catalysts

MDPI

Communication

Deep Eutectic Solvents as Smart Cosubstrate in Alcohol Dehydrogenase-Catalyzed Reductions

Santiago Nahuel Chanquia [1,2,†], Lei Huang [1,†], Guadalupe García Liñares [2], Pablo Domínguez de María [3] and Selin Kara [1,*]

1 Department of Engineering, Biocatalysis and Bioprocessing Group, Aarhus University, 8000 Aarhus, Denmark; santiago.chanquia@gmail.com (S.N.C.); lei.huang@eng.au.dk (L.H.)
2 Organic Chemistry Department, UMYMFOR-CONICET, Laboratory of Biocatalysis, Faculty of Exact and Natural Sciences, University of Buenos Aires, Buenos Aires C1428EGA, Argentina; linares@qo.fcen.uba.ar
3 Sustainable Momentum, SL. Av. Ansite 3, 4-6, Las Palmas de Gran Canaria, 35011 Canary Islands, Spain; dominguez@sustainable-momentum.net
* Correspondence: selin.kara@eng.au.dk; Tel.: +45-2237-8964
† The authors contributed equally.

Received: 20 July 2020; Accepted: 29 August 2020; Published: 3 September 2020

Abstract: Alcohol dehydrogenase (ADH) catalyzed reductions in deep eutectic solvents (DESs) may become efficient and sustainable alternatives to afford alcohols. This paper successfully explores the ADH-catalyzed reduction of ketones and aldehydes in a DES composed of choline chloride and 1,4-butanediol, in combination with buffer (Tris-HCl, 20% *v*/*v*). 1,4-butanediol (a DES component), acts as a smart cosubstrate for the enzymatic cofactor regeneration, shifting the thermodynamic equilibrium to the product side. By means of the novel DES media, cyclohexanone reduction was optimized to yield maximum productivity with low enzyme amounts (in the range of 10 g L^{-1} d^{-1}). Notably, with the herein developed reaction media, cinnamaldehyde was reduced to cinnamyl alcohol, an important compound for the fragrance industry, with promising high productivities of ~75 g L^{-1} d^{-1}.

Keywords: deep eutectic solvents; alcohol dehydrogenases; smart cosubstrate; reductions; cinnamyl alcohol

1. Introduction

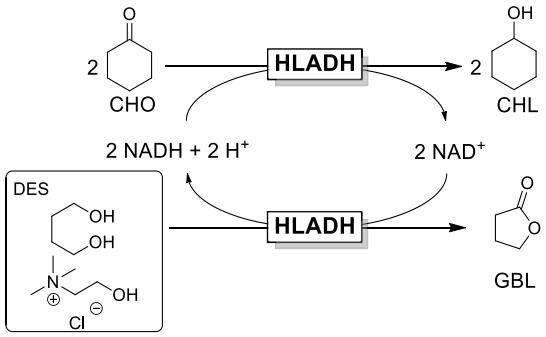
Oxidoreductases (EC1) are becoming increasingly important catalysts in organic synthesis given their high activity and selectivity when applied in redox processes. Reactions ranging from C = O and C = C reductions to hydroxylations, epoxidations, and Baeyer-Villiger reactions are catalyzed by these enzymes [1–5]. In particular, the application of alcohol dehydrogenases (ADHs, EC 1.1.1.1) represents a sustainable and efficient method for the synthesis of (optically active) alcohols starting from carbonyl compounds as inexpensive substrates [6,7].

ADHs are cofactor-dependent (nicotinamide cofactors (NAD(P)H)) and mediate as electron donors/acceptors in oxidations or reductions. For economic reasons, cofactors must be used in catalytic amounts combined with an in situ regeneration system [8], which normally implies a second enzymatic reaction, usually mediated by glucose dehydrogenase (GDH) [9] or formate dehydrogenase (FDH) and an adequate ancillary cosubstrate. Alternatively, chemical, electrochemical, or photochemical regeneration methods have been proposed [10–12], as well as synthetic nicotinamide cofactors in stoichiometric amounts [13]. In this area, the ADH mediated oxidation of simple alcohols for cofactor regenerations is a common and efficient approach, particularly useful when the same ADH is used in both reactions, conforming a biocatalytic version of the Meerwein–Ponndorf–Verley (MPV)

reduction [14–17]. However, these combined cycles often present drawbacks related to their reversibility and poor thermodynamic driving force, which forces the use of a surplus of the cosubstrate or the carrying out of a coproduct removal. Using an excess amount of cosubstrate (e.g., isopropanol, ethanol, etc.) might enhance the solubility of a main substrate/product; nevertheless, it is still not an ideal approach in terms of atom economy. To overcome these issues, the use of smart cosubstrates has been put forth. The main concept behind this relatively new development consists of shifting the equilibrium of a certain reaction by transforming one of the substrates into thermodynamically and/or kinetically inert coproducts [18–20].

