doi:10.2533/chimia.2023.390 Chimia 77 (2023) 390–394

Enantioselective Switch and Potential Applications in Biocatalysis

Lucia Robustini and Francesca Paradisi*

Abstract: Enantioselectivity has always been a key feature of enzymatic synthesis. In some cases, when enzymes are not strictly enantioselective, it is possible to induce an enantioselective switch by tuning the reaction conditions. A transaminase from *Halomonas elongata* (ω-HeWT), while generally *S*-selective, could be shifted towards generating the *R*-enantiomer at higher concentrations of amino acceptor or ionic strength, for example. Other enzymes are reported to have a similar behavior, and here we discuss some of them and their potential applications.

Keywords: Biocatalysis · Enantiopreference · Enzymes · Transaminase

Francesca Paradisi is the Chair of Sustainable Pharmaceutical Chemistry at the University of Bern since 2019. Biocatalysis as a sustainable approach to the synthesis of valuable products is the focus of her research. Her group developed several enzyme-based processes in continuous flow, with immobilized biocatalysts, reducing the gap between academic discovery and industrial application.

Lucia Robustini is a PhD candidate in the group of Prof. Francesca Paradisi at the University of Bern since April 2020. She graduated in 2015 as a Food Biotechnologist from the University of Ferrara (Italy), with a thesis in Biocatalysis at the University of Freiburg (Germany).

Introduction

Enantioselectivity is a highly regarded advantage of biocatalysts, often portrayed as an almost unbeatable feature when compared with less performing synthetic chiral catalysts.[1] This key characteristic, which is inherent in a vast majority of biological catalysts, often avoids tedious separations and enables easy reaction workup under mild conditions. Downstream purification of the desired product from contaminants, including heavy metals, side-products, solvents, and unwanted stereoisomers, is one of the most significant parameters that influence the cost of a process.[2] The presence of the unwanted enantiomer reduces the reaction yield making it a critical step in the production of Active Pharmaceutical Ingredients (APIs)[3,4] or in the fragrance industry, where one or the other enantiomer differ for olfactive properties such as character or intensity (Fig. 1).[5,6]

Enzymes, being chiral in nature, are usually highly selective when applied to their natural substrates. This selectivity tends to remain when the same biocatalysts are tested with non-natural substrates. In some cases, however, enzymes can be tested with particularly challenging substrates, which are not so easily recognized by the biocatalyst in an optical sense. ω-Transaminases (ω-Tas, PDB 6GWI) are pyridoxal-5'-phosphate (PLP)-dependent enzymes that catalyze the formal reductive amination of prochiral substrates, such as ketones to the corresponding chiral amines and are widely recognized for their high enantioselectivity.[7,8] Steric discrimination at the active site is accepted as the explanation for the specificity of this enzyme class.[9] Implementing chiral amine synthesis from small prochiral substrates using *Halomonas elongata* transaminase, an enantioselectivity inversion was observed under specific reaction conditions (Scheme 1).[10–12]

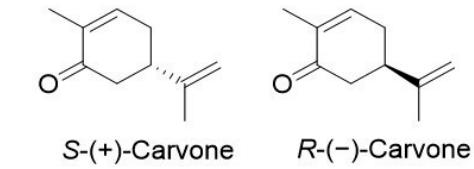


Fig. 1. Example of enantiomers with different features. On the left, *S*-(+)-carvone mentholated, spicy aroma with bready notes and medium strength. On the right, *R*-(–)-carvone, has herbal, minty and sweetish medium-strength fragrance.

Scheme 1. Oxidative transamination of pro-chiral substrate from *Halomonas elongata* Transaminase (HeWT).

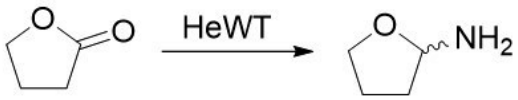
In nature, other enzymes were reported to have similar enantioswitching behavior: lipases from *Candida rugosa* (CRL),[13] ethionamide monooxygenase (EthA),[14] or phenylacetone monooxygenase (PAMO)[14] to name some of them. The enantioselectivity can clearly be influenced by several parameters, such as reaction time, ionic strength, temperature, solvent polarity (*log*P), water activity (a_w), pH, substrate concentration,[15] or biocatalyst concentration.[16]

Enantioselective Switch Triggered by Biocatalyst Concentration

The enantiopreference of HeWT has been previously tested also with cyclic prochiral ketones.[17] The standard reaction conditions include 1 mg/mL of purified enzyme, 10 mM tetrahydrofu-

*Correspondence: Prof. Dr. F. Paradisi, E-mail: francesca.paradisi@unibe.ch
Dept. Chemistry, Biochemistry and Pharmaceutical Sciences. University of Bern, Freiestrasse 3, CH-3012 Bern

ran-1-one (THF-ketone), 10 mM (S)-α-methylbenzylamine (S-MBA) and 0.1 mM PLP (Scheme 2).



