Tuning Enzyme Activity for Nonaqueous Solvents
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**Introduction**

The enzyme 4-oxalocrotonate tautomerase (4-OT) from *Pseudomonas putida* mt-2 catalyzes the tautomerization of 2-hydroxyhexa-2,4-dienedioate (1) to 2-oxohex-3-enedioate (2) as part of a metabolic pathway for the degradation of aromatic hydrocarbons (Scheme 1A).[1,2] In addition, 4-OT can promiscuously catalyze several C–C bond-forming reactions, including Michael-type additions and aldol condensations, yielding precursors for important classes of pharmaceuticals.[3–7] For instance, the 4-OT catalyzed Michael-type addition of acetaldehyde to nitrostyrene in 40% ethanol (permitting high substrate loading) to give the desired γ-nitroaldehyde product in excellent isolated yield (89%) and enantiopurity (ee = 98%). The present work demonstrates the power of mutability-landscape-guided enzyme engineering for efficient biocatalysis in non-aqueous solvents.

![Scheme 1](image)

Scheme 1. A) Tautomerization reaction naturally catalyzed by 4-OT. B) Michael-type addition of acetaldehyde (3) to nitroalkenes 4a and 4b, which are precursors for the γ-amino butyric acid analogues phenibut (6a) and pregabalin (6b), respectively (Scheme 1B).[5]

Enzymes have evolved to function under aqueous conditions and may not exhibit features essential for biocatalytic application, such as the ability to function in high concentrations of an organic solvent. Consequently, protein engineering is often required to tune an enzyme for catalysis in non-aqueous solvents. In this study, we have used a collection of nearly all single mutants of 4-oxalocrotonate tautomerase, which promiscuously catalyzes synthetically useful Michael-type additions of acetaldehyde to various nitroolefins, to investigate the effect of each mutation on the ability of this enzyme to retain its “Michaelase” activity in elevated concentrations of ethanol. Examination of this mutability landscape allowed the identification of two hotspot positions, Ser30 and Ala33, at which mutations are beneficial for catalysis in high ethanol concentrations. The “hotspot” position Ala33 was then randomized in a highly enantioselective, but ethanol-sensitive 4-OT variant (L8F/M45Y/F50A) to generate an improved enzyme variant (L8F/A33I/M45Y/F50A) that showed great ethanol stability and efficiently catalyzes the enantioselective addition of acetaldehyde to nitro styrene in 40% ethanol (permitting high substrate loading) to give the desired γ-nitroaldehyde product in excellent isolated yield (89%) and enantiopurity (ee = 98%). The present work demonstrates the power of mutability-landscape-guided enzyme engineering for efficient biocatalysis in non-aqueous solvents.

**Very Important Paper**

**Tuning Enzyme Activity for Nonaqueous Solvents: Engineering an Enantioselective “Michaelase” for Catalysis in High Concentrations of Ethanol**

Chao Guo,[a] Lieuwe Biewenga,[a] Max Lubberink,[a, b] Ronald van Merkerk,[a] and Gerrit J. Poelarends*[a]

Enzymes have evolved to function under aqueous conditions and may not exhibit features essential for biocatalytic application, such as the ability to function in high concentrations of an organic solvent. Consequently, protein engineering is often required to tune an enzyme for catalysis in non-aqueous solvents. In this study, we have used a collection of nearly all single mutants of 4-oxalocrotonate tautomerase, which promiscuously catalyzes synthetically useful Michael-type additions of acetaldehyde to various nitroolefins, to investigate the effect of each mutation on the ability of this enzyme to retain its “Michaelase” activity in elevated concentrations of ethanol. Examination of this mutability landscape allowed the identification of two hotspot positions, Ser30 and Ala33, at which mutations are beneficial for catalysis in high ethanol concentrations. The “hotspot” position Ala33 was then randomized in a highly enantioselective, but ethanol-sensitive 4-OT variant (L8F/M45Y/F50A) to generate an improved enzyme variant (L8F/A33I/M45Y/F50A) that showed great ethanol stability and efficiently catalyzes the enantioselective addition of acetaldehyde to nitrostyrene in 40% ethanol (permitting high substrate loading) to give the desired γ-nitroaldehyde product in excellent isolated yield (89%) and enantiopurity (ee = 98%). The presented work demonstrates the power of mutability-landscape-guided enzyme engineering for efficient biocatalysis in non-aqueous solvents.

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Hence, several enzyme engineering studies have been performed to improve the activity and enantioselectivity of 4-OT for this reaction.[8,9]

Solubilization of substrates 4a and 4b requires the use of cosolvents. Because enzymes have evolved to function under aqueous conditions, high concentrations of cosolvents can significantly affect their catalytic performance and eventually result in enzyme precipitation.[10] In this study, we used a collection of nearly all single-mutant variants of 4-OT to investigate the effect of each mutation on the ability of the enzyme to retain its “Michaelase” activity in elevated concentrations of

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ethanol. Ethanol was selected as cosolvent because it is readily accessible from bio-renewable sources and can also function as a precursor for 3.\(^{[11]}\) Randomization of the identified “hotspot” position Ala33 in the context of a previously engineered highly enantioselective, but ethanol-sensitive, 4-OT variant (L8F/M45Y/F50A) afforded an improved enzyme variant (4-OT L8F/A33I/M45Y/F50A) with high ethanol stability, allowing efficient and enantioselective Michael-type addition reactions in 40% (v/v) ethanol. As such, our work provides an interesting example of how targeted mutagenesis of a single amino acid can radically modify the cosolvent stability of an enzyme, allowing efficient catalysis in high concentrations of ethanol.

