General protein purification and immobilization method on controlled porosity glass: biocatalytic applications

Supplementary Information

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Contents

Enzymes used in this study

The pyridoxal-5-phosphate (PLP)-dependent ω-transaminase enzymes catalyze enantiospecific interconversions of amino and keto groups and generally have a wide substrate scope.[1] Their applicability in the synthesis of enantiopure chiral amines, pharmaceutical compounds, and intermediates thereof has been demonstrated.[2-5] Due to substrate and product inhibition, ω-transaminases have been subject to engineering.[6] To surpass the substrate or product concentration limit one may explore the possibility of altering the reaction media. An organic solvent may prevent inhibition by providing increased dissolution of hydrophobic substrates and products. Lyophilized cell extracts containing various ω-transaminases have been shown to function in organic solvents,[7] where increased reaction rates and substrate tolerance were observed. Similarly, condition promiscuity was observed with the engineered transaminase CDX-017 from Codexis immobilized to a porous methacrylic polymer support.[8]

Candida antarctica lipase B (CalB),[9] also known as *Pseudozyma antarctica* lipase B, is widely used in biocatalytic applications due to its high stability, tolerance to solvents, and high enantiospecificity in kinetic resolution of chiral alcohols.[10] CalB is efficiently immobilized via hydrophobic interactions,[11] due to its hydrophobic surface. Lipase A from the same species (CalA) displays interfacial activation, shows activity for bulky substrates such as tertiary alcohols, but often provides low enantiospecificity.[12-14] Both CalA and CalB have been subject to engineering to alter the enantiospecificity and to enable conversion of desired substrates.[15-24]

Regiospecific Baeyer-Villiger oxidation catalyzed by Baeyer-Villiger monooxygenases (BVMOs) is a promising approach in biocatalytic fine chemical synthesis.[25] Most investigations of this enzyme type have been performed on the so called single-component BVMOs,[26, 27] where $FADH_2$ is inherently regenerated by NADPH. [28]

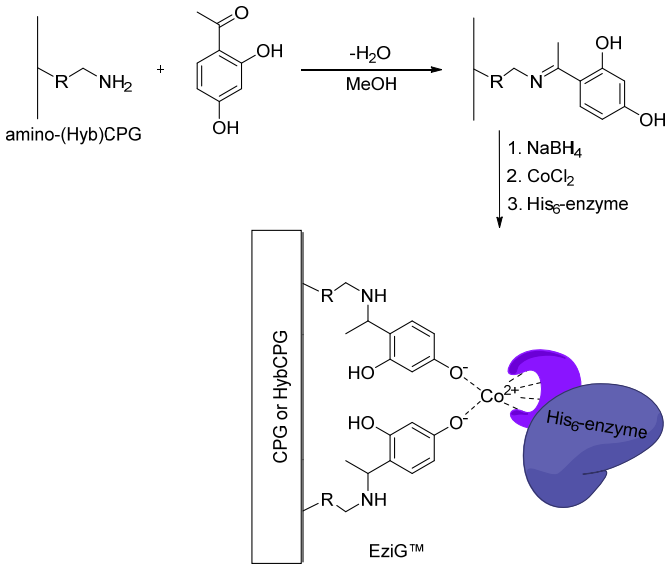
Two-component BVMOs, e.g. the $FMNH_2$-dependent 2,5-diketocamphane monooxygenase from *Pseudomonas putida* (2,5-DKCMO), have been shown to function as enantiospecific biocatalysts in aqueous buffer.[29] In contrast to their single-component homologues these BVMOs do not catalyze regeneration of the reduced flavin cofactor by NAD(P)H. Since $FMNH_2$-reconverting enzymes require NAD(P)H, a three-enzyme-based cascade reaction including regeneration of both cofactors is required. A workable cascade of this sort would demonstrate the usability of the two-component BVMOs for biocatalytic applications, e.g. with flavin reductase (FRE) from *E. coli*[30, 31] for $FMNH_2$– and alanine dehydrogenase (AlaDH) from *B. subtilis*[32] for NADH-recycling. The substrates of BVMOs are generally poorly soluble in aqueous media. However, the total amount of substrate has been increased by employment of a two-phase system, although time dependent deactivation was observed due to contact with the organic solvent interface.[33]