Biocatalysis in non-conventional reaction media, such as solvent-free systems, organic solvents, ionic liquids (ILs), or supercritical fluids, is becoming a well-established strategy to cope with the challenges that modern synthesis entails [21]. Herein, deep eutectic solvents (DESs), a new type of ILs [22], hold promising features as reaction solvents [23,24]. A DES is composed of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), typically a quaternary ammonium salt. The DES preparation is straightforward—both components must be gently mixed to form a liquid—which is advantageous over traditional ILs both in terms of simplicity and environmental impact. To reduce the inherent high viscosity of DESs, the addition of water as cosolvent (up to 20% *v*/*v*) enables a low viscous media, while keeping the non-conventional nature of it (i.e., a substrate-dissolving system) [25]. These low viscous DES-water mixtures are particularly useful when continuous processes are envisaged [26,27].

Based on the recent work of us and other groups [20,28–30], herein the combination of the smart cosubstrate concept with a DES, to set up an efficient and environmentally-friendly synthetic alternative, is addressed for the first time. ADH from horse liver (HLADH), which can accept a broad range of diols as substrates [31–34], was selected as the prototypical enzyme, as it also can catalyze reactions in DES-aqueous systems [35]. As reaction media, a DES consisting of choline chloride (ChCl) and 1,4-butanediol (1,4-BD) was chosen. Acting as smart cosubstrate, 1,4-BD shifts the equilibrium of the MPV reduction when being oxidized to γ-butyrolactone (GBL), a thermodynamically stable and kinetically inert coproduct (Scheme 1). Thus, the reaction solvent becomes a part of the reaction, avoiding the addition of any other reagents, which greatly simplifies the process and makes it much more efficient and sustainable. Analogous approaches have been reported in the literature for glycerol [25] and for glucose [30].



Scheme 1. Alcohol dehydrogenase from horse liver (HLADH)-catalyzed reduction of cyclohexanone (CHO) using deep eutectic solvent (DES)-buffer mixture while using 1,4-butanediol as a DES hydrogen bond donor component.

2. Results and Discussion

To validate the concept of using smart cosubstrates as part of the DES-water media, the HLADH-catalyzed reduction of cyclohexanone (CHO) to cyclohexanol (CHL) was chosen as model reaction (Scheme 1). A choline chloride—1,4-butanediol DES was formed and mixed with

20% buffer (*v*/*v*) to decrease viscosity and to provide the enzyme with the necessary water for its activity [35].

HLADH was produced and purified starting from the respective plasmid, and its activity was characterized for a range of CHO concentrations from 0 mM to 500 mM. A clear excess substrate inhibition effect was observed, whereby high K_i/K_M value (65.5) indicated no severe inhibition, K_i being the inhibition constant and K_M the Michaelis-Menten constant. Kinetic parameters for this enzyme are shown in Figure S1. In a previous work, the attractive concept of using DES both as solvent and for cofactor regeneration has been studied [30]. However, to the best of our knowledge, smart cosubstrates in combination with DES and redox biocatalysis have never been reported before.

Firstly, three different DESs of molar ratio of ChCl to 1,4-BD at 1:3, 1:4, and 1:5 were prepared and the formation of a liquid phase was visually evaluated. To perform the biocatalytic reactions, DES with proportions of 1:4 and 1:5 were used, as they formed a largely stable liquid phase over time. Conversely, the 1:3 combination formed two phases after being stored at room temperature for a few hours.

Once DES proportions were chosen, the enzymatic reaction was conducted to assess which DES was a more appropriate non-conventional media (1:4 or 1:5), in combination with an amount of buffer of 20% (*v*/(*v*) to keep the viscosity low [25]. Gratifyingly, both DES-water mixtures were enzyme-compatible, and quantitative product (CHL) formation was observed after 24 h. The medium DES (ChCl:1,4-BD/1:4) was chosen for further studies [36]. For the enzymatic reaction, full conversion was observed in both cases after 24 h reaction time (Figure 1). The inhibition of HLADH by 1,4-BD at higher concentrations that has been previously reported [37] did not alleviate to reach full conversions.

