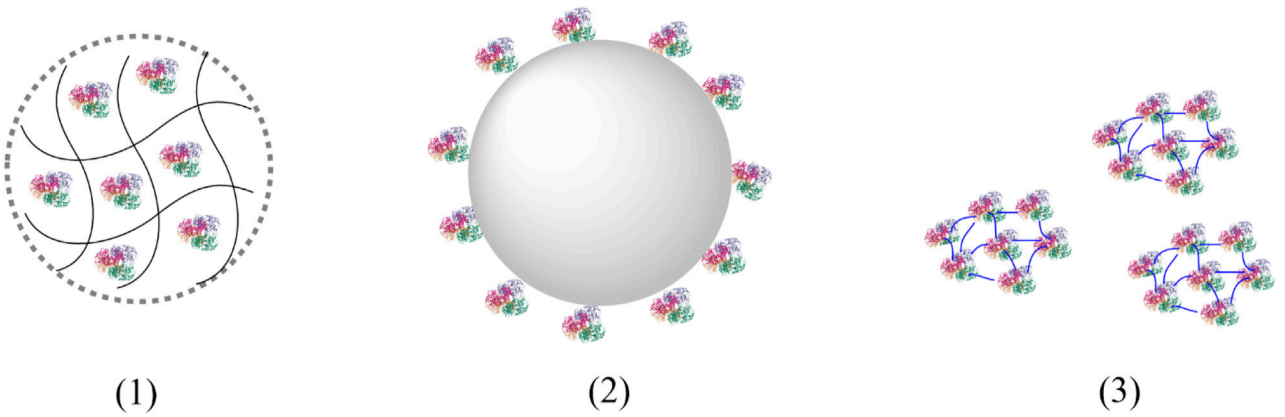


FIGURE 5 | Immobilisation principles. (1) Entrapment of enzymes in a polymeric matrix; (2) Immobilisation of enzymes to a carrier; (3) Cross-linked enzymes aggregates (CLEAs).

tolerance to organic solvents and to a wide pH range. Natural polymers have also been used and have the advantage of being biocompatible and biodegradable. They are mostly hydrophilic and thereby associated to a lower susceptibility to cause enzyme deactivation in comparison to hydrophobic carriers, which may induce detrimental conformational changes in proteins, leading to denaturation and activity loss.

Immobilisation onto solid carrier materials may occur *via* (reversible) physical adsorption based on electrostatic or hydrophobic interactions or (irreversibly) *via* covalent attachment. Physical adsorption can usually be achieved with the use of simple protocols not requiring chemical modification of the carrier or the enzyme. However, susceptibility to enzyme leaching, particularly in aqueous media, limits its applicability in most biocatalytic reactions under conditions employed in industrial processes. Nevertheless, some example of immobilisation of ADHs through adsorption for synthetic purposes have been reported, including immobilisation on raw inorganic materials, such as Al_2O_3 and TiO_2 (Sigurdardóttir et al., 2019), carbomethyl dextran-coated magnetic nanoparticles (Vasic et al., 2020) and silica gel (Liu et al., 2019). Adsorption of ADHs through ionic binding onto polymers bearing cationic or anioninc groups has also been described (Dreifke et al., 2017). Importantly, electrostatic adsorption is a promising strategy for the immobilisation of negatively-charged phosphorylated cofactors, such as NADPH, within porous materials modified with positively charged groups (Benítez-Mateos et al., 2017; López-Gallego et al., 2017; Velasco-Lozano et al., 2017). Because of the reversible nature of such binding, the cofactor molecules are allowed to diffuse across the intra-porous space without leaving the support, thus being available for a co-immobilised enzyme.

Recombinant enzymes bearing a His-tag, i.e., a terminal sequence of six to nine histidine residues, can be non-covalently immobilised on polymers containing chelating groups loaded with metals such as Ni(II), Fe(III), Zn(II), Cu(II) and Co(II) (Sheldon and Pereira, 2017), polymers decorated with nitrile tri-acetic acid molecules and loaded with Ni(II) (Ni-NTA) (Homaei et al., 2013) and controlled porosity glass-based materials bearing chelated Fe(III), such as EziG^{TM} (Homaei et al., 2013; Cassimjee et al., 2014; Thompson et al., 2019) being commonly used carriers. This strategy usually leads to relatively high immobilisation yields in short times and also offers the possibility of performing the immobilisation directly from a crude cell extract, since it also serves as a purification step. As a disadvantage, metal leaching may be an issue in large scale processes. Examples of immobilisation of ADHs by this approach comprehend co-immobilisation of ADH and GDH on sepharose charged with Ni(II) to give reusable bienzymatic heterogeneous biocatalysts with improved operational and storage stability for bioreductions in flow reactors in semi-continuous mode (Plž et al., 2020); immobilisation of Co(II)-containing magnetic beads for use in minituarised packed-bed flow reactors (Peschke et al., 2019) and co-immobilisation of ADHs together with non-ADH enzymes in Co(II)-charged agarose microbeads (Velasco-Lozano et al., 2020) and EziG^{TM} (Böhmer et al., 2018) for cascade reactions.

Attaching enzymes to a carrier *via* covalent bonds provides a more stable binding in comparison to adsorption, preventing enzyme leaching and also leading to improved chemo- and thermostability due to multipoint attachment to the carrier. In addition, reactive groups on the surface of carriers used for this kind of immobilisation enable their modification by insertion of additional moieties or polymer-coating to confer them desirable properties such as hydrophilicity to improve enzyme stability (H. Orrego et al., 2020) On the other hand, conformational changes imposed to ADHs by covalent immobilisation and inappropriate enzyme orientation, together with mass transfer limitations, decreases activity retention and immobilisation efficiency. Interestingly, either improvement or decrease in stereoselectivity and even switch in enantiopreference have been reported to be caused by conformational changes after covalent immobilisation (Petkova et al., 2012). The covalent bonds between the enzyme and the carriers usually involve the reaction of nucleophilic amino acid residues on the surface of the protein, such as lysine or cysteine residues, with electrophilic

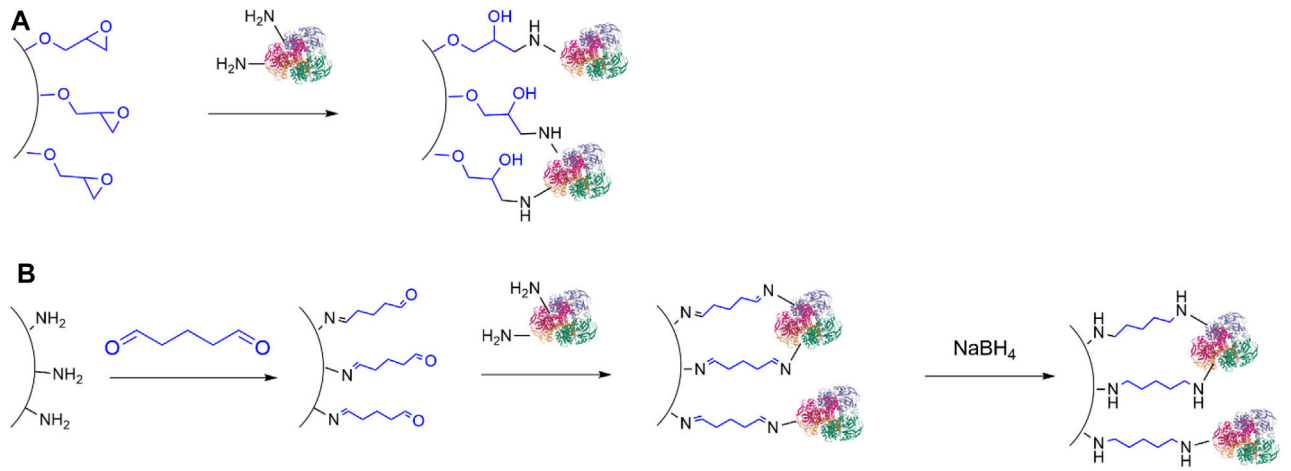


FIGURE 6 | Immobilisation of enzymes on solid carriers *via* covalent bond. **(A)** Attachment of enzymes to epoxy resins; **(B)** Attachment of enzymes to carriers bearing formyl moieties.

moieties on the surface of the carrier, such as Michael acceptors and oxirane and formyl groups (Homaei et al., 2013). Attention must be given to the tolerance of the enzyme to the reaction conditions required for some immobilisation procedures, such as alkaline pH, and to possible cross-linking between particles resulting in a lower available surface area and thereby increasing mass transfer limitations.

Covalent immobilisation of ADHs have been achieved with epoxy resins (**Figure 6A**), (Sole et al., 2019) including the commercially available Eupergit^{TM} (Boller et al., 2002). As an example, Truppo and co-workers (Li et al., 2015) described the immobilisation of an ADH on a commercial amino-epoxy-functionalised poly methacrylate resin to give a heterogeneous biocatalyst with improved tolerance to organic solvents and good recyclability, which could be used to perform the synthesis of 1-(3,5-bis(trifluoromethyl)phenyl)ethenone at 50g scale.

Another commonly used method for covalent immobilisation of ADHs (and enzymes in general) relies on the treatment of amino-functionalised carriers with a di-aldehyde, typically glutaraldehyde, which is then followed by reaction with lysine residues, so that both carrier and enzymes become bonded to a spacer through imine groups (**Figure 6B**). Alternatively, the use of a carrier bearing formyl groups, such as glyoxyl-functionalised supports (Mateo et al., 2006; H. Orrego et al., 2020) obviates the addition of a di-aldehyde. Further reduction of the imines to secondary amine moieties with sodium borohydride or the milder 2-picoline borane (Orrego et al., 2018) provides a more stable attachment between the enzymes and the carrier, though this step is not always performed. Importantly, these immobilisation methods require alkaline conditions, so that denaturation of enzymes may occur. This strategy has been used to immobilise ADHs on amino-functionalised titania nanoparticles (Ghannadi et al., 2019), silicon carbide (Zeuner et al., 2018) and nanoporous silica (Engelmann et al., 2020).

Similarly, ADHs have been successfully immobilised in agarose bearing formyl groups (Mateo et al., 2006; Velasco-Lozano et al., 2017; H. Orrego et al., 2020). For instance, Serra and coworkers (Dall'Oglio et al., 2017) described the immobilisation of an ADH and GDH (for cofactor regeneration) in glyoxyl-agarose. The immobilisates were used as a blend in a packed-bed flow reactor, which could be operated for 15 days for continuous bioreduction of ketones with only a slight decrease in conversion, thus demostrating the robustness of the immobilised biocatalysts.