The EziG™ carrier, details and examples

Controlled porosity glass (CPG) is a suitable immobilization matrix for enzymes for employment in biocatalytic reactions due to its inert nature and fluid permeable properties. Conventional CPG exhibits a ligand loading capacity that is inversely related to its pore size, the surface accessible silanol groups serving as functionalization moieties have a limited density per unit of surface area of approximately 4.5 µmol/m^2.[34] Due to the nature of the CPG surface many of the functionalization moieties suffer from steric hindrance and their distribution is not uniform. By coating the interior and exterior surfaces of the CPG with an approximately 10 nm film of an organic polymer, creating a hybrid CPG (HybCPG), the dependence between loading and pore size is minimized by increased control of the spacing between functionalization sites. HybCPG is stable in an aqueous environment above pH 10 in contrast to CPG. The porous surface tailoring by the choice of the organic polymer used in HybCPG gives the possibility to create a more favorable environment for an immobilized enzyme which otherwise may be denatured by contact with a silica-like surface.[35]

The His$_6$-affinity tag enables immobilized metal affinity chromatography (IMAC) for protein purification.[36] By insertion of six to eight histidine residues (N-terminally or C-terminally) on the gene sequence of a target protein it can after overexpression be separated from the native host cell proteins by binding to a matrix of chelated metal ions. Because of its efficiency and ease of use IMAC purification has become common practice. The purified target protein can be obtained after elution e.g. with imidazole solution.[37] Use of the His$_6$-tag for enzyme immobilization has been demonstrated with mesoporous silica and silica oxide beads as carrier matrices.[38-40] Although active silica immobilized CalB was reported,[39] other less stable enzymes were found to be deactivated in the presence of silica oxide, especially so when applied to organic solvents.[35]

EziG™, the CPG based materials described herein, can be used for combined immobilization and purification of one or more His$_6$-tagged enzymes. This general method, based on the well-established IMAC technique, is effective for heterogeneous biocatalysis in a variety of solvents. The preparation of EziG™ is described in Scheme S1. EziG™ could also be used for purification of dissolved enzyme, elution with buffer containing imidazole yielded virtually similar purity to IMAC with a sepharose resin when visualized by SDS-PAGE.



Scheme S1: Preparation of EziG™ from LCAA CPG or amino HybCPG followed by binding of His$_6$-enzyme. Chelation of Co(II) by 2,4-dihydroxybenzyl residues has been described,[41] and is here schematically depicted. Alternative binding modes of Co(II) is plausible (not shown). The structure of the R-group is not revealed, and varies between the CPG products.

Table S1 contains additional data regarding the immobilized preparations used as catalyst for the model transamination reaction depicted in Scheme 1 (main article). The amount of bound Co(II) exceeds the amount of bound enzyme in all cases, presumably due to space limitations; a monolayer of enzyme on the porous surface is assumed. This reaction was chosen to visualize the enantiospecificity of the transamination and to compare the reactivity in MTBE, as demonstrated by Mutti *et al.* for lyophilized cell lysates of overexpressed ω-transaminases,[7] with three different types of ω-TA-EziG™. The E value (*E*) for all three preparations was 1.1 (*S*) for the amino donor (**1**), while the product amine (**4**) was obtained in virtually

enantiopure (*S*) form (>99% ee). The conversion was not reproducible, and varied between 10 and 75%; solvent screening and optimization of the water activity, which has been shown to be influential,[7] was not performed. Continued testing, including the use of other more stable ω-transaminases, are ongoing.

Table S1: Immobilization yield, loading data and activity of ω-TA immobilized on EziG™ prepared from different types of CPG.