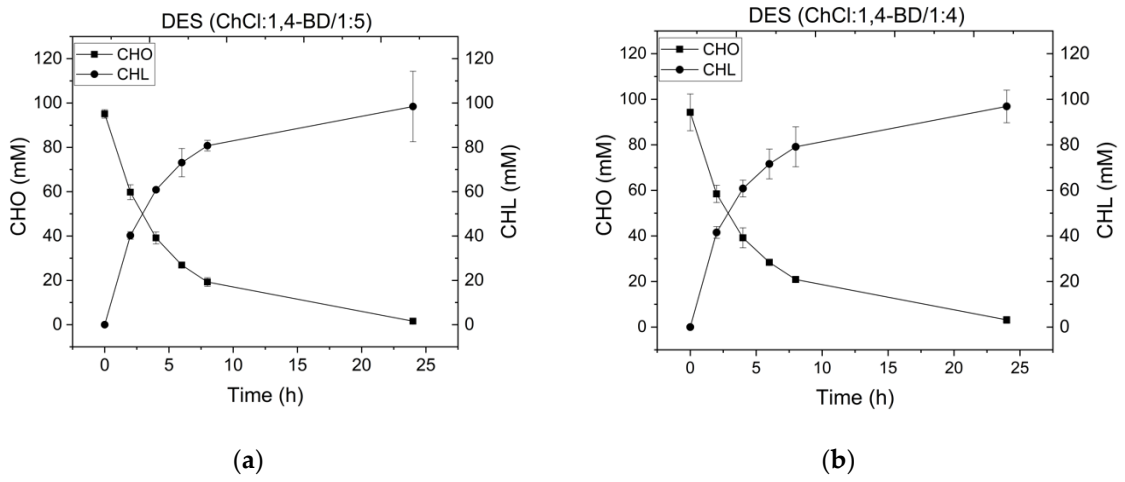


Figure 1. HLADH-catalyzed CHO reduction in two different mixtures of DES-buffer (80:20, *v*/*v*): (**b**) DES (ChCl:1,4-BD/1:4); (**a**) DES (ChCl:1,4-BD/1:5). Reaction conditions: 100 mM CHO, 1 mM NAD^+, 1 mg/mL purified HLADH. 25 °C, and 1200 rpm.

Encouraged by the promising results in the new DES-buffer media containing a smart cosubstrate, the optimum HLADH loading for the system was subsequently evaluated. To that end, increasing concentrations of the purified enzyme were used (0.05 to 2 mg/mL) to find out the best reaction rate with the minimum amount possible of enzyme, as shown in Figure 2. All progress curves using different enzyme loadings are shown in Figure S2. Based on these results we have chosen the following reaction conditions for HLADH-catalyzed reduction of CHO for the further experiments: ChCl:1,4-BD 1:4 as DES; HLADH, 0.25 mg/mL; NAD^+, 1 mM; temperature: 25 °C; stirring: 1200 rpm; Tris-HCl buffer volume percentage, and 20% *v*/*v*.

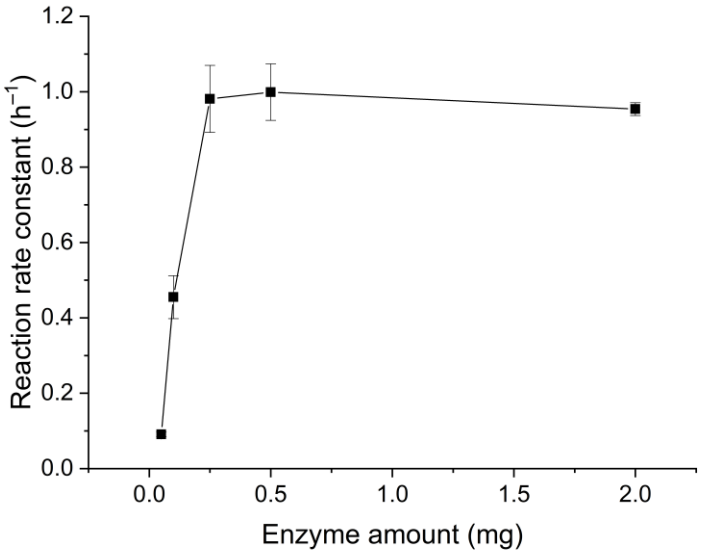
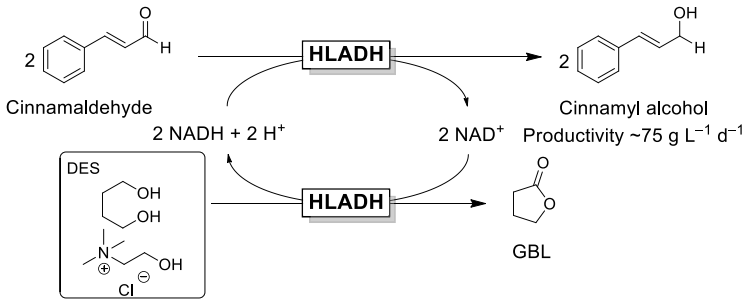