López-Gallego and coworkers reported a self-sufficient heterogeneous biocatalyst by coimmobilising both enzyme and cofactor on glyoxyl-agarose macroporous beads (Orrego et al., 2021). After covalent immobilisation of a thermophilic (*S*)-2-hydroxybutyryl-CoA dehydrogenase from *Thermus thermophilus* HB27 (Tt27-HBDH), the remaining formyl groups on the carrier allowed for a subsequent coating with polyethyleneimine (PEI) to give a cationic layer in which NADH molecules could be embedded and reversibly immobilised through ionic interactions. The resulting biocatalyst could be used for ten cycles to produce (*S*)-ethyl 3-hydroxybutyrate with a productivity in the range of 0.066–0.027 g L^{-1} h^{-1} and a TTN of 145 for NADH (in constrast to a TTN limited to 10 when soluble, non-coimmobilised NADH was used).

CLEAs: CLEAs are prepared through precipitation of the enzymes as physical aggregates in aqueous media, without disturbing their tertiary structure, with the aid of salts or non-ionic polymers or by the addition of an organic water-soluble solvent (Sheldon, 2019; Sheldon et al., 2021). The enzyme molecules in the aggregates are then covalently cross-linked by the addition of a di-aldehyde. Typically, ammonium sulphate is used as an aggregant and glutaraldehyde as a cross-linking agent. They can be added together in the enzyme solution, since physical aggregation takes place much faster than the cross-linking reaction. As an advantage, immobilisation through CLEAs do not require a carrier, thus resulting in lower cost and in high productivities and space-time yields in comparison to previously discussed strategies, since "activity dilution" due to the addition

of a large amount of non-catalytic ballast is avoided. In addition, CLEAs are especially effective to improve stability of multimeric enzymes, have low susceptibility to enzyme leaching and usually present good diffusional properties due to their high porosity. Low mechanical robustness and the difficult to produce particles with uniform and suitable size, however, account for their disadvantages (Sheldon, 2019; Sheldon et al., 2021). An example of immobilisation of ADHs by producing CLEAs was provided by Shao and coworkers (Wu K. et al., 2021) who succeeded thereby in improving storage and thermo- and chemostability as well as tolerance to pH of a mutant short-chain dehydrogenase of *Novosphingobium aromaticivorans*. The immobilisate was used for the bioreduction of a chloroketone to give a chiral intermediate of the drug atazanavir with high stereoselectivity and a space-time yield of 226.6 g L^{-1} day^{-1} keeping 85.3% of conversion after being reused for six batches.

Two or more different enzymes can be immobilised in a single CLEA to give the so-called combi-CLEAs. This approach has been employed for the obtaining of bi-enzymatic immobilisates, including magnetic combi-CLEAs (Chen et al., 2018) bearing the main ADH and the cofactor regenerating enzyme (Ning et al., 2014; Zhang J. et al., 2020; Xu et al., 2020). As an example, Su et al. (2018) described the preparation of combi-CLEAs comprised of ketoreductase and GDH enzymes embedded with magnetic Fe_3O_4 nanoparticles and their use in the obtaining of 1.98 g of enantiopure ethyl (*S*)-4-chloro-3-hydroxybutyrate from its corresponding ketoester in 15 h with a TTN of NADH of 11.880. The bifunctional biocatalyst presented improved chemo- and mechanical stability, showing better recyclability than the non-magnetic combi-CLEAs.

3.3 Reaction Medium Engineering

Today, mostly aqueous reaction media are used for ADH-catalysis. Despite common perception, water is a sub-optimal solvent for many biocatalytic reactions. Many of the reagents of interest are rather hydrophobic and therefore rather poorly water-soluble. Today, a very common way around this challenge still is to utilise rather dilute reagent concentrations in the reaction mixtures (Holtmann and Hollmann, 2022). As a result product titres of less than 10 g L^{-1} and less are the rule rather than the exception in biocatalysis. This is unsustainable both from an economic (Huisman et al., 2010) and an environmental (Ni et al., 2014) point of view. Therefore, increasing the reagent concentrations represents a prime target en route to environmentally benign and economically attractive ADH-catalysed reactions.

Fortunately, in recent years, the pioneering works by Klibanov and coworkers are receiving a renewed interest from the research community (Zaks and Klibanov, 1984, 1985; Dordick et al., 1986). As early as the 1980s these authors demonstrated the applicability of various enzyme classes in non-aqueous media. Next to a larger choice of solvents and the increased solubility of the reagents of interest, non-aqueous media also offer advantages with respect to enzyme stability (especially at elevated temperatures). In case of ADH-catalysis the immobilisation of the nicotinamide cofactor within the ADH active site induced by the non-aqueous surrounding also eliminates a range of water-related inactivation mechanisms. Therefore, the number of ADH-reactions occurring in non-aqueous or micro-aqueous media is steadily increasing (de Gonzalo et al., 2007; Jakoblinnert et al., 2011; Musa and Phillips, 2011; Kara et al., 2014b; Heidlindemann et al., 2014; Spickermann et al., 2014; Popłoński et al., 2018).

In cases where non-aqueous reaction media are not straightforward to implement (e.g., because of issues with biocatalyst stability or –formulation) multiphase reaction systems offer an attractive alternative to improve the reagent payloads. Slurry-to-slurry reactions, for example, apply dispersions of solid, poorly water soluble reagents in aqueous (biocatalyst-containing) media. Provided the affinity of the enzyme for the starting material is sufficiently high (K_M in the range of the equilibrium solubility or lower) the rate of the biocatalytic transformation is not impaired severely. An elegant example was reported by researchers from Codexis converting a very poorly starting ketone (solubility <0.5 g L^{-1}) into the corresponding (not much more soluble) alcohol product; using the slurry-to-slurry concept substrate loadings of up to 100 g L^{-1} were converted efficiently into the desired product at 200 kg-scale (Liang et al., 2010a). An attractive feature of slurry-to-slurry reactions is of course that in principle a simple filtration step suffices for product isolation.

More commonly, two liquid phase systems are applied. Here, a hydrophobic, water-immiscible organic phase serves as substrate reservoir and product sink at the same time (**Figure 7**). 2LPS exhibit a range of advantages over simple monophasic reaction media): First, they enable overall higher reagent loadings and thereby increase the economic attractiveness and reduce water wastes. Furthermore, product separation from the catalysts is principally straightforward after phase separation (Wu et al., 2009; Kara et al., 2013c; Ou et al., 2019). Second, water-reactive substrates and products can (to some extent) be protected in the organic layer from, e.g., hydrolysis. Another attractive feature is that, in some cases the organic solvent can help to control the selectivity of multi-step reactions. For example, whole cell-catalysed oxidations of primary alcohols can be plagued by (undesired) overoxidation of the desired aldehyde due to the presence of endogeneous aldehyde dehydrogenases. This issue has been addressed using the 2 LPS approach, e.g., by Bühler et al. (2002), Bühler et al. (2003), Bühler and Schmid (2004) and Molinari et al. (1997), Gandolfi et al. (2001). In the presence of hydrophobic organic phases, the intermediate, hydrophobic aldehydes were efficiently extracted into the organic layer and were thereby not available for aldehyde-dehydrogenase-catalysed overoxidation to the acids.

3.4 Cofactor Regeneration

Ever since ADHs have moved into the focus of biocatalysis, cofactor regeneration strategies have been investigated (Chenault and Whitesides, 1987). The majority of ADHs considered today depend on either nicotinamide cofactor, either the non-phosphorylated (NAD^+/NADH) or the phosphorylated ($NADP^+$/NADPH) cofactor (**Figure 1**). Commercial nicotinamide cofactors are rather expensive, making their use in sub-stoichiometric amounts and *in situ* regeneration of the active redox state mandatory. Over the

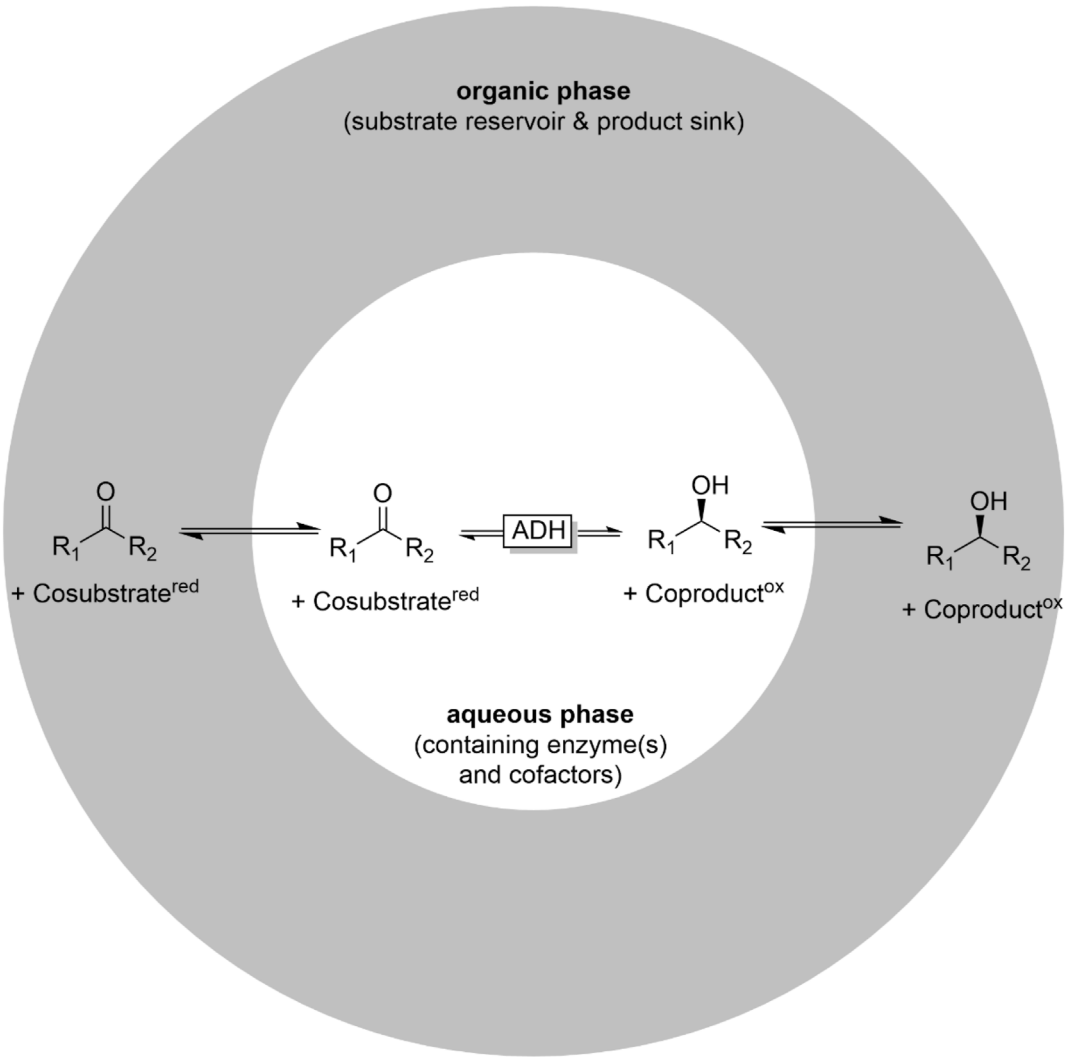


FIGURE 7 | The two liquid phase system (2LPS) approach combining an organic phase (hydrophobic solvent containing the reagents in high concentrations) and an aqueous (biocatalyst-containing) reaction phase.

decades (Chenault and Whitesides, 1987), a broad variety of *in situ* regeneration systems has been developed, which has been reviewed extensively (Wichmann and Vasic-Racki, 2005; Kara et al., 2014a; Zhang and Hollmann, 2018). Therefore, in this contribution we focus on the most relevant ones with a particular focus on practicability and environmental impact.