Scheme 2. Transamination of THF-ketone into THF-amine with HeWT. Reaction conditions: 10 mM THF-ketone, 50 mM amine (S-MBA or isopropyl amine (IPA), 1 mg/mL HeWT, in KPi buffer (50 mM, pH 8.0), and 30 °C.

The *ee* (%), calculated after three hours, indicated a decrease in enantiopreference from 18 to 6% when the concentration of HeWT was increased from 0.1 to 5 mg/mL. In fact, as reported by Hanefeld *et al.*[18] and Gröger *et al.*[19] the thermodynamic product, the racemate in this case, is predominant in reaction with high biocatalyst concentrations or when the reaction is performed for longer time. The explanation proposed involved the dissipation of the initial driving forces toward one enantiomer rather than the other one, and the equilibrium established between the two enantiomers. On the other hand, the kinetic product, (*S*)-enantiomer in our case, is favored when the reaction occurs in short period of time or at low enzyme concentration, as the enantiopreference, even if not absolute, is predominant (Fig. 2A). Furthermore, after 24 h with 2 mg/mL of catalyst, the product in solution was already converted into a racemic mixture (Fig. 2B). It was postulated that a plausible reason for this behavior is related to a thermodynamic equilibrium, even though the mechanism at molecular level is not yet fully understood.

Enantioselective Switch Triggered by Co-/Substrate Concentration

The enantiopreference switch was first observed during the scale up of the THF-ketone amination reaction. In this case, the *ee* % switched from 11% (*S*) to 17% (*R*) when the concentration of the amino acceptor was increased from 10 mM to 300 mM, with 5 equivalents of amino donor. To determine whether the switch was due to one of the substrates or their combination, further studies were done. The concentration of amino donor (isopropyl amine, IPA) was first kept constant at 50 mM, while the amino acceptor was increased from 10 to 300 mM. The result showed that the (*S*)-preference of HeWT was enhanced by increasing the concentration of THF-ketone. However, when the amino donor concentration was increased from 50 mM to 1.5 M, the selectivity curiously shifted from (*S*) to (*R*) (Table 1).

Enantioselective Switch Triggered by Alternative Substrates

To better understand the mechanism behind the inversion, alternative amino donors and amino acceptors were screened.[20] Specifically, 2-butanone and cadaverine were tested as potential alternatives. The rationale behind screening alternative substrates was primarily related to the potential interaction between the substrates and the catalytic residues in the pocket. A similar trend was shown in *Humicola lauginosa* lipase, where the enantioselectivity changed accordingly to the bulkiness of the substrate: specifically, from an (*R*)-enantiomer, with 2-phenoxypropanoic acid ester, to an (*S*)-enantiomer analogue ester with longer acyl moieties.[21] In line with this principle, HeWT was tested with 2-butanone as an alternative amino acceptor to THF-ketone. The results showed that the (*S*)-selectivity of the enzyme was enhanced by 3 to 11-fold compared to THF-ketone (Table 2).

In addition, the effect of alternative amino donors was also investigated. Along with IPA, cadaverine was screened as amino