Figure 2. Relation between reaction rate constants and amount of HLADH used.

Using these chosen reaction conditions, a quantitative conversion of CHO to CHL was achieved after 5 h leading to ~10 g L^{-1} d^{-1}. Performing the reaction at higher concentrations of CHO (e.g., 500 mM or 1000 mM) led, however, to unsuccessful results, presumably due to substrate inhibition (Figure S1) and the cosubstrate 1,4-BD inhibition [20]. The fact that adding water to the DES system leads to a non-conventional but low viscous media may open the option of setting up continuous processes in which low substrate loadings can be pumped in an efficient manner [26,27].

The synthetic concept including DES and a smart cosubstrate was extended to other substrates as well. Whereas benzaldehyde and ethyl 4-chloro-3-oxobutanoate did not lead to any conversion, presumably due to low solubility in the DES system, cinnamaldehyde was reduced to cinnamyl alcohol (~25% conversion, 48 h reaction time, Scheme 2).



Scheme 2. HLADH-catalyzed cinnamaldehyde reduction in DES-buffer (80:20, *v*/*v*). DES: ChCl:1,4-BD/1:4.

Cinnamyl alcohol is a valuable industrial compound used as aroma chemical, e.g., derivatized as esters [38]. Given the inherent instability of the substrate and the product [39,40], biocatalytic synthesis of cinnamyl alcohol are put forth (using natural catalysts during the process). Remarkably, very promising productivities (~75 g L^{-1} d^{-1}) were obtained with the novel low viscous DES-smart cosubstrate system, suggesting that a continuous process might be envisaged for the production of this type of compounds, for which environmentally-friendly media and reaction conditions are needed. For work-up procedures, the addition of an excess of water to the media breaks the DES structure, and would allow the extraction of the cinnamyl alcohol with solvents (e.g., ethyl acetate). The evaporation of the water excess would create the DES mixture again [41].

3. Materials and Methods

3.1. Chemicals and Materials

Chemicals, cultivation media components, and reagents were purchased from Sigma–Aldrich (Søborg, Denmark), Carl Roth (Karlsruhe, Germany), and VWR (Søborg, Denmark) and used as received. Ni-NTA affinity resin was ordered from Expedeon (Cambridgeshire, UK) and BCA protein quantification kit (PierceTM) was obtained from Thermo Fischer Scientific (Schwerte, Germany).

3.2. Enzyme Production

HLADH gene was present in a Novagen pET-28b plasmid. For transformation, 1 µL of plasmid solution was added to 50 µL of Agilent BL21-Gold (DE3) competent cells in a 1.5 mL plastic micro-centrifuge tube, mixed gently and then the mixture was incubated on ice for half an hour. Afterwards, the cells were incubated for 30 s at 42 °C and put on ice again for five minutes. Next, 800 µL of lysogeny broth (LB) liquid medium was added and left to incubate for 1 h at 37 °C and 180 rpm.

Afterwards, 200 µL of the above-given preparation was added to Petri dishes with LB agar plates (+50 µg/mL kanamycin) and left to incubate overnight at 37 °C. Sterilized baffled flasks were filled with 20 mL of LB liquid medium (+50 µg/mL kanamycin). Next, a colony was picked from the agar plate and added to each flask containing the liquid medium. The liquid cultures were left to incubate overnight at 150 rpm and 37 °C in a New Brunswick™ Innova® 44 (Eppendorf AG, Juelich, Germany) incubator.