3.5 Regeneration of Reduced Nicotinamide Cofactors

The so-called substrate-coupled regeneration approach represents a very simple approach to regenerate NAD(P)H in ADH-catalysed reduction reactions (Wichmann and Vasic-Racki, 2005; Kara et al., 2013a). In this approach, an oxidisable cosubstrate (frequently isopropanol) is added to the reaction mixture enabling an overall biocatalytic variant of the Meerwein-Ponndorf-Verley reduction (**Figure 8**).

From a practical point-of-view this approach is very attractive as the production enzyme at the same time also serves as regeneration enzyme and that—in principle—the nicotinamide cofactor can stay bound to the enzyme active site. The latter is of interest for circumventing solvent-related degradation of the cofactor (Chenault and Whitesides, 1987) and enables using the enzyme under non aqueous conditions. Since the ADH reaction is reversible, the same approach can also be used to promote ADH-catalysed oxidation reactions (e.g., using isopropanol as cosubstrate).

On the downside, the reversibility of the reaction results in a rather unfavourable equilibrium of the overall reaction. Chemically, the substrate/cosubstrate couple is very similar to the product/coproduct couple (essentially consisting of an alcohol and a carbonyl compound) resulting in an equilibrium constant around one. To shift the equilibrium to the desired side, Le Chatelier measures such as the removal of one product from the reaction mixture can be taken. But most frequently, the

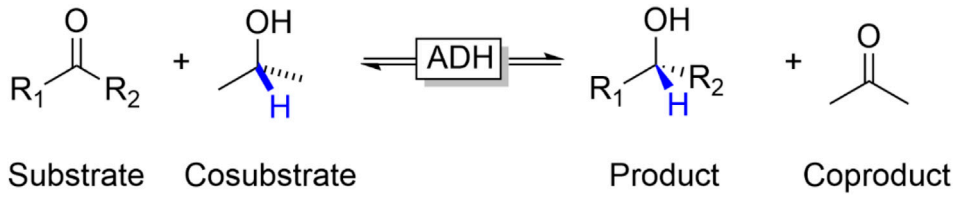


FIGURE 8 | Substrate-coupled reduction of carbonyl groups (aldehydes, ketones) to the corresponding alcohols using a sacrificial alcohol cosubstrate (such as isopropanol) as stoichiometric reductant.

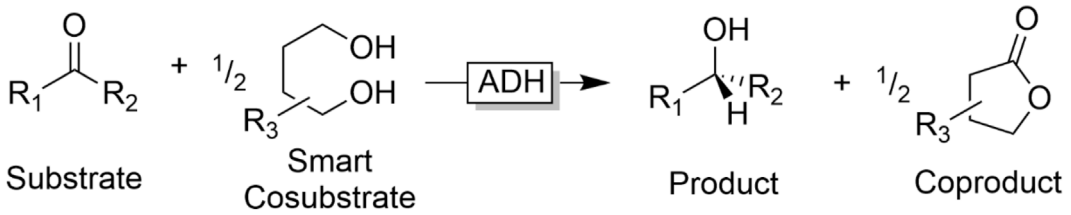
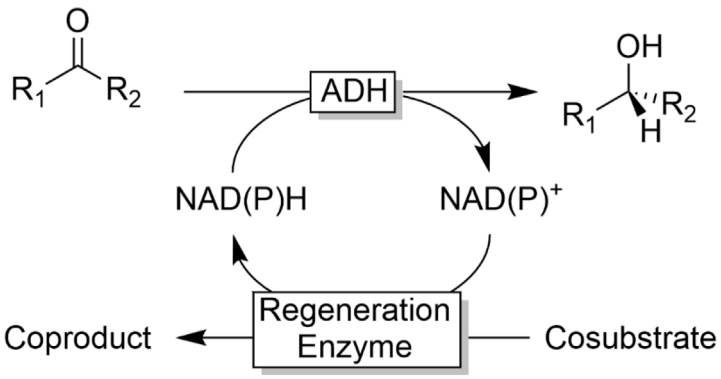


FIGURE 9 | Smart Cosubstrates to turn ADH-catalysed reduction kinetically and thermodynamically irreversible.



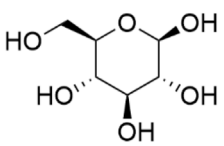
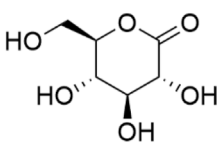
Cosubstrate	Coproduct	Regeneration Enzyme
		Glucose dehydrogenase
HCO_2H	CO_2	Formate dehydrogenase
H_2	-	Hydrogenase

FIGURE 10 | Selection of some common enzyme-coupled NAD(P)H regeneration approaches.

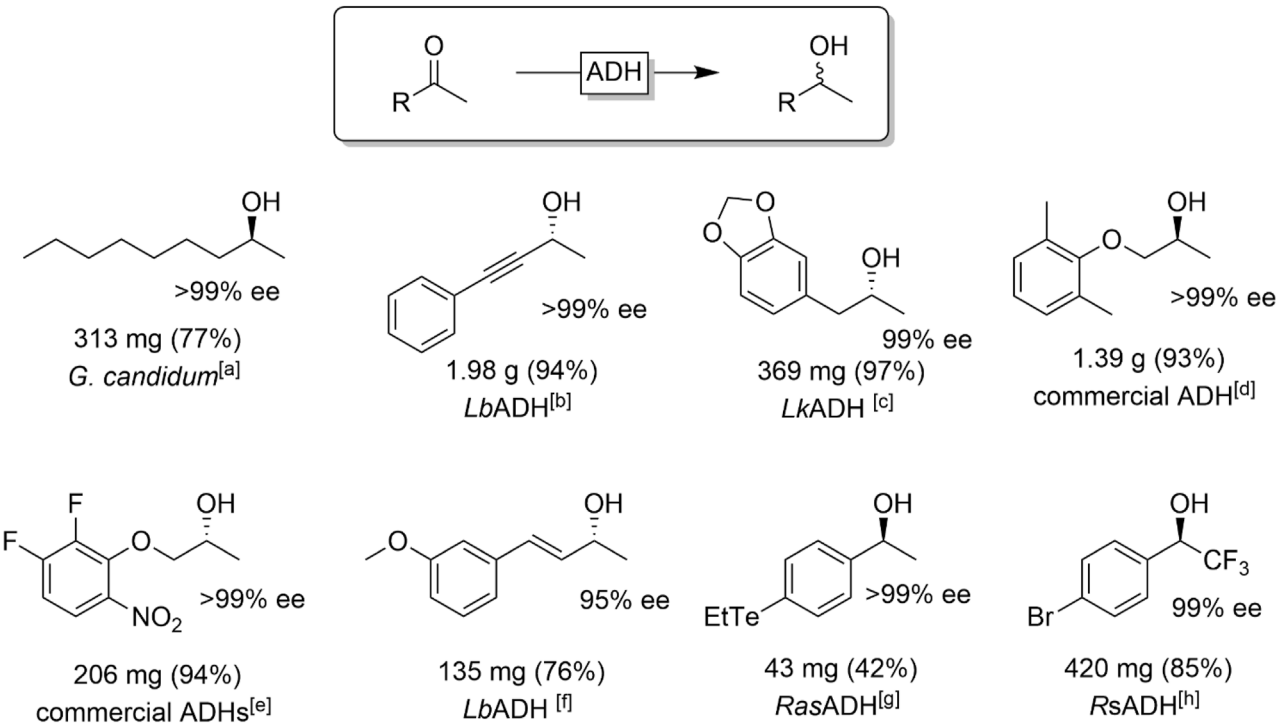


FIGURE 11 | Selection of ADH-catalysed reductions of methyl ketones. [a] *Geotrichum candidum* acetone preparations (Nakamura and Matsuda, 1998), [b] *Lb*ADH (from *Lactobacillus brevis*) (Schubert et al., 2001), [c] *Lk*ADH (from *Lactobacillus kefir*) (Simon et al., 2014), [d] ADH from the commercial ADH screening KIT 'Chiralscreen OH' (Nagai et al., 2018), [e]ADH-A (López-Iglesias et al., 2015), [f] *Lb*ADH (Albarrán-Velo et al., 2020) [g] *Ras*ADH (from *Ralstonia* sp.) (Bandeira et al., 2020), [h] *Rs*ADH (from *Rhodococcus* sp.) (Rosen et al., 2006).

cosubstrate is used in significant (more than 20-fold) excess to shift the equilibrium. "Smart cosubstrates" represent an interesting solution to this thermodynamic challenge. Kara et al., for example, developed a system comprising lactonisable diols as 'smart cosubstrates' (**Figure 9**) (Kara et al., 2013b; Zuhse et al., 2015; Huang et al., 2018).

"Smart cosubstrates" enable a dramatic reduction in the cosubstrate loading for several reasons: on the one hand, the lactone coproduct is very unreactive for thermodynamic and kinetic reasons. On the other hand, as the "smart cosubstrate" is oxidised twice by the ADH, only 0.5 equivalents are needed to obtain full conversion of the substrate.

The reversibility issue of ADH-catalysed reduction reactions is also frequently solved via the so-called enzyme-coupled NAD(P)H regeneration approach (**Figure 10**) (Wichmann and Vasic-Racki, 2005; Kara et al., 2013a) Here, a second, irreversible $NAD(P)^+$-dependent oxidation reaction is used to regenerate the reduced nicotinamide cofactor. The most popular enzyme systems right now are glucose-dehydrogenase (Sorgedrager et al., 2008; Liang et al., 2010b; Huisman et al., 2010; Gong et al., 2017; Chen et al., 2021) and formate dehydrogenase (Shaked and Whitesides, 1980; Seelbach et al., 1996; Gröger et al., 2004; Jiang et al., 2020). Very recently, Vincent and coworkers reported significant advances in the use of hydrogenases (Reeve et al., 2012; Reeve et al., 2017; Zor et al., 2017; Zhao et al., 2021) enabling H_2 as cosubstrates.