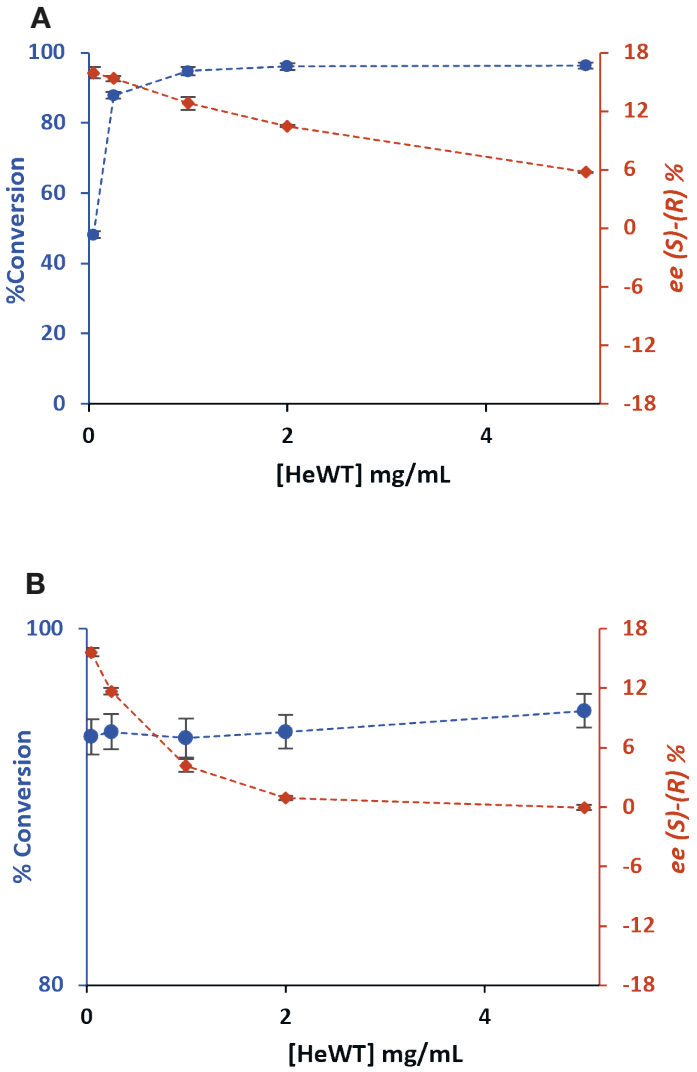


Fig. 2. Conversion and ee (%) of THF-ketone into THF-amine catalysed by different concentrations of HeWT. Samples of the reactions were taken after (A) 3 hours and (B) 24 hours.

donor. This bulky diamine cyclizes upon reaction and therefore the issue of requiring a large excess of amino donor in the reaction to shift the equilibrium is resolved.[22] Moreover, this low-cost substrate was used in previous study, resulting in an enhanced *ee* with excellent conversions.[23]

Fig. 3 shows how the behavior of the biocatalyst is similar, when increasing concentration of either cadaverine (Fig. 3A) or IPA (Fig. 3B). In both cases, as the ratio of amino donor vs amino acceptor increases leading to a racemic mixture (Fig. 3A), the concentration of the (*R*)-enantiomer increases, or even is preferred over the (*S*) (Fig. 3B). This shared trend indicates that the concentration of amine in solution strongly affects the enantiopreference of the HeWT, independently of the specific amine donor.

Table 1. Enantioselectivity inversion of HeWT at different substrate concentration.

Substrate	Concentration (M)	*ee%*
THF-ketone[a]	0.01–0.3	5–15 *(S)*
IPA[b]	0.05–1.5	10 *(S)*–7 *(R)*

[a]IPA constant at 0.05 mM; [b]THF-ketone constant at 0.1 mM

Table 2. Alternative amino acceptor: at the same concentration of 10, 100 and 300 mM the (*S*)-selectivity of the enzyme is enhanced when 2-butanone is used as substrate. The amino donor (IPA) and cofactor were fixed at 50 mM and 100 mM respectively, in KPi buffer (50 mM, pH 8).

Substrate Concentration (M)	Substrate THF-ketone (ee%)	Substrate 2-Butanone (ee%)
10	6 (S)	69 (S)
100	9 (S)	58 (S)
300	14 (S)	53 (S)

Enantioselective Switch Triggered by Co-solvents

Co-solvents have been reported to have an impact on enantioselectivity and there are indeed many examples in the literature. For instance, α-chymotrypsin exhibits a change of selectivity in anhydrous organic media, suggesting that the concentration of water molecules is critical to maintain the specific conformation of the enzyme active site, hence its specificity.[24] Klibanov *et al.* noted an inversion in enantiopreference when the protease from *Aspergillus oryzae* (AoP) catalyses the transesterification between