Later, 400 mL of Terrific Broth (TB) medium (+50 µg/mL kanamycin), and 4 mL of the previous pre-culture were added to 2-L baffled flasks and incubated at 37 °C and 150 rpm for 2.5 h. Cells were induced by adding 200 µL of a 1 M solution of isopropyl β-d-1-thiogalactopyranoside (IPTG) followed by incubation for 24 h at 25 °C and 150 rpm.

The cultures were then divided in centrifuge plastic bottles and centrifuged at 9000 rpm and 4 °C for 10 min in a Sorvall LYNX 4000 Superspeed Centrifuge (Thermo Fischer Scientific, Schwerte, Germany). The cell pellets were next suspended in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and sonicated using a Labsonic M (Sartorius, Goettingen, Germany) with MS 73 probe (60% amplitude: 4 × 4 min, 0.4 cycles on ice, 4 min break in between). Then, cell debris and the soluble fraction were separated by centrifugation at 16,000 rpm for 45 min at 4 °C, obtaining a cell free extract (CFE), which was purified using a Ni-TNA column to obtain a solution of the pure HLADH, which was lyophilized and stored at −20 °C.

3.3. Kinetic Assay

Enzyme kinetics were measured for lyophilized purified HLADH using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Glostrup, Denmark) and thermostated at 25 °C with a PCB 1500 Water Peltier System (Agilent Technologies, Glostrup, Denmark) at 340 nm, using a NADH concentration of 0.1 mM and 0–500 mM model substrate CHO. Enzyme concentration ranged from 0.0005 to 0.005 mg/mL. The assays for the negative controls were performed without the substrate and without the enzyme.

3.4. DES Preparation

DESs were prepared by adding the correspondent components, ChCl and 1,4-BD, to a flask, which was magnetically stirred at 400 rpm IKA Magnetic stirrer (IKA, Staufen, Germany) and heated with a water bath at 80 °C for 1 h.

3.5. Reduction of Cyclohexanone

To perform the reactions, stock solutions of NAD^+ and HLADH were prepared by dissolving both the cofactor and the lyophilized enzyme in Tris-HCl (50 mM, pH 7.5), and incubated at 25 °C for 30 min. Then, 800 µL of the selected pure DES, 100 µL of Tris-HCl (50 mM, pH 7.5), 10 µL of

cyclohexanone (CHO), and 100 µL of the stock solution of NAD^+ and HLADH were added in glass vials and incubated at 25 °C and 1200 rpm in a heating thermo shaker (Hettich Benelux, ProfiLab24 GmbH, Berlin, Germany). This methodology was also followed for other substrates (benzaldehyde, 4-chloro-3-oxobutanoate, and cinnamaldehyde) screened.

3.6. GC Analysis

Conversion of the substrates and formation of the products were followed by gas chromatography (GC) (Schimadzu GC2030, Holm & Halby, Brøndby, Denmark) analysis. Samples of 50 µL were taken from the reaction vials and added to 1.5 mL plastic micro-centrifuge tubes, followed by the addition of 250 µL of ethyl acetate (containing 2 mM methyl benzoate as an internal standard), vortexed and centrifuged for 1 min at 13,000 rpm. Then, 200 µL was taken from the upper organic phase and transferred to another plastic micro-centrifuge tube. A small amount of $MgCl_2$ was added for drying, the tubes were agitated manually, centrifuged for 1 min at 13,000 rpm, and 150 µL was taken and transferred to GC glass vials with a micro-insert for low volume. The GC method used can be found in the Supplementary Materials section.

4. Conclusions

The successful use of a DES as solvent and as a smart cosubstrate for cofactor regeneration in redox systems has been successfully explored for the first time. Promising results for the reduction of a series of carbonyl compounds were achieved, leading to full conversions when enzyme loadings were properly optimized. The particular composition of the used DES enabled a highly efficient process system, as the formed by-product (GBL) is inert, pushing the equilibrium to the formation of the desired alcohol. Of particular relevance is the production of cinnamyl alcohol, for which proof-of-concept productivities (~75 g L^{-1} d^{-1}) are highly promising. Further optimizations for this DES media may include the assessment of higher substrate loadings (e.g., step-wise addition), enzyme immobilization, and set-up of continuous (non-viscous) DES-water media. Moreover, expanding the substrate range, as well as using other ADH enzymes (e.g., variants with higher robustness for the DES media), may become an important line of research. In a broader sense, the combination of DES and smart cosubstrate systems may create powerful synergies to be applied for efficient sustainable chemical processes.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/10/9/1013/s1, Figure S1: Enzyme kinetic assay of HLADH toward CHO, Table S1: Kinetic parameters for the obtained HLADH, Table S2: Details of GC method used in this study, Figure S2: HLADH-catalyzed CHO reduction progress in DES (ChCl:1,4-BD/1:4)-Buffer (80:20, *v*/*v*) media with different enzyme loading. Figure S3: Example of a GC chromatography spectrum.