3.6 Regeneration of Reduced Nicotinamide Cofactors

Regeneration of oxidised nicotinamide cofactors. Similarly to reductive ADH reactions, also for oxidative ADH processes, a range of enzyme- and substrate coupled approaches have been reported. The enzyme-coupled approach principally suffers from the same thermodynamic challenges as discussed above. Kroutil and coworkers therefore devised a "smart cosubstrate" approach based on α-halo substituted ketones as sacrificial electron acceptors (Lavandera et al., 2008b; Kurina-Sanz et al., 2009). The corresponding *vic*-halo alkanols are thermodynamically stabilised through intramolecular H-bonds thereby efficiently shifting the overall equilibrium.

In contrast to the regeneration of the reduced nicotinamide cofactors, the opposite reaction (regeneration of $NAD(P)^+$ from NAD(P)H) is not plagued by regioselectivity issues and the aromatic $NAD(P)^+$ can be formed. This is also why "chemical" regeneration systems represent a viable alternative to enzymatic counterparts. The preferred stoichiometric electron acceptor is molecular oxygen because of the high thermodynamic driving force of O_2 reduction on the one hand and because of the favorable byproducts (H_2O or H_2O_2, which is generally

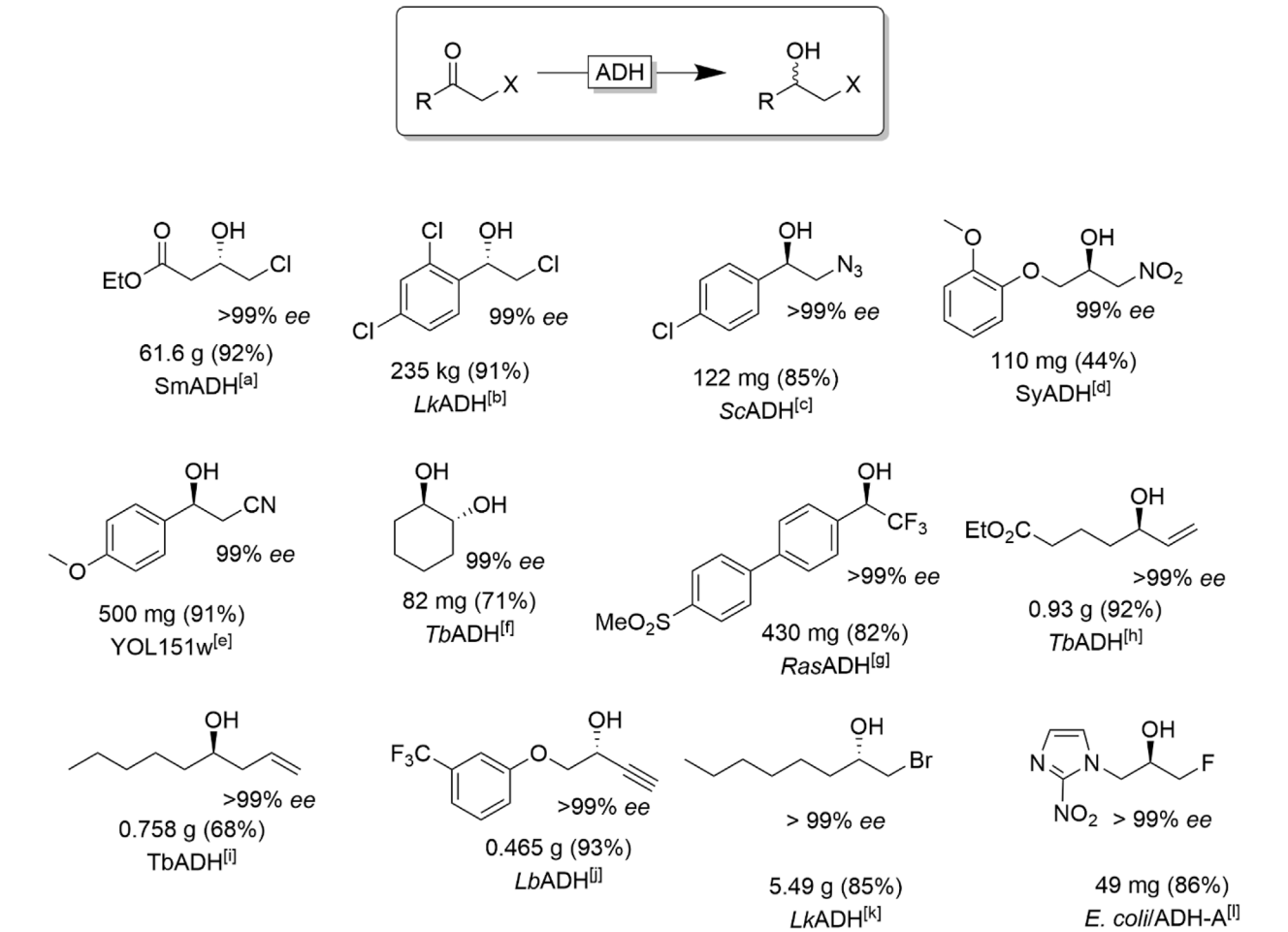


FIGURE 12 | Selection of chiral α-functionalised alcohols obtained *via* stereoselective reduction of the corresponding ketone. [a] *Sm*ADH from *Stenotrophomonas maltophilia* (Yang et al., 2020), [b] engineered *Lk*ADH (from *Lactobacillus kefir*) (Zheng et al., 2021), [c] *Sc*ADH (from *Saccharomyces cerevisiae*) (Ankati et al., 2008), [d] (Wang et al., 2019), [e] YOL151w: Yeast ADH expressed in *E. coli* (Nowill et al., 2011), [f] (Zhang et al., 2018) [g] (Gonzalez-Martinez et al., 2019) [h] (Fischer and Pietruszka, 2012) [i] *Tb*ADH (from *Thermoanaerobacter brokii*) (Bisterfeld et al., 2017), [j] *Lb*ADH (from *Lactobacillus brevis*)(Holec et al., 2015). [k] engineered *Lk*ADH (from *Lactobacillus kefir*), (Berkessel et al., 2007), [l] overexpressed ADH-A (from *Rhodococcus ruber*) (Borzecka et al., 2013).

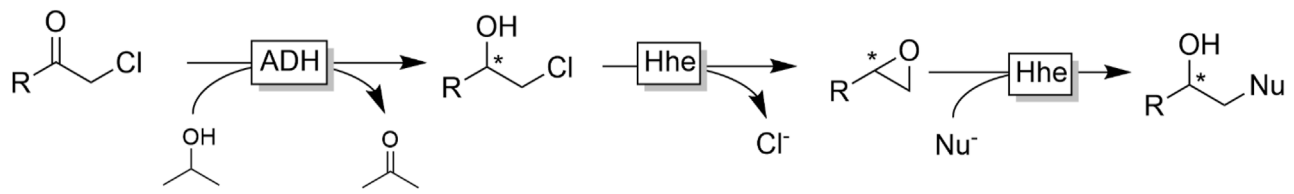


FIGURE 13 | Bienzymatic cascade to transform α-chloroketones into β-substituted chiral alcohols combining stereoselective ADH-catalysed reduction of the chloroketone with a halohydrin dehalogenase (Hhe)-catalysed substitution of the chlorine with a nucleophile (occurring *via* an intermediate epoxide).

dismutated into O_2 and H_2O). Amongst the enzymatic $NAD(P)^+$ regeneration systems, clearly the so-called NAD(P)H oxidases dominate (Riebel et al., 2002; Riebel et al., 2003; Jiang and Bommarius, 2004; Park et al., 2011; Gao et al., 2012; Dias Gomes et al., 2019; Anderson et al., 2021). But also some monooxygenases (at first sight unexpected) can be used for the regeneration of $NAD(P)^+$ exploiting the (usually undesired) uncoupling reaction (Holtmann and Hollmann, 2016). For example, flavin-dependent monooxygenases (Ni et al., 2016) and ene reductases (Pesic et al., 2019) or heme-enzymes (Holec et al., 2016; Jia et al., 2017) can be used as $NAD(P)^+$ regeneration catalysts.

Amongst the chemical $NAD(P)^+$ regeneration catalysts redox-active dyes such as ABTS (Schröder et al., 2003; Aksu et al., 2009)

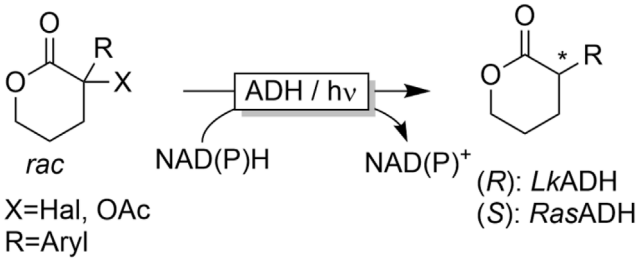


FIGURE 14 | Photoenzymatic reductive dehalogenation of α-substituted carbonyl compounds.

or quinoid dyes such as Meldola's blue (Kochius et al., 2012; Könst et al., 2013; Kochius et al., 2014) and others can be used. Most frequently used are the natural flavin cofactors (riboflavin, flavin adenine mononucleotide and flavin adenine dinucleotide) (Jones and Taylor, 1973; Jones and Taylor, 1976; Boratynski et al., 2010; Piantini et al., 2011; Boratynski et al., 2013), whose photoresponsiveness can be exploited to dramatically accelerate the oxidation of NAD(P)H to $NAD(P)^+$ (Gargiulo et al., 2011; Rauch et al., 2017).

4 ADH-CATALYSED REDUCTION REACTIONS

The stereoselective reduction of prochiral ketones is by far the most popular application of ADHs in organic synthesis. Especially in the synthesis of pharmaceutically active ingredients, ADHs are very popular due to their high selectivity (Raynbird et al., 2020). In the past years, thousands of publications have appeared reporting an ADH-catalysed ketoreduction reaction. Therefore, an exhaustive coverage of this vast literature landscape is not possible, and we restrict this passage to some representative examples and interesting concepts.

Ketones comprising one large and one small substituent at the carbonyl group are very common substrates for ADH-catalysed reductions. Especially methyl ketones are popular starting materials and a broad range of aliphatic (Sinha and Keinan, 1997), aromatic (Hamada et al., 2001; Stampfer et al., 2002; Hummel et al., 2003; Gonzalez-Martinez et al., 2019; Bandeira et al., 2020), and conjugated ketones (Schubert et al., 2001; Sgalla et al., 2007; Albarrán-Velo et al., 2020; González-Granda et al., 2021) have been reported. **Figure 11** shows a representative selection of chiral alcohols obtained from the reduction of methyl ketones.