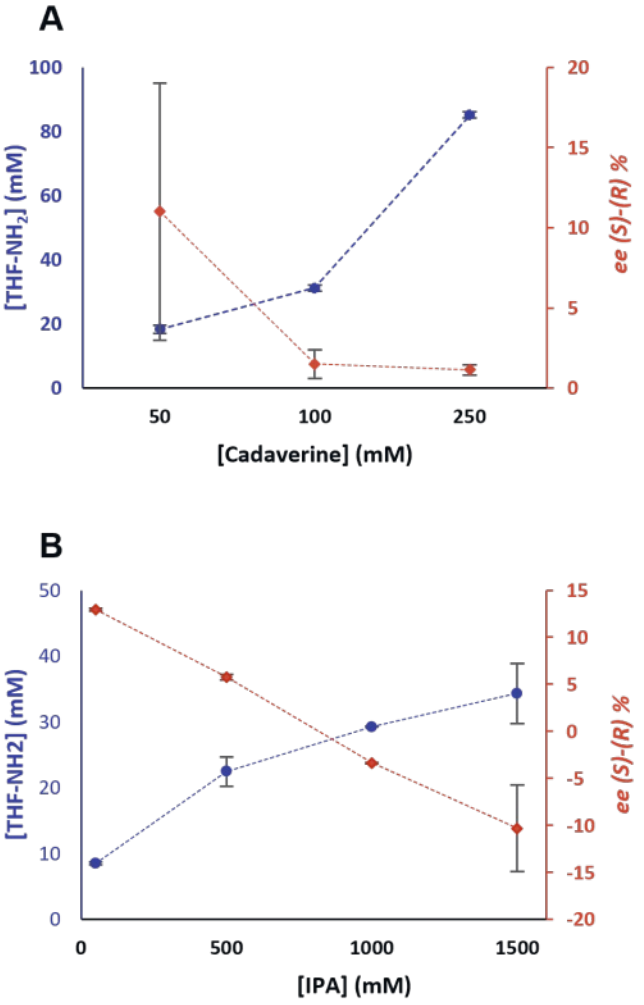
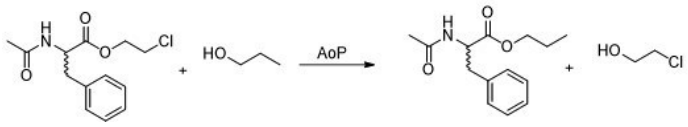


Fig. 3. Changes of the enantiopreference (*ee* (%)) with different amino donors. In both cases the amino acceptor THF-ketone was 10 mM. The biotransformations were performed for 3 hours with 0.1 mM PLP in KPi buffer (100 mM at pH 8), at 37 °C.



Scheme 3. Transesterification catalysed by AoP between N-acetyl-phenyl alanine 2-chloroethyl ester and 1-propanol.

N-acetyl-L-or D-phenyl alanine 2-chloroethyl ester and 1-propanol in presence of 18 different anhydrous solvents (Scheme 3).[25]

Mesiano *et al.* observed a similar behavior of the protease, interestingly, also in relation to changes in pressure.[26] This enzyme in fact (together with subtilisin which was also included in the study) becomes more stereoselective as the pressure increased. In our example, the enantiopreference (*ee* (%)) of the catalyst improved considerably from yielding an almost racemic mixture in presence of 1.5 M DMSO, up to a significant predominance of the (*S*)-enantiomer when isopropanol was added to the reaction mix at the same concentration (Fig. 4).

Currently, it is not fully understood whether this trend is due to the polarity or the bulkiness of solvent molecules interacting with the active site.

Other Parameters with a Key Role in the Behavior

As mentioned above, the protease from *Aspergillus oryzae* inverts its enantiopreference in response to changes in pressure. A similar case was reported for benzoylformate decarboxylase and respective mutants, where the hydrostatic pressure promoted the production of the (*R*)-enantiomer.[27] Other enzymes, however, are reported to exhibit a change in their stereopreference when other, less obvious, reaction parameters are changed. *Thermoanaerobiurn brockii* sec-ADH[28] is an enzyme that catalyzes the reduction of aliphatic acyclic ketones, resulting in the formation of alcohols such as 2-butanol. Interestingly, the enantioselectivity of the resulting alcohol depends on the temperature at which the reaction takes place. Above 26 °C, the enzyme preferentially produces the (*R*)-enantiomer, while below this temperature, the (*S*)-enantiomer is favored. Another thermo-depending enantiospecificity was presented in 2022 by Alphand *et al.* for a type II Baeyer-Villiger monooxygenase (BVMO) that loses enantiopreference at low temperatures.[29] It is also worth mentioning another curious case of an iminoreductase from *Amycolatopsis orientalis* (AoIRED).[30] Its specificity towards the (*S*)-enantiomer is extremely high, with *ee*s up to >99%, when the enzyme is freshly prepared, but this changes in favor of the (*R*)-product when the enzyme is 74 hours old.