Author Contributions: Conceptualization: S.K.; experimental work: S.N.C. and L.H.; writing: S.N.C., L.H., G.G.L., P.D.d.M., and S.K. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by *Deutsche Forschungsgemeinschaft* (DFG) grant number: KA 4399/3-1 and *Aarhus Universitets Forskningsfond* (AUFF) Starting Grant.

Acknowledgments: The authors thank Diederik Johannes Opperman (University of the Free State, South Africa) for the recombinant plasmid containing HLADH gene. Furthermore, we would also like to thank Kim Møller Johansen and Thomas Dyekjær from Department of Engineering at Aarhus University for their technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Monti, D.; Ottolina, G.; Carrea, G.; Riva, S. Redox Reactions Catalyzed by Isolated Enzymes. *Chem. Rev.* **2011**, *111*, 4111–4140. [CrossRef]
2. Brenna, E. *Synthetic Methods for Biologically Active Molecules: Exploring the Potential of Bioreductions*; John Wiley & Sons Inc.: Hoboken, NJ, USA, 2013.

3. Faber, K. *Biotransformations in Organic Chemistry*; Springer Science and Business Media LLC: Berlin/Heidelberg, Germany, 2018; pp. 133–204.
4. Hollmann, F.; Arends, I.W.C.E.; Holtmann, D. Enzymatic reductions for the chemist. *Green Chem.* **2011**, *13*, 2285. [CrossRef]
5. Younus, H. Oxidoreductases: Overview and Practical Applications. In *Biocatalysis*; Springer Science and Business Media LLC: Berlin/Heidelberg, Germany, 2019; pp. 39–55.
6. Kratzer, R.; Woodley, J.M.; Nidetzky, B. Rules for biocatalyst and reaction engineering to implement effective, NAD(P)H-dependent, whole cell bioreductions. *Biotechnol. Adv.* **2015**, *33*, 1641–1652. [CrossRef] [PubMed]
7. Huisman, G.W.; Liang, J.; Krebber, A. Practical chiral alcohol manufacture using ketoreductases. *Curr. Opin. Chem. Boil.* **2010**, *14*, 122–129. [CrossRef] [PubMed]
8. Weckbecker, A.; Gröger, H.; Hummel, W. Regeneration of Nicotinamide Coenzymes: Principles and Applications for the Synthesis of Chiral Compounds. In *Biosystems Engineering I*; Springer Science and Business Media LLC: Berlin/Heidelberg, Germany, 2010; Volume 120, pp. 195–242.
9. Chenault, H.K.; Whitesides, G.M. Regeneration of nicotinamide cofactors for use in organic synthesis. *Appl. Biochem. Biotechnol.* **1987**, *14*, 147–197. [CrossRef]
10. Wu, H.; Tian, C.; Song, X.; Liu, C.; Yang, N.; Jiang, Z. Methods for the regeneration of nicotinamide coenzymes. *Green Chem.* **2013**, *15*, 1773–1789. [CrossRef]
11. Hummel, W.; Gröger, H. Strategies for regeneration of nicotinamide coenzymes emphasizing self-sufficient closed-loop recycling systems. *J. Biotechnol.* **2014**, *191*, 22–31. [CrossRef]
12. Quinto, T.; Köhler, V.; Ward, T.R. Recent Trends in Biomimetic NADH Regeneration. *Top. Catal.* **2013**, *57*, 321–331. [CrossRef]