Very recently, some of us proposed an extension of the ADH-catalysed stereoselective ketone reduction by a generation of the ketone starting material from non-functionalised starting materials (Xu et al., 2022). For this, we combined the peroxygenase-catalysed "through oxidation" of several alkylbenzenes to the corresponding acetophenones followed by the enantioselective reduction into the desired *R*- or *S*-alcohols. Instead of the peroxygenase also P450 monooxygenases (Both et al., 2016) or (photo)catalytic oxyfunctionalisation (Zhang et al., 2017; Gacs et al., 2019; Zhang et al., 2019; Albarrán-Velo et al., 2021) have been reported.

α-functionalised ketones represent another important class of ADH-starting materials (**Figure 12**). The resulting halohydrin products represent versatile building blocks *via* nucleophilic substitution reactions of the halides giving access to, e.g., epoxides (intramolecular substitution), amino alcohols or hydroxyl nitriles. Next to the often very high stereoselectivity of the reduction reaction another advantage of ADH-catalysis is that reductive dehalogenation reactions, frequently observed with transition metal catalysts, is generally not an issue (Erian et al., 2003; Mori et al., 2004).

Several aryl, benzyl and alkyl chlorohydrins (Hanson et al., 2005; Poessl et al., 2005; Berkessel et al., 2007; Schrittwieser et al., 2009a; Schrittwieser et al., 2009b; Zheng et al., 2021), and, to a lesser extent, bromo-(Mangas-Sánchez et al., 2011) and fluorohydrins (Borzecka et al., 2013) have been synthesised on (semi-)preparative scale.

The large repertoire of halohydrins derived from ADH-catalysed reduction of the corresponding ketones (**Figure 12**) also includes propargylic chlorohydrins (Schubert et al., 2002) and compounds bearing functional groups such as esters (Yang et al., 2020), amino-protected groups (Patel, 2001; de Miranda et al., 2015) and heteroaryl moieties (Borzecka et al., 2013). Importantly, some relevant chlorohydrins have been produced by ADH in practical scale, such as the enantiopure ethyl (*S*)-chloroacetoacetate (Yang et al., 2020), a chiral synthetic intermediate of the "blockbuster" drug atorvastatin, and (S)-2-chloro-1-(2,4-dichlorophenyl) ethanol (Zheng et al., 2021), an intermediate of the antifungal agent luliconazole.

In addition of halo-substituted alcohols also ß-azido- (Edegger et al., 2006a; Cuetos et al., 2013), ß-nitro alcohols (Tentori et al., 2018; Wang et al., 2019) and ß-nitrile alcohols (Ankati et al., 2008) are accessible. Reduction of α-hydroxy carbonyls results in chiral vicinal diols (Kihumbu et al., 2002; Wachtmeister et al., 2014; Kulig et al., 2019; Muschallik et al., 2020). Chiral 2,2,2-trifluoro-1-arylethanols are important motifs in medicinal chemistry and drug development and therefore have also extensively been investigated as targets for ADH-catalysis (Rosen et al., 2006; Hussain et al., 2008; Adebar et al., 2019; Gonzalez-Martinez et al., 2019).

An interesting application of the ADH-catalysed reduction of α-haloketones was established by Kroutil and coworkers (**Figure 13**) (Schrittwieser et al., 2009a) In the first step ADH-catalysed reduction of α-chloro ketones gave access to enantiomerically pure β-chloro alcohols. The latter are substrates for halohydrine dehalogenases (Hhe's) which reversibly dehalogenate them into epoxides. Utilising the reversibility of this step and shifting the equilibrium by an excess of alternative nucleophiles (such as azide or cyanide) a range of optically pure β-azido- and β-cyano-alcohols could be obtained in 50 mg scale.

A very unconventional conversion of α-substituted carbonyl compounds was developed by Hyster and coworkers (Emmanuel et al., 2016; Biegasiewicz et al., 2018). Upon illumination with blue light, ADH-bound NAD(P)H can serve as single electron donor. In case of α-halo- or α-acetoxy-substituents, serving as leaving groups, light-induced dehalogention or deacetoxylation reactions have been observed (**Figure 14**).

Dicarbonyl starting materials have found significant attention in the past years as substrates for ADH-catalysed reduction reactions (**Figure 15**).

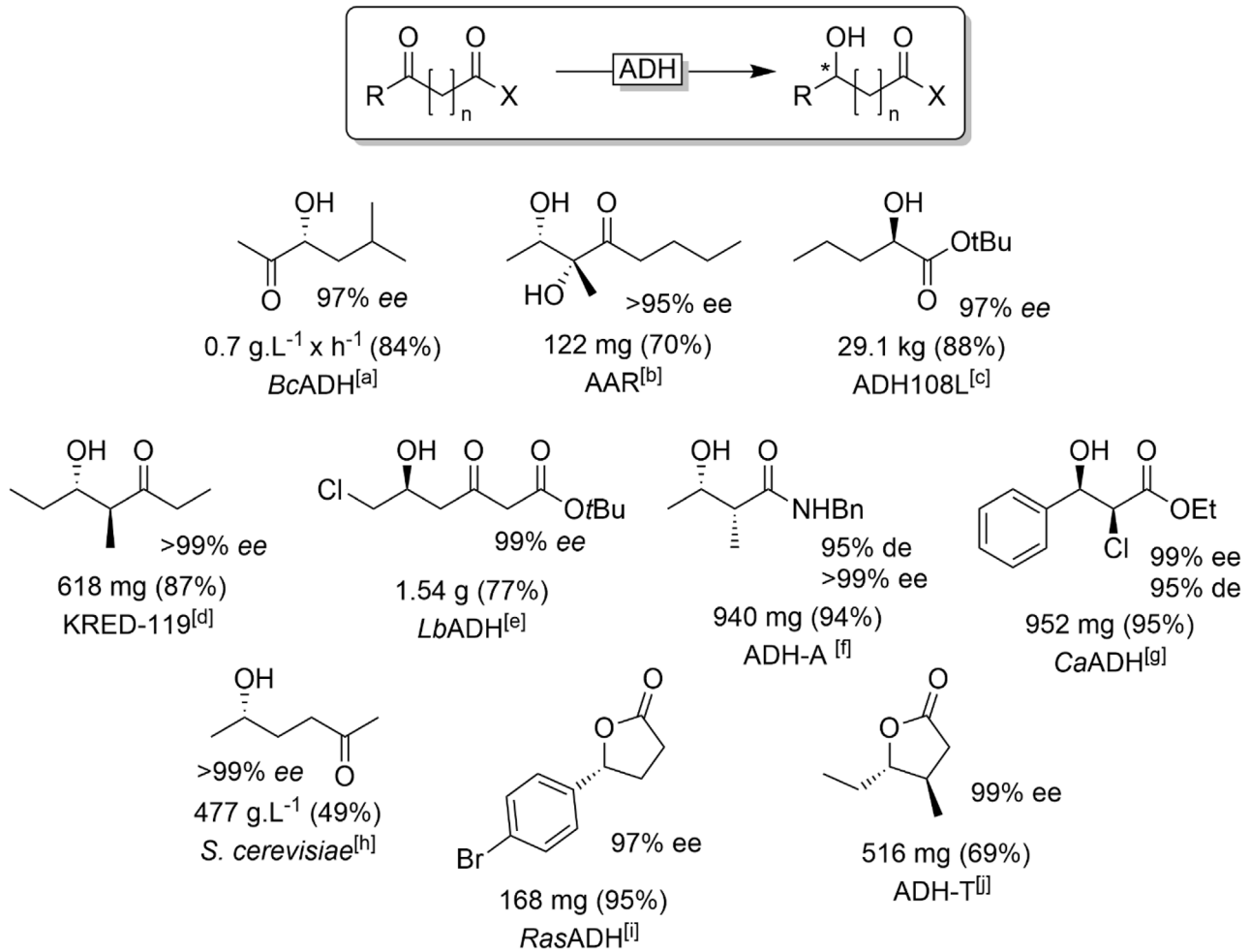


FIGURE 15 | Representative examples of products obtained from ADH-catalysed reduction of dicarbonyl compounds. [a] *Bc*ADH (from *Bacillus clausii*) (Muschallik et al., 2020), [b] AAR (acetylacetoinreductase) (Di Carmine et al., 2018), [c] commercial ADH (Burns et al., 2017), [d] KRED-119: commercial ADH (Kalaitzakis et al., 2006), [e] *Lb*ADH (from *Lactobacillus brevis*) (Wolberg et al., 2000), [f] ADH-A (from *Rhodococcus ruber*) (Méndez-Sánchez et al., 2019), [g] *Ca*ADH (from *Clostridium acetobutylicum*) (Applegate et al., 2011), [h] Baker's yeast (Katzberg et al., 2009), [i] *Ras*ADH (from *Ralstonia*) (Díaz-Rodríguez et al., 2014), [j] ADH-T (from *Thermoanaerobacter* sp.) (Kumru et al., 2018).

α-hydroxy acids have been synthesised from the corresponding keto acids (Zhu et al., 2006a; Pennacchio et al., 2008; Applegate et al., 2011; Burns et al., 2017). Starting from 1,2-diketo compounds, selective monoreduction to the corresponding α-hydroxyketone is challenging due to the frequently observed overreduction to the dialcohol (Hoyos et al., 2008; Monsalve et al., 2010; Pal et al., 2015) necessitating reaction engineering approaches to maximise the yield in the desired monoreduction product (Muschallik et al., 2020). Some new ADHs showing some potential for the selective monoreduction have been identified recently (Shanati et al., 2019). Similarly, β-hydroxy esters are accessible via various ADHs (Hummel et al., 2003; Muller, 2005; Zadlo et al., 2016; Wang et al., 2020) as well as ß-hydroxy ketones (Ludeke et al., 2009). The bioreduction of 1,4-diketones has been investigated for a long time already (Haberland et al., 2002a; Haberland et al., 2002b; Katzberg et al., 2009; Muller et al., 2010; Mourelle-Insua et al., 2018). Similar to 1,2-diketones, selective monoreduction so far has been achieved via kinetic control of the overall reaction.

α-substituted ß-diketones are prone to *in situ* racemisation of the enolisable C-H bond. This opens up the possibility of generating two chiral centers at the same time in a single ADH-catalysed reduction step provided the ADH exhibits stereoselectivity for both positions, i.e., preferentially converting one substrate isomer and performing the carbonyl reduction reaction stereoselectively (Ji et al., 2001; Kalaitzakis et al., 2006; Kalaitzakis and Smonou, 2010b; a; Giovannini et al., 2011; Kalaitzakis and Smonou, 2012; Méndez-Sánchez et al., 2019).