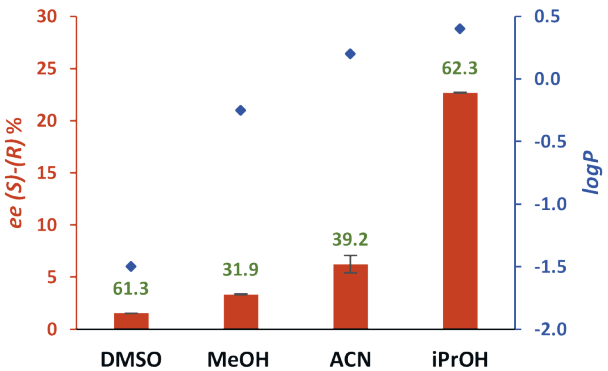


Fig. 4. Impact of co-solvent on the enantiopreference (*ee* (%)), and dependence on their logP. Each biotransformation contains purified HeWT (0.25 mg/mL),10 mM THF-ketone, 1 eq. S-MBA, 0.1 mM PLP and 1.5 M of co-solvent in KPi buffer (50 mM, pH 8), at 30 °C.

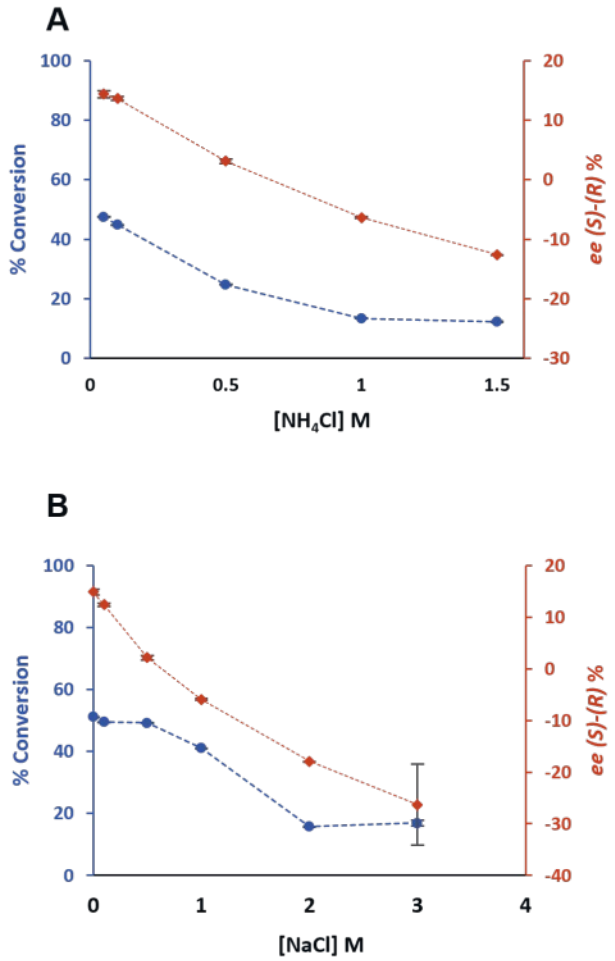


Fig. 5. Enantiopreference shift in dependence of the ionic strength. Each biotransformation contained THF-ketone (10 mM), S-MBA (1 eq.), purified HeWT (0.25 mg/mL), PLP (0.1 mM), and KPi-buffer (50 mM); pH 8, at increasing concentration of (A) NH_4Cl (0.05–1.5 M) and (B) NaCl (0.01–3 M).

In previous experiments, the impact of ionic strength on the enantiopreference of HeWT was investigated. Remarkably, this type of dependence has not been extensively reported in the literature. The screening involved different concentrations of NaCl (0.01–3 M) and NH_4Cl (0.05–1.5 M) and the outcome presented a consistent trend where the (*S*)-selectivity changed in favor of the (R) as the salt concentration increased (Fig. 5).