CPG type[1]	Porosity[2] (Å)	Amino derivati-zation[3] (μmol/g)	Cobalt(II) loading[4] (μmol/g)	Yield of immobilization (% active enzyme in MTBE)	Loading, active enzyme in MTBE (% w/w)	Loading, active enzyme in buffer[5] (% w/w)	ee_p (%, GC)	Initial rate in MTBE[6] (μmol/min/g EziG™)	Initial rate in MTBE[6] (μmol/min/g active enzyme)
LCAA CPG	533	166	2.8	>99%	24	19	>99 (*S*)	0.38	1.58
HybCPG VBC	526 (^{2})	398	18.7	>99%	29	25	>99 (*S*)	0.70	2.41
HybCPG copo	590 (^{2})	360	25.5	>99%	21	13	>99 (*S*)	0.06	0.29

[1] Particle size 120-200 mesh [2] Measured by mercury porosimetry, after polymer derivatization the accessible pore diameters of the HybCPGs is reduced by 160-200 Å. [3] Nitrogen content before preparation of EziG™ according to Scheme 1. [4] Elemental analysis of the EziG™ without bound enzyme. [5] No PLP was added after immobilization, dissociation thereof is a possible cause of the lower amount of active enzyme compared to buffer. [6] Initial rate of the reaction depicted in Scheme 2.

Although porous hydrophobic supports are workable for immobilization of CalB, testing of EziG™ as carrier was performed to demonstrate its general applicability. The catalytic activity was approximately six times higher with Accurel®-immobilized CalB compared to the corresponding EziG™ immobilized preparation, when comparing the obtained initial rate per mass unit. The CPG materials are denser than Accurel® and show no significant bulk swelling in solvent. Therefore the volumetric activity of CalB-EziG™ is expected to be constant whereas the volumetric activity of the Accurel®-preparations will decrease *in situ* as the material swells in the solvent. The volumetric activity of the dry Accurel®-immobilized CalB was approximately two times higher than the best EziG™ preparation (Accurel® versus HybCPG copo, Table S2) based on the difference of the bulk density. In the solvent the Accurel® swelled to approximately double the volume; therefore the final volumetric activity of EziG™- and Accurel®-immobilized CalB is more or less equal. The results from the immobilized lipase preparations in the chosen model reaction (Scheme 2, main article) are shown in Table S2.

CalB could also be bound to unmodified amino-HybCPG copo (without modification according to Scheme S1, to form EziG™), assumingly by hydrophobic interactions analogous to binding to Accurel®. When performing the same immobilization protocol as done with EziG™, with unmodified amino-HybCPG copo as carrier, approximately one third of the activity as that obtained with the corresponding EziG™ material was reached (Table S2). In this case the enzyme was desorbed when the particles were incubated in buffer, after which no activity could be measured; whereas the EziG™ retained activity (48 h, 50 mM MOPS, pH 7.5).

Immobilization on Accurel® or similar supports is efficient and suitable for CalB, e.g. as commercially available Novozym 435. However, immobilization with EziG™ may still be an attractive choice for this enzyme since it can be performed in a shorter time frame from cell culture supernatant without prior purification. CalB as enantiospecific acylation catalyst in combination with Pd-nanoparticles for amino racemization bound in the same compartments in a mesoporous silica carrier has been shown to be effective for dynamic kinetic resolution.[42] Exploration of other catalysts in combination with CalB, such as metal ions, metal nanoparticles, or other enzymes, is ongoing in our laboratory, for which EziG™ and variants thereof may prove useful. Such experiments cannot be done with Accurel® in a straightforward manner since the majority of available enzymes do not bind to this material.

The Accurel® preparation of the CalB variant Trp104Ala had a different *E* value compared to the EziG™ preparation (Table S2); this may be a result of diffusion effects or structural distortion of the adsorbed enzyme.

Table S2: Summary of data for immobilized preparations of CalB and CalA, with activities for the chosen model reaction (Scheme 3).