ɣ- and δ-ketoesters are attractive targets for ADH-catalysed reduction reactions as the corresponding alcohols easily (often spontaneously) undergo intramolecular esterification. The resulting lactones are common motifs in natural products (Korpak and Pietruszka, 2011; Fischer and Pietruszka, 2012; Classen et al., 2014; Díaz-Rodríguez et al., 2014; Kumru et al.,

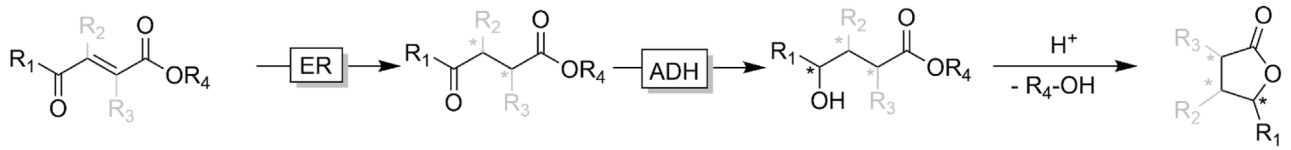


FIGURE 16 | Bienzymatic cascade transforming conjugated ketoacid esters into lactones. In the first step, an ene reductase (ER) catalysed the reduction of the conjugated C=C-bond followed by ADH-catalysed keto reduction and acid-catalysed intramolecular lactonisation. For reasons of simplicity, enzyme cofactors and absolute stereochemistries have been omitted.

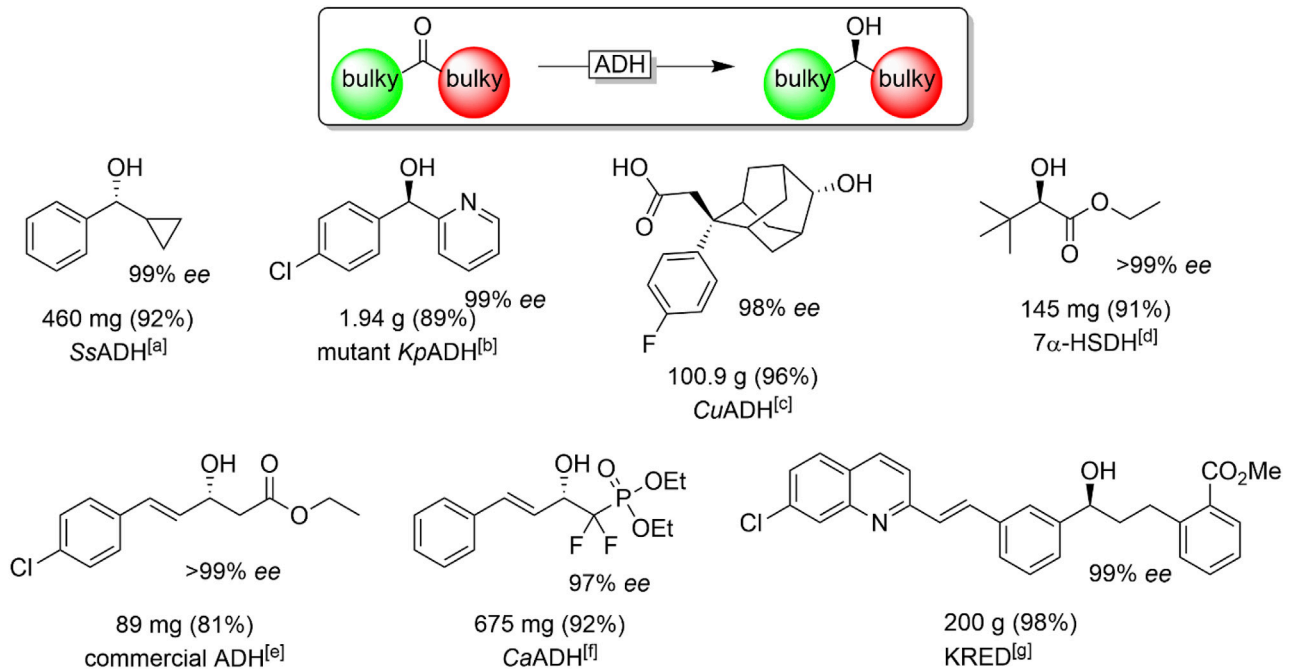


FIGURE 17 | Chiral alcohols synthesised through ADH-catalysed asymmetric reduction of "bulky-bulky" ketones. [a] *Ss*ADH (from *Sporobolomyces salmonicolor*) (Zhu and Hua, 2006), [b] *Kp*ADH (mutant of the ADH from *Kluyveromyces polysporus*) (Xu et al., 2018), [c] *Cu*ADH (from *Candida utilis*) (Hanson et al., 2014), [d] 7-αHSDH (from *Bacteroides fragilis*) (Zhu et al., 2006a), [e] (Dai et al., 2013) [f] *Ca*ADH (from *Clostridium acetobutylicum*) (Panigrahi et al., 2015), [g] KRED: evolved ADH from the company CODEXIS (Liang et al., 2010a).

2018; Borowiecki et al., 2020). Starting from synthetically easily accessible substituted conjugated ketoesters bienzymatic cascades comprising ene reductases (ERs) and ADHs enable access to a broad range of chiral ɣ-butyrolactone products (**Figure 16**) (Korpak and Pietruszka, 2011; Classen et al., 2014; Brenna et al., 2015).

α,β-unsaturated carbonyl groups are popular starting materials for ADH-ER-cascades. Successful realisation of such cascades critically depends on the suitable selection of selective ADHs as principally both, the α,β-unsaturated starting material and the saturated carbonyl compound can be converted by ADHs. Hence, utilising unselective ADHs results in complex product mixtures (Paul et al., 2013) necessitating sequential arrangement of the individual reduction steps. Using the "right" combination of selective ADHs and ERs, however, enables efficient one-pot one-step cascades (Brenna et al., 2012a; b).

Finally, the reduction of so-called "bulky-bulky"-ketones, i.e., sterically very demanding starting materials, are worth discussing here. These substrates have been a challenge for stereoselective reductions mediated by ADHs for quite some time. Nevertheless, some useful enzymes have been reported comprising the ADHs from *Sphingobium yanoikuyae* (*Sy*ADH) (Lavandera et al., 2008a; Cuetos et al., 2012; Man et al., 2014), *Ralstonia* sp. (*Ras*ADH) (Lavandera et al., 2008a; Cuetos et al., 2012; Man et al., 2014), *Sporobolomyces salmonicolor* (*Ss*ADH) (Zhu and Hua, 2006; Li et al., 2009; Li et al., 2010; Chen et al., 2012) *Kluyveromyces polysporus* (*Kp*ADH) (Xu et al., 2018) *Clostridium acetobutylicum* (*Ca*ADH) (Applegate et al., 2011), hydroxysteroid dehydrogenases (Zhu et al., 2006a; Ferrandi et al., 2020) and enzymes from *Kluyveromyces marxianus* (Li et al., 2019) or *Kluyveromyces polysporus* (Xu et al., 2018; Zhou et al., 2020). A selection of bulky ketones reduced by ADHs is given in **Figure 17**.

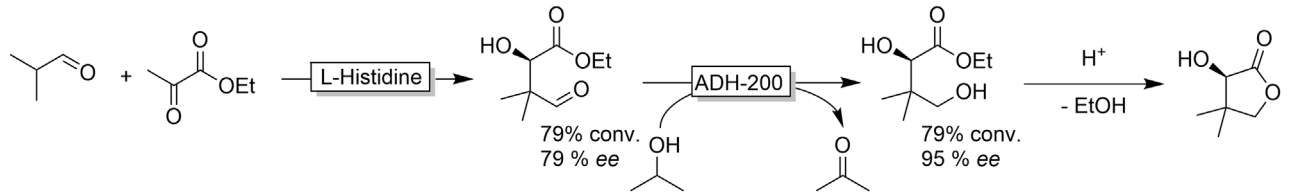


FIGURE 18 | Cascade reaction combining organocatalytic (L-histidine-catalysed aldol reaction) C-C-bond formation and ADH-catalysed aldehyde reduction followed by acid-catalysed lactonisation to form (*R*)-pantolactone.

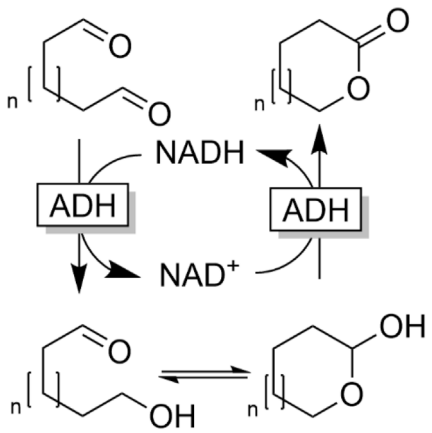


FIGURE 19 | Tishchenko-type dismutation of dialdehydes by ADH-catalysed redox-neutral conversion of dialdehydes into lactones.

Reduction of aldehydes. In contrast to the reduction of ketones, aldehyde reductions are far less popular, which can largely be attributed to the fact that generally the reduction of an aldehyde group does not result in the formation of a chiral centre (chiral alcohol).

Nevertheless, some interesting applications of ADH-catalysed aldehyde reduction have been reported. Gröger and coworkers, for example, established an organo/biocatalytic cascade to access (*R*)-pantolactone from simple starting materials (**Figure 18**) (Heidlindemann et al., 2015) The ADH-step partially functioned as kinetic resolution thereby upgrading the comparably poor optical purity of the organocatalytic product.

Hall and coworkers reported an ADH-catalysed, overall redox-neutral cascade reaction transforming dialdehydes into lactones (Tishchenko-type reaction, **Figure 19**) (Tassano et al., 2020).

Also starting from dialdehydes Zhang et al. established an elegant synthesis of optically pure cyclic diols by combining the pyruvate decarboxylase (PDC)-catalysed aldol-type ring closure and ADH-catalysed reduction of the intermediate cyclic α-hydroxy ketone (**Figure 20**) (Zhang et al., 2018).

ADHs often not only exhibit enantioselectivity with respect to the hydride transfer (yielding *R*- or *S*-alcohols) but can also discriminate between enantiomers at other positions. This has, for example, been exploited for the kinetic resolution of racemic aldehydes such as binaphthyls (Kawahara et al., 1988), biaryls (Yuan et al., 2010; Staniland et al., 2014) or ferrocenes (Yamazaki and Hosono, 1988). Aldehydes bearing a chiral centre in α-position represent attractive targets for stereoselective ADH-catalysed reduction especially under α-racemising conditions. Particularly the dynamic kinetic resolution of profene aldehydes to enantiomerically pure profene alcohols has been investigated by several groups (Giacomini et al., 2007; Friest et al., 2010; Rapp et al., 2021).

Reduction of acids. Carboxylic acids cannot be reduced by ADHs; the carboxylate group is thermodynamically and kinetically inert towards serving as hydride acceptor from NAD(P)H. Carboxylate reductases (CARs) circumvent this limitation by activating the carboxylate group as thioester (Winkler, 2018). Inspired by this strategy, we have evaluated the general possibility of reducing (chemically synthesised) thioesters with common ADHs (**Figure 21**) (Younes et al., 2017).