13. Zachos, I.; Nowak, C.; Sieber, V. Biomimetic cofactors and methods for their recycling. *Curr. Opin. Chem. Boil.* **2019**, *49*, 59–66. [CrossRef]
14. Baer, K.; Kraußer, M.; Burda, E.; Hummel, W.; Berkessel, A.; Gröger, H.; Kraußer, M.; Gröger, H. Sequential and Modular Synthesis of Chiral 1,3-Diols with Two Stereogenic Centers: Access to All Four Stereoisomers by Combination of Organo- and Biocatalysis. *Angew. Chem. Int. Ed.* **2009**, *48*, 9355–9358. [CrossRef]
15. Meerwein, H.; Schmidt, R. Ein neues Verfahren zur Reduktion von Aldehyden und Ketonen. *Eur. J. Org. Chem.* **1925**, *444*, 221–238. [CrossRef]
16. Ponndorf, W. Der reversible Austausch der Oxydationsstufen zwischen Aldehyden oder Ketonen einerseits und primären oder sekundären Alkoholen anderseits. *Angew. Chem.* **1926**, *39*, 138–143. [CrossRef]
17. De Graauw, C.F.; Peters, J.A.; Van Bekkum, H.; Huskens, J. Meerwein-Ponndorf-Verley Reductions and Oppenauer Oxidations: An Integrated Approach. *Synthesis* **1994**, *1994*, 1007–1017. [CrossRef]
18. Ni, Y.; Holtmann, D.; Hollmann, F. How Green is Biocatalysis? To Calculate is To Know. *ChemCatChem* **2014**, *6*, 930–943. [CrossRef]
19. Wang, B.; Land, H.; Berglund, P. ChemInform Abstract: An Efficient Single-Enzymatic Cascade for Asymmetric Synthesis of Chiral Amines Catalyzed by ω-Transaminase. *Chem. Commun.* **2013**, *49*, 161–163. [CrossRef]
20. Kara, S.; Spickermann, D.; Schrittwieser, J.H.; Leggewie, C.; Van Berkel, W.J.; Arends, I.W.C.E.; Hollmann, F. More efficient redox biocatalysis by utilising 1,4-butanediol as a 'smart cosubstrate'. *Green Chem.* **2013**, *15*, 330. [CrossRef]
21. Pätzold, M.; Siebenhaller, S.; Kara, S.; Liese, A.; Syldatk, C.; Holtmann, D. Deep Eutectic Solvents as Efficient Solvents in Biocatalysis. *Trends Biotechnol.* **2019**, *37*, 943–959. [CrossRef] [PubMed]
22. Zhang, Q.; Vigier, K.D.O.; Royer, S.; Jerome, F. Deep eutectic solvents: Syntheses, properties and applications. *Chem. Soc. Rev.* **2012**, *41*, 7108. [CrossRef] [PubMed]
23. Guajardo, N.; Mueller, C.R.; Schrebler, R.; Carlesi, C.; De Maria, P.D. ChemInform Abstract: Deep Eutectic Solvents for Organocatalysis, Biotransformations, and Multistep Organocatalyst/Enzyme Combinations. *ChemCatChem* **2016**, *47*, 1020–1027. [CrossRef]
24. Alonso, D.A.; Baeza, A.; Chinchilla, R.; Guillena, G.; Pastor, I.M.; Ramon, D.J. Deep Eutectic Solvents: The Organic Reaction Medium of the Century. *Eur. J. Org. Chem.* **2016**, *2016*, 612–632. [CrossRef]
25. Guajardo, N.; De Maria, P.D.; Ahumada, K.; Schrebler, R.A.; Ramírez-Tagle, R.; Crespo, F.A.; Carlesi, C. Water as Cosolvent: Nonviscous Deep Eutectic Solvents for Efficient Lipase-Catalyzed Esterifications. *ChemCatChem* **2017**, *9*, 1393–1396. [CrossRef]