His_6-lipase	Carrier[1]	Bulk density[2] (g/cm^3)	Incubation time for immobilization	Reaction temperature (°C)	*E*	Activity in toluene[3] (μmol/min/g carrier)	Bulk volumetric activity in toluene[3] (μmol/min/cm^3 carrier)
CalB	EziG™ (LCAA CPG)	0.28	30 min	22	>300 (*R*)	0.83	0.23
CalB	EziG™ (HybCPG VBC)	0.23	30 min	22	>300 (*R*)	0.90	0.21
CalB	EziG™ (HybCPG copo)	0.245	30 min	22	>300 (*R*)	1.08	0.26
CalB	Accurel®	0.10	8 h	22	>300 (*R*)	5.99	<0.60[4]
CalB Trp104Ala	EziG™ (LCAA CPG)	0.28	30 min	50	n/a	n/d	n/d
CalB Trp104Ala	EziG™ (HybCPG VBC)	0.23	30 min	50	1.3 (*R*)	0.19	0.04
CalB Trp104Ala	EziG™ (HybCPG copo)	0.245	30 min	50	1.0	0.04	0.01
CalB Trp104Ala	Accurel®	0.10	10 days	50	7.1 (*R*)	0.23	<0.023[4]
CalB	Amino-HybCPG copo[5]	0.245	30 min	22	>300 (*R*)	0.36	0.083
CalA	EziG™ (LCAA CPG)	0.28	30 min	22	1.3 (*R*)	16.18	4.53

[1] See Table 1 for EziG™ data. [2] Dry carrier before binding of enzyme. [3] Initial rate of consumption of 1-phenylethanol. [4] Without inclusion of swelling which occurs in contact with solvent. [5] Amino-HybCPG copo was used without modification according to Scheme 1.

With the aim of creating a scalable biocatalytic BVMO reaction a multi-phase system was tested (Figure S1). The reaction set-up consisted of an aqueous phase for dissolution of alanine and the cofactors, to which oxygen gas was added with a syringe, and an organic phase with (+)-camphor (**9**) (100 mM in cyclohexane). It has previously been demonstrated that in a two-phase reaction system of water and cyclohexane, an interface can deactivate biocatalysts such as the single-component BVMO phenylacetone monooxygenase.[33] The system described here required EziG™-immobilization for conversion, and the immobilized preparation effectively protected the enzymes from contact with the solvent interface while mild orbital shaking was applied.