Considerations and Open Opportunities

Despite significant efforts to elucidate the mechanism behind the stereopreference of HeWT, it is still unclear how the switch from (*S*) to (*R*) under different conditions occurs. It is not unlikely that various factors could contribute to how the substrate enters the active site, which we expect to be the key step that discriminates the conversion into one enantiomer over the other. The factors that appear to influence the enantioselectivity of HeWT include the presence of solvents (with varying degrees of influence), the ionic strength of the solution, and the ratio of amino donor over the amino acceptor. This was clear, especially when both cadaverine and IPA were used in excess compared to substrate THF-ketone. Other factors, such as temperature, pressure, and the 'aging' of the enzyme, have also been shown to affect the stereoselectivity in other enzymes, whereby these factors are not all well understood. These observations suggest that interactions involving H-bonds in the catalytic site, as well as differences in substrate solvation, are critical in determining the stereochemistry of the final product. The main examples reported in literature, with few exceptions, primarily attribute the inversion to the presence of an organic solvent. This principle is in line with what we reported, but in most cases, the investigated enzymes are lipases or esterases.

There are a few reported cases of inversion under unusual conditions with other enzymes, but these are scattered and quite unique, not enough to envisage a general trend. With our work we postulated that the activity of HeWT is heavily influenced by the orientation of the substrate presented to the active site, with one orientation directing towards the carbonyl moiety and the other towards the C5 position, resulting in the transfer of the amino group to yield either the (*R*) or (*S*) configuration. Understanding this phenomenon implies further investigation, including single point mutations, molecular dynamic simulation and modelling. The ability to master the mechanism would certainly provide a valuable tool for the finetuning of biocatalysis, enabling the tailored synthesis of specific enantiomers.

Acknowledgements

The authors acknowledge the Swiss National Science Foundation (SNSF) (grant number200021_192274).