26. Guajardo, N.; Schrebler, R.A.; De María, P.D. From batch to fed-batch and to continuous packed-bed reactors: Lipase-catalyzed esterifications in low viscous deep-eutectic-solvents with buffer as cosolvent. *Bioresour. Technol.* **2019**, *273*, 320–325. [CrossRef] [PubMed]
27. Guajardo, N.; De María, P.D.; De María, H.P.D. Continuous Biocatalysis in Environmentally-Friendly Media: A Triple Synergy for Future Sustainable Processes. *ChemCatChem* **2019**, *11*, 3128–3137. [CrossRef]
28. Kara, S.; Spickermann, D.; Weckbecker, A.; Leggewie, C.; Arends, I.W.C.E.; Hollmann, F. Bioreductions Catalyzed by an Alcohol Dehydrogenase in Non-aqueous Media. *ChemCatChem* **2014**, *6*, 973–976. [CrossRef]
29. Lavandera, I.; Kern, A.; Resch, V.; Silva, B.F.; Glieder, A.; Fabian, W.M.F.; De Wildeman, S.; Kroutil, W. One-Way Biohydrogen Transfer for Oxidation ofsec-Alcohols. *Org. Lett.* **2008**, *10*, 2155–2158. [CrossRef]
30. Mourelle-Insua, Á.; Lavandera, I.; Gotor-Fernandez, V. A designer natural deep eutectic solvent to recycle the cofactor in alcohol dehydrogenase-catalysed processes. *Green Chem.* **2019**, *21*, 2946–2951. [CrossRef]
31. Irwin, A.J.; Jones, J.B. Asymmetric syntheses via enantiotopically selective horse liver alcohol dehydrogenase catalyzed oxidations of diols containing a prochiral center. *J. Am. Chem. Soc.* **1977**, *99*, 556–561. [CrossRef]
32. Lok, K.P.; Jakovac, I.J.; Jones, J.B. Enzymes in organic synthesis. 34. Preparations of enantiomerically pure exo- and endo-bridged bicyclic [2.2.1] and [2.2.2] chiral lactones via stereospecific horse liver alcohol dehydrogenase catalyzed oxidations of meso diols. *J. Am. Chem. Soc.* **1985**, *107*, 2521–2526. [CrossRef]
33. Jakovac, I.J.; Goodbrand, H.B.; Lok, K.P.; Jones, J.B. Enzymes in organic synthesis. 24. Preparations of enantiomerically pure chiral lactones via stereospecific horse liver alcohol dehydrogenase catalyzed oxidations of monocyclic meso diols. *J. Am. Chem. Soc.* **1982**, *104*, 4659–4665.
34. Kara, S.; Spickermann, D.; Schrittwieser, J.H.; Weckbecker, A.; Leggewie, C.; Arends, I.W.C.E.; Hollmann, F. Access to Lactone Building Blocks via Horse Liver Alcohol Dehydrogenase-Catalyzed Oxidative Lactonization. *ACS Catal.* **2013**, *3*, 2436–2439. [CrossRef]
35. Huang, L.; Bittner, J.P.; De María, P.D.; Jakobtorweihen, S.; Kara, S. Modeling Alcohol Dehydrogenase Catalysis in Deep Eutectic Solvent/Water Mixtures. *ChemBioChem* **2019**, *21*, 811–817. [CrossRef] [PubMed]
36. All calibration curves for the substrates and product were conducted in the corresponding DES (80%, *v*/*v*) and buffer (20%, *v*/*v*), in which the reaction takes place. The sample calibration for DES (ChCl:1,4-BD/1:4) at 80% (*v*/*v*) and Tris-buffer at 20% (*v*/*v*) is shown in the supplementary information.
37. Huang, L.; Romero, E.; Ressmann, A.K.; Rudroff, F.; Hollmann, F.; Fraaije, M.W.; Kara, S. Nicotinamide Adenine Dinucleotide-Dependent Redox-Neutral Convergent Cascade for Lactonizations with Type II Flavin-Containing Monooxygenase. *Adv. Synth. Catal.* **2017**, *359*, 2142–2148. [CrossRef]
38. Klumbys, E.; ZebeC, Z.; Weise, N.J.; Turner, N.J.; Scrutton, N.S. Bio-derived production of cinnamyl alcohol via a three step biocatalytic cascade and metabolic engineering. *Green Chem.* **2018**, *20*, 658–663. [CrossRef]
39. Chamouleau, F.; Hagedorn, C.; May, O.; Gröger, H. Biocatalytic aldehyde reduction using tailor-made whole-cell catalysts: A novel synthesis of the aroma chemical cinnamyl alcohol. *Flavour Fragr. J.* **2007**, *22*, 169–172. [CrossRef]
40. Pennacchio, A.; Rossi, M.; Raia, C.A. Synthesis of Cinnamyl Alcohol from Cinnamaldehyde with Bacillus stearothermophilus Alcohol Dehydrogenase as the Isolated Enzyme and in Recombinant E. coli Cells. *Appl. Biochem. Biotechnol.* **2013**, *170*, 1482–1490. [CrossRef] [PubMed]
41. Maugeri, Z.; Leitner, W.; De Maria, P.D. Practical separation of alcohol–ester mixtures using Deep-Eutectic-Solvents. *Tetrahedron Lett.* **2012**, *53*, 6968–6971. [CrossRef]