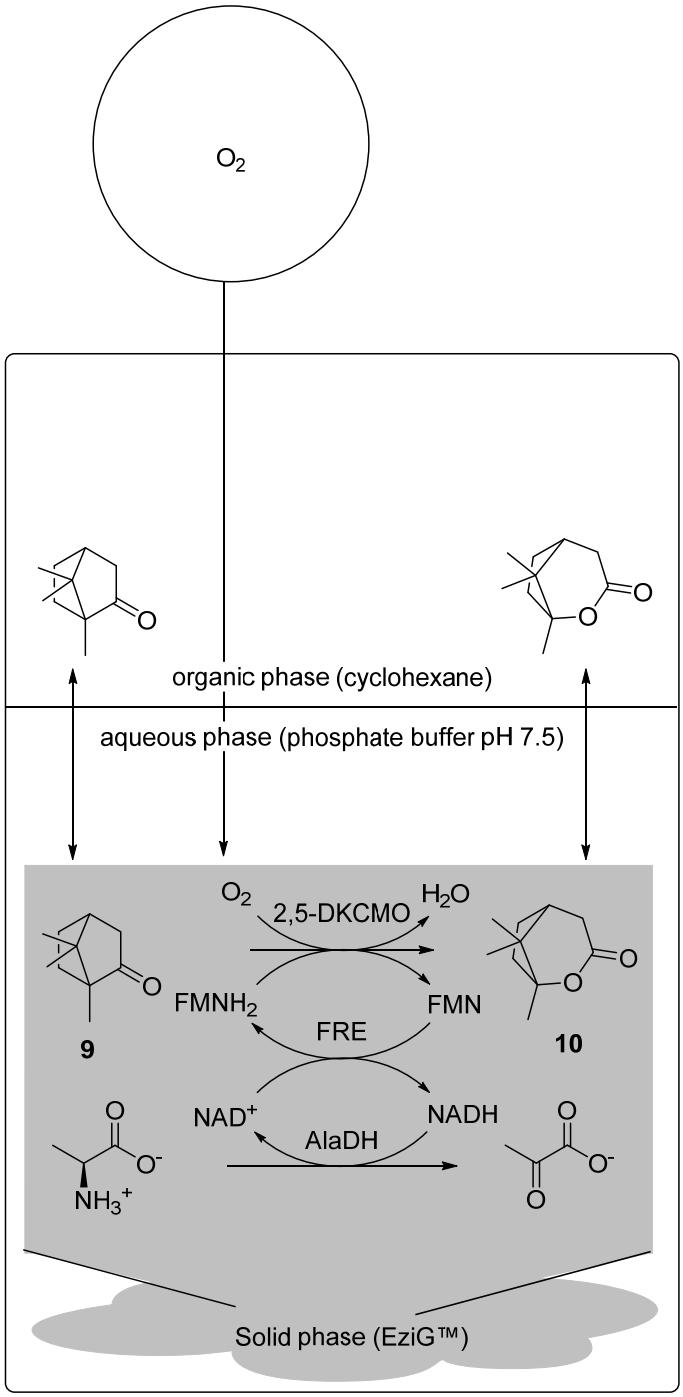


Figure S1: Three-phase reaction system with an EziG™ immobilized single-component BVMO (2,5-DKCMO) in a cascade reaction with co-immobilized flavin reductase (FRE) and alanine dehydrogenase (AlaDH) for co-factor regeneration.

Materials and Methods

EziG™ Synthesis

The EziG™, chelating (Hyb)CPG for His_6-protein immobilization and purification, was constructed by treating amino-(Hyb)CPG (5.0 g) of desired type (Prime Synthesis, Inc) with 2,4-dihydroxyacetophenone (1.5 eq to amino functionalities) in methanol (200 mL) with continuous stirring for 60 min. The formed imine was reduced by addition of $NaBH_4$ (2 eq.) over 30 min, followed by washing with $NaCO_3$ (sat. aq.), water and then ethanol, followed by drying (80 °C, 2 h). The beads were then immersed in $CoCl_2$ (sat. aq. 100 mL), followed by rinsing with water and ethanol and drying (80 °C, 2 h).

Enzyme Preparation and Immobilization

Cultivations were performed as previously described.[17, 22, 43-45] The plasmid containing the gene encoding AlaDH was a gift from Prof. Wolfgang Kroutil at the University of Graz. When absent, His_6-tags were added to the genes by PCR.

The cell lysates were prepared by cell resuspension in HEPES buffer (50 mM, 500 mM NaCl, pH 8.3) for ω-TA and in potassiumphosphate buffer (50 mM, 500 mM NaCl, pH 7.5) for the others. After addition of BugBuster™ 10X cell debris was removed by centrifugation. The cell culture supernatants containing CalA or CalB were used without buffering. EziG™ was immersed in the lysates or supernatants followed by stirring on an orbital shaker (150 rpm). Bradford analyzed samples of the solutions during immobilization confirmed the completion of the binding and saturation of the EziG™ support as the protein concentration seized to decrease; activity assays where also performed with the solutions after removal of the EziG™ by filtration. The immobilized preparations were then rinsed with the appropriate buffer (MOPS 20 mM pH 7.5 for CalA and CalB, see above for the others) and then dried under vacuum for 16 h. Extraction of enzyme from the EziG™ was done by immersing the wet particles (no prior drying under vacuum) in elution buffer (50 mM sodiumphosphate, 500 mM imidazole, pH 7.5) and incubation on an orbital shaker for 20 min. The presence and purity of the extracted enzymes were visualized by SDS-PAGE. Immobilization of CalB and CalB Trp104Ala on ethanol activated Accurel® (Accurel MP1001, particle size <1000 µm, Membrana GmbH (Wuppertal, Germany)) was performed by adding the porous material to concentrated supernatant in a ratio of 50:1 to the amount of enzyme (protein content was measured by Bradford), followed by incubation for at least eight hours.