Received: March 14, 2023

[1] 'Asymmetric Synthesis with Chemical and Biological Methods', Eds D. Enders, K. E. Jaeger, **2007**, https://doi.org/10.1002/9783527610648.
[2] M. M. C. H. van Schie, J. D. Spöring, M. Bocola, P. Domínguez de María, D. Rother, *Green Chem*. **2021**, *23*, 3191, https://doi.org/10.1039/D1GC00561H.
[3] J. McConathy, M. J. Owens, *Prim. Care Companion CNS Disord*. **2003**, *5*, https://doi.org/10.4088/PCC.v05n0202.
[4] J. Yan, B. Xiang, D. Wang, S. Tang, M. Teng, S. Yan, Z. Zhou, W. Zhu, *J. Agric. Food Chem*. **2019**, *67*, 1784, https://doi.org/10.1021/acs.jafc.8b04536.
[5] C. Caillet, L. Chauvelot-Moachon, J.-L. Montastruc, H. Bagheri, *Br. J. Clin. Pharmacol*. **2012**, *74*, 886, https://doi.org/10.1111/j.1365-2125.2012.04262.x.
[6] R. Bentley, *Chem. Rev*. **2006**, *106*, 4099, https://doi.org/10.1021/cr050049t.
[7] N. van Oosterwijk, S. Willies, J. Hekelaar, A. C. Terwisscha van Scheltinga, N. J. Turner, B. W. Dijkstra, *Biochemistry* **2016**, *55*, 4422, https://doi.org/10.1021/acs.biochem.6b00370.
[8] M. D. Patil, G. Grogan, A. Bommarius, H. Yun, *Catalysts* **2018**, *8*, 254, https://doi.org/10.3390/catal8070254.
[9] V. Tseliou, T. Knaus, M. F. Masman, M. L. Corrado, F. G. Mutti, *Nat. Commun*. **2019**, *10*, 1, https://doi.org/10.1038/s41467-019-11509-x.
[10] E. Hegarty, F. Paradisi, *CHIMIA* **2020**, *74*, 890, https://doi.org/10.2533/chimia.2020.890.
[11] L. Cerioli, M. Planchestainer, J. Cassidy, D. Tessaro, F. Paradisi, *J. Mol. Catal. B Enzym*. **2015**, *120*, 141, https://doi.org/10.1016/j.molcatb.2015.07.009.
[12] C. M. Heckmann, L. Robustini, F. Paradisi, *ChemBioChem* **2022**, *23*, https://doi.org/10.1002/cbic.202200335.
[13] A. Cipiciani, F. Bellezza, F. Fringuelli, M. G. Silvestrini, *Tetrahedron Asymm*. **2001**, *12*, 2277, https://doi.org/10.1016/S0957-4166(01)00416-5.
[14] G. de Gonzalo, G. Ottolina, F. Zambianchi, M. W. Fraaije, G. Carrea, *J. Mol. Catal. B Enzym*. **2006**, *39*, 91, https://doi.org/10.1016/J.MOLCATB.2006.01.010.
[15] C. Zheng, V. T. Pham, R. S. Phillips, *Catal. Today* **1994**, *22*, 607, https://doi.org/10.1016/0920-5861(94)80126-6.
[16] F. Hollmann, L. G. Otten, in 'Wiley Encyclopedia of Chemical Biology', John Wiley & Sons, Inc., Hoboken, NJ, USA, **2009**, https://doi.org/10.1002/9780470048672.wecb150.
[17] C. M. Heckmann, L. Robustini, F. Paradisi, *ChemBioChem* **2022**, *23*, https://doi.org/10.1002/cbic.202200335.
[18] S. R. Marsden, L. Mestrom, D. G. G. McMillan, U. Hanefeld, *ChemCatChem* **2020**, *12*, 426, https://doi.org/10.1002/cctc.201901589.
[19] G. Rulli, N. Duangdee, K. Baer, W. Hummel, A. Berkessel, H. Gröger, *Angew. Chem. Int. Ed*. **2011**, *50*, 7944, https://doi.org/10.1002/ANIE.201008042.
[20] C. M. Heckmann, B. Dominguez, F. Paradisi, *ACS Sustain. Chem. Eng*. **2021**, *9*, 4122, https://doi.org/10.1021/acssuschemeng.0c09075.
[21] P. Berglund, I. Vallikivi, L. Fransson, H. Dannacher, M. Holmquist, M. Martinelle, F. Björkling, O. Parve, K. Hult, *Tetrahedron Asymm*. **1999**, *10*, 4191, https://doi.org/10.1016/S0957-4166(99)00438-3.
[22] C. A. McKenna, M. Štiblariková, I. De Silvestro, D. J. Campopiano, A. L. Lawrence, *Green Chem*. **2022**, *24*, 2010, https://doi.org/10.1039/D1GC02387J.
[23] A. Gomm, W. Lewis, A. P. Green, E. O'Reilly, *Chem. Eur. J*. **2016**, *22*, 12692, https://doi.org/10.1002/chem.201603188.
[24] M. Staahl, U. Jeppsson-Wistrand, M. O. Maansson, K. Mosbach, *J. Am. Chem. Soc*. **1991**, *113*, 9366, https://doi.org/10.1021/ja00024a051.

[25] S. Tawaki, A. M. Klibanov, *J. Am. Chem. Soc*. **1992**, *114*, 1882, https://doi.org/10.1021/ja00031a054.
[26] A. J. Mesiano, E. J. Beckman, A. J. Russell, *Chem. Rev*. **1999**, *99*, 623, https://doi.org/10.1021/cr970040u.
[27] S. Kara, W. S. Long, M. Berheide, S. Peper, B. Niemeyer, A. Liese, *J. Biotechnol*. **2011**, *152*, 87, https://doi.org/10.1016/J.JBIOTEC.2011.01.020.
[28] D. L. Graham, H. D. Simpson, D. A. Cowan, *Ann. N. Y. Acad. Sci.* **1996**, *799*, 244, https://doi.org/10.1111/j.1749-6632.1996.tb33208.x.
[29] R. Röllig, C. E. Paul, K. Duquesne, S. Kara, V. Alphand, *ChemBioChem* **2022**, *23*, https://doi.org/10.1002/CBIC.202200293.
[30] G. A. Aleku, H. Man, S. P. France, F. Leipold, S. Hussain, L. Toca-Gonzalez, R. Marchington, S. Hart, J. P. Turkenburg, G. Grogan, N. J. Turner, *ACS Catal*. **2016**, *6*, 3880, https://doi.org/10.1021/acscatal.6b00782.

License and Terms

The definitive version of this article is the electronic one that can be found at https://doi.org/10.2533/chimia.2023.390