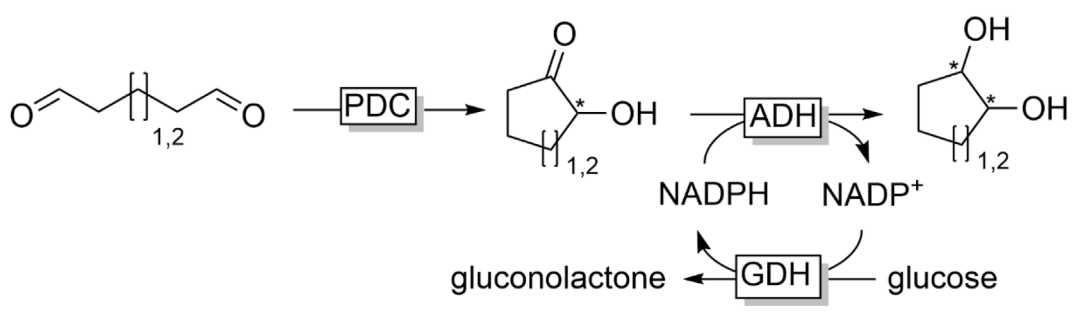


FIGURE 20 | Combination of pyruvate decarboxylase-catalysed aldol-type ring closure of dialdehydes with ADH-catalysed reduction of the α-hydroxyketone intermediate.

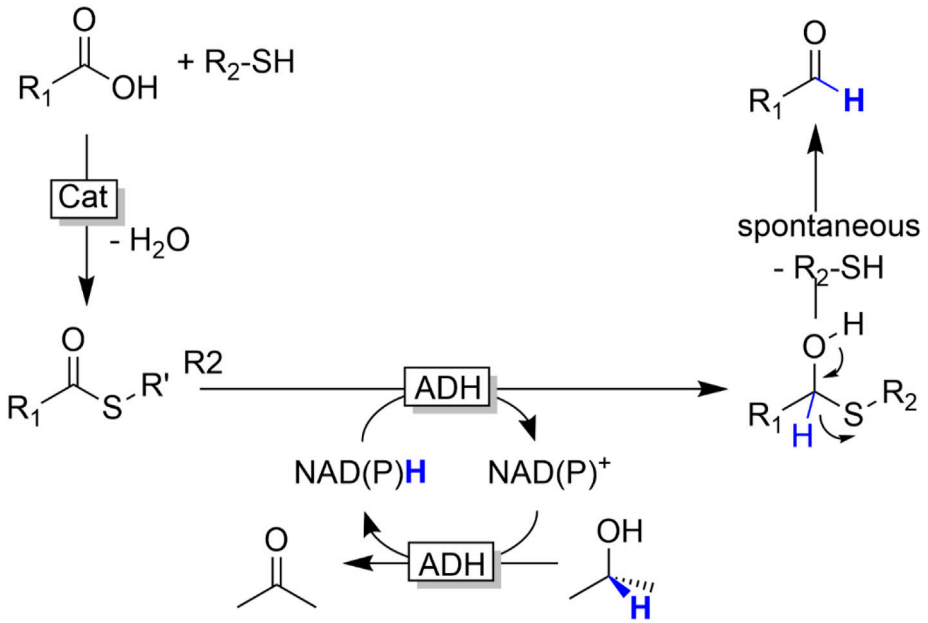


FIGURE 21 | ADH-catalysed reduction of carboxylic acids activated as thioesters.

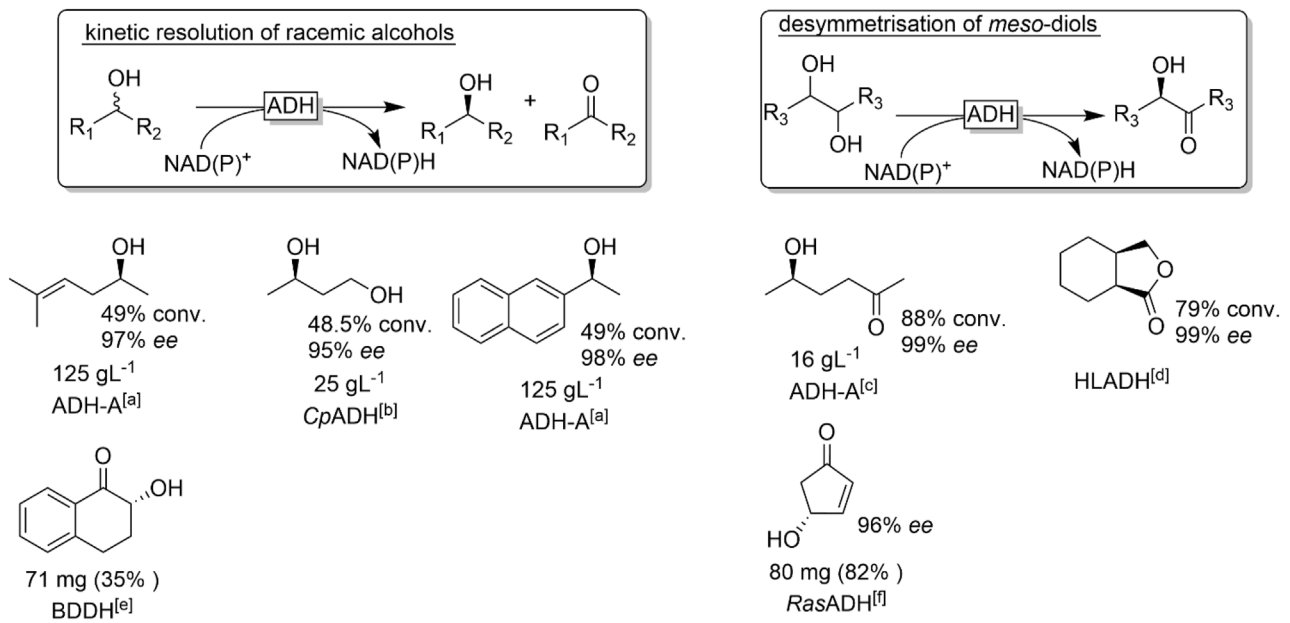


FIGURE 22 | Representative examples of chiral alcohol oxidation products derived from the kinetic resolution of racemic alcohols or the desymmetrisation of *meso*-diols. [a] ADH-A (from *Rhodococcus ruber*) (Stampfer et al., 2002), [b] *Cp*ADH (from *Candida parapsilosis*) (Matsuyama et al., 2001) [c] (Edegger et al., 2006b) [d] *via* intramolecular lactonisation, HLADH: horse liver ADH (Irwin and Jones, 1977a; Irwin and Jones, 1977b) [e] 2,3-butanediol dehydrogenase from *Bacillus subtilis* (Zhang et al., 2013), [f] (Holec et al., 2015).

5 ADH-CATALYSED OXIDATION REACTIONS

The oxidation of an alcohol to the corresponding carbonyl group is accompanied by the destruction of a (potential) chiral centre rather than the generation of one as in case of carbonyl reduction reactions. This seeming limitation has for a long time limited ADH-oxidation reactions to the kinetic resolution of racemic secondary alcohols. Some examples are shown in **Figure 22**.

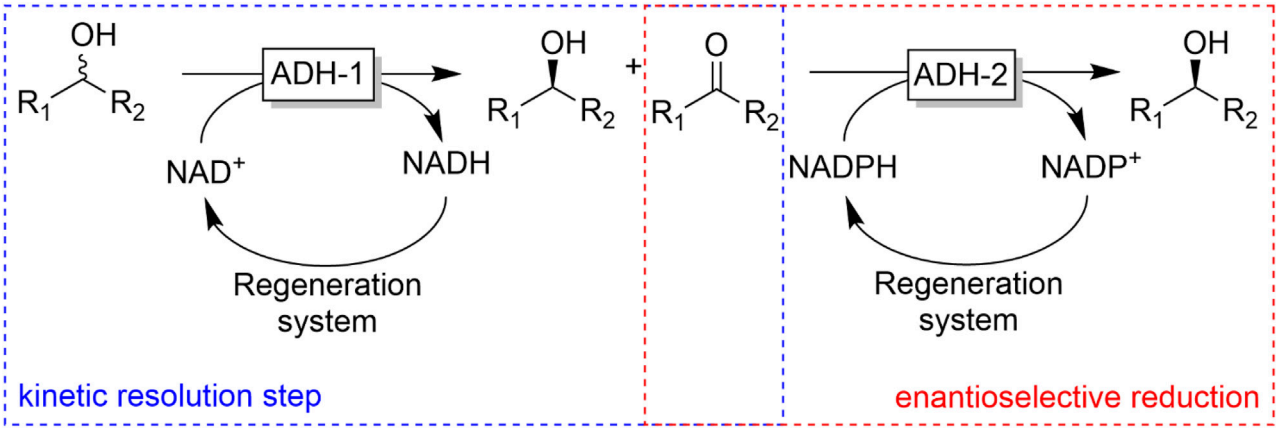


FIGURE 23 | Bienzymatic deracemisation of racemic alcohols using two enantiocomplementary ADHs.