Enzymatic assays

Active site quantification of ω-TA in aqueous buffer was performed as previously described.[43] Active site quantification of immobilized ω-TA in solvent was performed by adding ω-TA-EziG™ to 1-phenylethylamine (**1**) (1 mM, 1 mL, MTBE a_w=0.6, 1 mM pentadecane). The reaction mixture was stirred on an orbital shaker (150 rpm, 24 h, 22 °C). The water activity of the solvent was set by salt hydrate pairs (Na_2HPO_4, $2H_2O/7H_2O$) but not controlled after addition of the ω-TA-EziG™ or during the reaction. Conversions were measured by GC (200 µL samples to EtOAc, 3 drops of acetic anhydride and triethylamine, 8 h incubation at 22 °C), with pentadecane as internal standard.

Active site quantification of immobilized ω-TA in buffer was performed by adding ω-TA-EziG™ to 1-phenylethylamine (**1**) (1 mM, 1 mL, 50 mM HEPES, pH 7.0). The reaction mixture was stirred on an orbital shaker (150 rpm, 24 h, 22 °C). Samples (400 µL) were treated with NaOH (1%), extracted with DCM and analyzed by GC after addition of pentadecane (1 mM, EtOAc). The conversions were in all cases compared to blank reactions with EziG™ (no enzyme bound).

Reactions catalyzed by ω-TA-EziG™ were performed by addition of ω-TA-EziG™ (20 mg) to the reaction mixture (3 mL, 100 mM 1-phenylethylamine (**1**), 50 mM 2-phenoxypropanone (**2**), MTBE a_w=0.6, 50 mM pentadecane). The conversion and enantiomeric excess of 1-phenylethylamine (**1**) was followed by chiral GC, samples (50 µL) taken at recorded time points were treated as described above; formation of 1-phenoxypropan-2-amine (**4**) was measured without derivatization.

Lipase catalyzed kinetic resolution reactions were performed by addition of immobilized preparations (59.9 mg CalB-EziG™ LCAA CPG, 39.5 mg CalB-EziG™ HybCPG VBC, 38.4 mg CalB-EziG™ HybCPG copo, 13.0 mg CalA-EziG™ LCAA CPG or 21.4 mg CalB-Accurel) to the reaction mixture (3 mL, 10 mM 1-phenylethanol (**5**), 100 mM vinyl butyrate (**6**), 5 mM pentadecane, toluene a_w=0.1) followed by incubation with orbital shaking (200 rpm, 22 °C or 50 °C). The conversion and enantiomeric excess of 1-phenylethanol (**5**) and 1-phenylethyl butyrate (**7**) was measured by chiral GC by taking samples (50 µL) at recorded time points, with pentadecane as internal standard.

BVMO reactions were performed by adding 2,5-DKCMO, FRE and AlaDH (cell lysates or 65 mg immobilized preparation) to the reaction mixture (5.0 mL total, 2.0 mM (+)-camphor (**9**), 5.0 mM L-alanine, 0.3 mM FMN, and 0.5 mM NADH, in phosphate buffer (100 mM, pH 7.5)). Oxygen was then dissolved (bubbling for 30 s) followed by sealing of the vessel; the mixture was incubated on an orbital shaker (150 rpm, 22 °C). Samples (500 µL) were extracted to EtOAc with ethylbenzoate as internal standard and analyzed by GC. Equally treated blank reactions with EziG™ with no enzyme bound were also performed. The proportions and amounts of the enzymes were not measured.

Multi-phase BVMO reactions were performed by adding 2,5-DKCMO, FRE and AlaDH (cell lysates or 1.0 g immobilized preparation) to phosphate buffer (100 mM, pH 7.5) with L-alanine (160 mM), FMN (0.3 mM) and NADH (0.5 mM) with a total liquid volume of 5.0 mL. 5 mL cyclohexane with (+)-camphor (**9**) (100 mM) was then added as a second liquid phase. The sealed vessel was stirred on an orbital shaker (100 rpm, 22 °C) with continuous oxygen addition to the aqueous phase. Samples (50 μL) from the organic phase were taken at recorded time points and analyzed by GC after addition of ethylbenzoate (2.0 mM in EtOAc) as internal standard; the conversion after 72 h was measured after extraction of all components with EtOAc (20 mL), and compared with blank reactions (EziG™ without enzymes).

References

1. J. S. Shin and B. G. Kim, *Biosci Biotechnol Biochem*, 2001, **65**, 1782-1788.
2. C. K. Savile, J. M. Janey, E. C. Mundorff, J. C. Moore, S. Tam, W. R. Jarvis, J. C. Colbeck, A. Krebber, F. J. Fleitz, J. Brands, P. N. Devine, G. W. Huisman and G. J. Hughes, *Science*, 2010, **329**, 305-309.
3. J. S. Shin and B. G. Kim, *Biotechnol Bioeng*, 1999, **65**, 206-211.
4. M. Höhne and U. T. Bornscheuer, *ChemCatChem*, 2009, **1**, 42-51.
5. D. Koszelewski, I. Lavandera, D. Clay, D. Rozzell and W. Kroutil, *Adv Synth Catal*, 2008, **350**, 2761-2766.
6. P. Tufvesson, J. Lima-Ramos, J. S. Jensen, N. Al-Haque, W. Neto and J. M. Woodley, *Biotechnol Bioeng*, 2011, **108**, 1479-1493.
7. F. G. Mutti and W. Kroutil, *Adv Synth Catal*, 2012, **354**, 3409-3413.
8. M. D. Truppo, H. Strotman and G. Hughes, *ChemCatChem*, 2012, **4**, 1071-1074.
9. J. Uppenberg, N. Oehrner, M. Norin, K. Hult, G. J. Kleywegt, S. Patkar, V. Waagen, T. Anthonsen and T. A. Jones, *Biochemistry*, 1995, **34**, 16838-16851.
10. E. M. Anderson, K. M. Larsson and O. Kirk, *Biocatal Biotransform*, 1998, **16**, 181-204.
11. J. A. Bosley and A. D. Peilow, *J Am Oil Chem Soc*, 1997, **74**, 107-111.
12. R. Kourist and U. T. Bornscheuer, *Appl Microbiol Biotechnol*, 2011, **91**, 505-517.
13. S. H. Krishna, M. Persson and U. T. Bornscheuer, *Tetrahedron: Asymmetry*, 2002, **13**, 2693-2696.
14. M. Martinelle, M. Holmquist and K. Hult, *Biochim Biophys Acta*, 1995, **1258**, 272-276.
15. A. O. Magnusson, J. C. Rotticci-Mulder, A. Santagostino and K. Hult, *ChemBioChem*, 2005, **6**, 1051-1056.
16. A. O. Magnusson, M. Takwa, A. Hamberg and K. Hult, *Angew Chem Int Ed Engl*, 2005, **44**, 4582-4585.
17. K. Engström, M. Vallin, P. O. Syrén, K. Hult and J. E. Bäckvall, *Org Biomol Chem*, 2011, **9**, 81-82.
18. A. Hamberg, S. Maurer and K. Hult, *Chem Commun (Camb)*, 2012, **48**, 10013-10015.
19. J. Nyhlén, B. Martín-Matute, A. G. Sandström, M. Bocola and J. E. Bäckvall, *ChemBioChem*, 2008, **9**, 1968-1974.