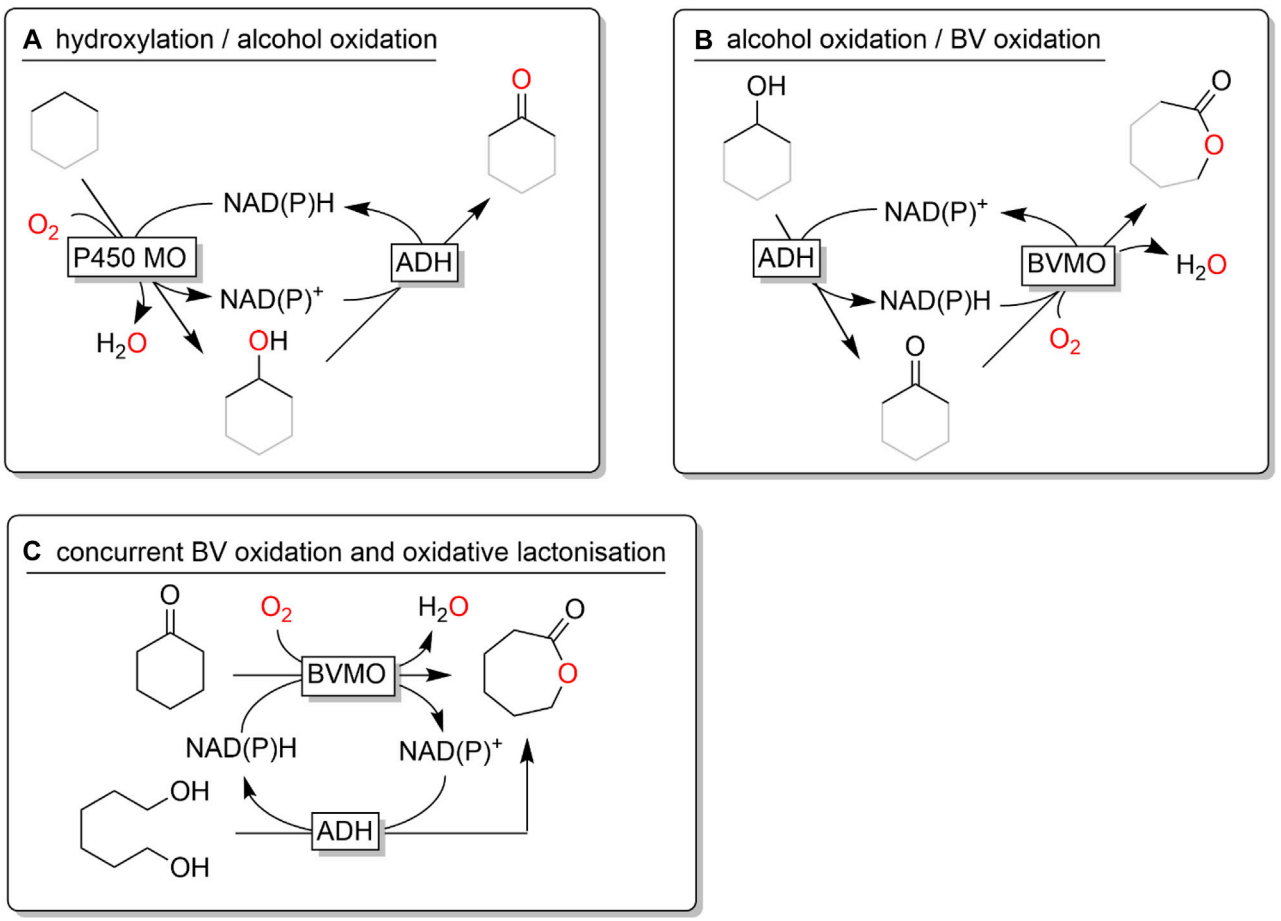


FIGURE 24 | Examples of redox-neutral cascades involving ADH-catalysed alcohol oxidation reactions. **(A)** combining monooxygenase-catalysed hydroxylation with ADH-catalysed alcohol oxidation; **(B)** combining ADH-catalysed alcohol oxidation with monooxygenase-catalysed Baeyer-Villiger oxidation; **(C)** concurrent lactone formation comining monooxygenase-catalysed Baeyer-Viliger oxidation with ADH-catalysed oxidative lactonisation.

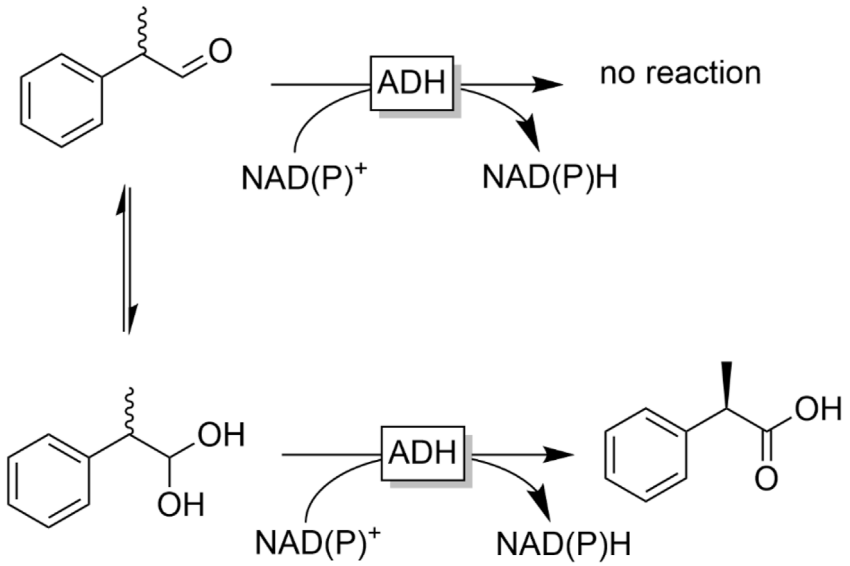


FIGURE 25 | Oxidation of aldehyde-gem-diols to the corresponding acid. Under enolising conditions the reaction can also be performed as dynamic kinetic resolution of chiral α-substituted aldehydes. This strategy also allows for the full oxidation of alcohols to carboxylic acids as recently demonstrated by Paradisi and coworkers (Contente et al., 2020).

The intrinsic limitation of kinetic resolution reactions of having a theoretical yield of only 50% (provided the catalyst exhibits high enantioselectivity) can be overcome making use of the meso-trick. Here, a prochiral meso-alcohol is oxidised completely to (ideally) one product enantiomer. Obviously, the scope of this approach is rather limited to *meso*-starting materials. A more general approach to obtain enantiomerically pure alcohols has been proposed by Kroutil and coworkers by combining two enantiocomplementary ADHs (one for the kinetic resolution of the racemic alcohol and the other one for the stereoselective reduction of the ketone intermediate into the desired alcohol enantiomer). If both ADHs also exhibit exclusivity for either the phosphorylated and non-phosphorylated nicotinamide cofactor, the overall reaction can be conducted as a one-pot one-step reaction (**Figure 23**) (Voss et al., 2008a; Voss et al., 2008b; Voss et al., 2010).

ADH-catalysed oxidations also play a key role in various multi-step cascades valorising simple starting materials into more complex products. For example, monooxygenase-catalysed hydroxylation reactions of non-functionalised C-H-bonds yield alcohols that can further be converted into the corresponding ketones (**Figure 24A**) (Müller et al., 2013; Staudt et al., 2013; Tavanti et al., 2017a; Tavanti et al., 2017b). Similarly, starting from alcohols, redox-neutral ADH-monooxygenase cascades producing lactones are possible (**Figure 24B**) (Schmidt et al., 2015a; Schmidt et al., 2015b; Scherkus et al., 2016; Scherkus et al., 2017; Wedde et al., 2017). Also combining both approaches has been demonstrated by Opperman and coworkers (Pennec et al., 2015).

An interesting convergent cascade combining Baeyer-Villiger monooxygenase-catalysed synthesis of lactones with ADH-catalysed cofactor regeneration yielding the desired lactone as (co-)product has been proposed by Kara and coworkers (**Figure 24C**) (Bornadel et al., 2016; Huang et al., 2017).

Other cascades involving ADH-catalysed alcohol oxidations have also been reported in combination with ene-reductase-catalysed C=C-bond reductions (Gargiulo et al., 2012; Oberleitner et al., 2013), transaminase-catalysed (Tauber et al., 2013; Corrado et al., 2021) or amine reductase-catalysed (Tavanti et al., 2017a; Dennig et al., 2019) reductive aminations or obtaining the alcohol via hydratation of an existing C=C-bond (Koppireddi et al., 2016; Zhang W. et al., 2020; Cha et al., 2020).

As far as primary alcohols are concerned, ADHs generally catalyse the selective oxidation to the aldehyde stage. Further oxidation generally does not occur as the aldehyde proton is not abstractable as a hydride. If desired, this situation can be changed by transiently converting the aldehyde into its corresponding gem-diol (preferentially by adjusting the reaction pH to alkaline values). The latter does contain a hydridically abstractable H-atom and therefore can also be oxidised to the corresponding acid (**Figure 25**) (Könst et al., 2012).

6 ENVIRONMENTAL CONSIDERATIONS

Biocatalytic reactions are frequently labelled as "green" (i.e., environmentally benign). To substantiate this claim, arguments such as enzymes being bio-based and bio-degradable (in contrast to transition metal catalysts), the mild reaction conditions (in contrast to frequently high reaction temperatures needed for chemical reactions) and the use of water as reaction medium (in contrast to organic solvents generally used in chemical reactions) are used.

Such qualitative arguments, however, are too short-sighted. First of all, one should be aware that any chemical transformation, irrespective if it is enzyme-catalysed or based on "traditional" chemical technologies, represents an environmental burden. Reactions consume energy to heat/cool and stir/pump the reaction mixtures, which still causes greenhouse gas emissions albeit indirectly and not always obvious for the user. The production of enzymes consumes resources and also biocatalytic reactions generate wastes (Tieves et al., 2019). Finally, water can be considered as green solvent only until after its usage as then it is generally contaminated with reactants and cannot be simply disposed into the environment and has to be considered as waste (Holtmann and Hollmann, 2022). Overall, a chemical transformation does not *per se* turn environmentally if performed using an enzyme catalyst.

Enzymatic reactions can exhibit significant environmental benefits (i.e., they can be *greener*) over traditional chemical syntheses. But it is not enough just to repeat the boilerplate arguments mentioned above. A quantitative, comparative assessment of the environmental impact of alternative routes is necessary to claim greenness. Ideally, such comparisons include the entire production chain from raw materials to the final product, as full life cycle assessments typically aim at (Eissen et al., 2009; Tufvesson et al., 2012). The extensive data basis required for such LCAs usually significantly goes beyond the possibilities of lab researchers. Therefore, simplified mass-based metrics such as Sheldon's E-Factor (Sheldon et al., 2022) (E for Environmental) and its derivates (Sheldon, 2017; 2018) represent a doable compromise to compare different synthetic routes based on their waste generation.

We encourage the readers of this contribution to utilise the E-Factor to assess the wastes generated in their own reactions and to utilise the result as a starting point for further improvements. In other words, an honest E-Factor analysis will reveal the real issues of a given reaction instead of perceived ones and can serve as a guiding principle to plan new experiments. A "bad" E-Factor can be a good starting point for improvements.

7 CONCLUSIONS AND FUTURE OUTLOOK

Alcohol dehydrogenases can nowadays be considered as established catalysis in organic synthesis. ADHs enable the selective oxidation of alcohols (and aldehydes) as well as reduction of aldehydes and ketones on preparative scale under mild reaction conditions.

Limitations stressed in the past such as limited substrate scope, limited stability or their dependence on costly nicotinamide cofactors have been solved in the past decades. Protein engineering has become a standard technique to tailor the substrate scope, selectivity and stability of a given ADH. Also ADH stability under non-natural conditions (such as high reagent concentrations or elevated temperatures) does not represent a preparative hurdle any more as, e.g., engineered ADH mutants are available and/or reaction engineering measures (such as immobilisation of the enzymes) can be utilised.

So far, ADHs have been valued mostly for their enantioselectivity giving access to optically pure chiral fine chemicals and pharmaceutical intermediates. We do expect that the scope of ADH-catalysis will expand also to the synthesis of commodity and bulk chemicals. The tighter cost requirements for such products will necessitate to focus more on performance indicators such as space-time yields, final product titres and turnover numbers of the enzymes and cofactors. Given the rapid development of ADH catalysis in the past, we are convinced that soon ADHs will have become indispensable catalysts for all chemical synthesis routes.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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