20. D. Rotticci, J. C. Rotticci-Mulder, S. Denman, T. Norin and K. Hult, *ChemBioChem*, 2001, **2**, 766-770.
21. A. G. Sandström, K. Engström, J. Nyhlén, A. Kasrayan and J. E. Bäckvall, *Protein Eng Des Sel*, 2009, **22**, 413-420.
22. A. G. Sandström, Y. Wikmark, K. Engström, J. Nyhlén and J. E. Bäckvall, *Proc Natl Acad Sci U S A*, 2012, **109**, 78-83.
23. Q. Wu, P. Soni and M. T. Reetz, *J Am Chem Soc*, 2013, **135**, 1872-1881.
24. K. Engström, J. Nyhlén, A. G. Sandström and J. E. Bäckvall, *J Am Chem Soc*, 2010, **132**, 7038-7042.
25. D. E. Torres Pazmino, H. M. Dudek and M. W. Fraaije, *Curr Opin Chem Biol*, 2010, **14**, 138-144.
26. K. Balke, M. Kadow, H. Mallin, S. Sass and U. T. Bornscheuer, *Org Biomol Chem*, 2012, **10**, 6249-6265.
27. H. Leisch, K. Morley and P. C. Lau, *Chem Rev*, 2011, **111**, 4165-4222.
28. C. Rodriguez, I. Lavandera and V. Gotor, *Curr Org Chem*, 2012, **16**, 2525-2541.
29. R. Gagnon, G. Grogan, E. Groussain, S. Pedragosamoreau, P. F. Richardson, S. M. Roberts, A. J. Willetts, V. Alphand, J. Lebreton and R. Furstoss, *J Chem Soc Perk T 1*, 1995, 2527-2528.
30. M. Fontecave, R. Eliasson and P. Reichard, *J Biol Chem*, 1987, **262**, 12325-12331.
31. M. Kadow, K. Balke, A. Willetts, U. Bornscheuer and J. E. Bäckvall, *Appl Microbiol Biotechnol*, 2014**, 98,** 3975–3986**.**.
32. F. G. Mutti, C. S. Fuchs, D. Pressnitz, N. G. Turrini, J. H. Sattler, A. Lerchner, A. Skerra and W. Kroutil, *Eur J Org Chem*, 2012, 1003-1007.
33. F. Schulz, F. Leca, F. Hollmann and M. T. Reetz, *Beilstein J Org Chem*, 2005, **1**, 10.
34. K. K. Unger, N. Becker and P. Roumeliotis, *J Chromatogr*, 1976, **125**, 115-127.
35. M. Lundqvist, I. Sethson and B. H. Jonsson, *Langmuir*, 2004, **20**, 10639-10647.
36. J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature*, 1975, **258**, 598-599.
37. E. Sulkowski, *Trends Biotechnol*, 1985, **3**, 1-7.
38. K. E. Cassimjee, M. Trummer, C. Branneby and P. Berglund, *Biotechnol Bioeng*, 2008, **99**, 712-716.
39. K. E. Cassimjee, R. Kourist, D. Lindberg, M. Wittrup Larsen, N. H. Thanh, M. Widersten, U. T. Bornscheuer and P. Berglund, *Biotechnol J*, 2011, **6**, 463-469.
40. D. A. Gaffney, S. O'Neill, M. C. O'Loughlin, U. Hanefeld, J. C. Cooney and E. Magner, *Chem Commun (Camb)*, 2010, **46**, 1124-1126.
41. A. Goswami and A. K. Singh, *Anal Chim Acta*, 2002, **454**, 229-240.
42. K. Engström, E. V. Johnston, O. Verho, K. P. J. Gustafson, M. Shakeri, C.-W. Tai and J.-E. Bäckvall, *Angew Chem Int Ed Engl*, 2013, **52**, 14006–14010.
43. K. E. Cassimjee, M. S. Humble, V. Miceli, C. G. Colomina and P. Berglund, *ACS Catal*, 2011, **1**, 1051-1055.
44. M. Kadow, S. Saß, M. Schmidt and U. T. Bornscheuer, *AMB Express*, 2011, **1**, 13.
45. F. G. Mutti, C. S. Fuchs, D. Pressnitz, J. H. Sattler and W. Kroutil, *Adv Synth Catal*, 2011, **353**, 3227-3233.