**Results**

In order to identify “hotspot” positions of 4-OT at which mutations are beneficial for catalysis in high concentrations of ethanol, a defined collection of 1040 single-mutant variants of 4-OT\(^{[21]}\) was screened using cell-free extracts (CFEs) prepared from cultures each expressing a different 4-OT mutant. The Michael-type addition of 3 to 4a was used as a model reaction in screening owing to the marked absorbance of 4a at 320 nm. Control experiments demonstrated no significant difference between the effect of ethanol on the reaction catalyzed by purified 4-OT or 4-OT present in CFE (Figure S1). The “Michaelase” activity of each single-mutant variant of 4-OT was measured using either 5 or 25% ethanol as cosolvent and the remaining activity at 25% ethanol, compared to that at 5% ethanol, was graphically represented in a mutability landscape for solvent tolerance (Figure 1). Increasing the concentration of ethanol from 5 to 25% reduced the “Michaelase” activity of wild-type 4-OT by approximately 50% (Figures 1 and 2A). Interestingly, analysis of the ethanol-tolerance mutability landscape revealed two “hotspot” positions, Ser30 and Ala33, at which single mutations resulted in enzyme variants that showed more than 70% residual “Michaelase” activity at 25% ethanol (Figure 1). Notably, the crystal structure of wild-type 4-OT does not provide an immediate explanation for the improved ethanol tolerance caused by mutations at these two positions (Figure S2), illustrating the importance of mutability-landscape navigation to identify functional “hotspot” positions. Three single mutants, 4-OT S30C, S30Y and A33D, which showed high ethanol tolerance, were purified and the effect of ethanol and other cosolvents on the “Michaelase” activity was tested (Figure 2). Interestingly, 4-OT S30C, S30Y and A33D also showed tolerance towards other cosolvents such as DMSO and isopropanol, suggesting that these mutations convey general cosolvent resistance. Notably, while the 4-OT variants perform well up to 40% DMSO, visible protein precipitation with concomitant loss of activity was observed at DMSO concentrations ≥ 50% (v/v).

We next investigated if we could use the information from the solvent-tolerance mutability landscape to engineer a previously constructed highly enantioselective 4-OT variant, L8F/M45Y/F50A (4-OT FYA),\(^{[19]}\) to function in high concentrations of ethanol. As single mutants at “hotspot” position Ala33 generally exhibited higher “Michaelase” activity than those at “hotspot” position Ser30, we focused our mutagenesis strategy on position Ala33.\(^{[20]}\) In the context of 4-OT FYA, residue Ala33 was mutated to all possible amino acids and the nineteen enzyme variants were expressed and purified to homogeneity. Initially, we tested all variants for visible precipitation upon incubation (1 h) of the enzyme with increasing concentrations of ethanol (up to 50%). The parental enzyme 4-OT FYA rapidly precipitated when incubated with ethanol concentrations equal to or greater than 10% (Figure 3D). Interestingly, enzyme variants with isoleucine (A33I/FYA), leucine (A33L/FYA) or valine (A33V/FYA) at position 33 could tolerate up to 50% ethanol without any visible protein precipitation after 1 hour of incubation. Notably, substitution of Ala33 to aspartate, glutamate or cysteine in the context of 4-OT FYA also strongly improved the stability of the enzyme in high concentrations of ethanol, tolerating up to 40% ethanol without visible protein precipitation.

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**Figure 1.** Ethanol-tolerance mutability landscape of 4-OT. The horizontal axis of the data matrix represents the residue positions of 4-OT. The vertical axis represents all 20 canonical amino acids. The wild-type amino acid at each position is indicated with a bold square. White squares indicate that this mutant is not present in the collection. The color of the square indicates the residual “Michaelase” activity of a specific single-mutant variant of 4-OT for the addition of 3 to 4a in 25% ethanol, compared to that in 5% ethanol. Grey boxes indicate that the “Michaelase” activity was too low to determine the remaining activity.
Next, we tested the activity and enantioselectivity of these six quadruple mutants in the presence of 5, 30 or 50% ethanol. The Michael-type addition of 3 to 4b was used as model reaction because the optical purity of product 5b can easily be analyzed by gas chromatography. All six quadruple mutants proved to be highly enantioselective giving nearly enantiopure product 5b, and, importantly, increasing ethanol concentrations do not negatively affect enzyme enantioselectivity (Table 1). In the presence of 30% ethanol, the reactions with the six quadruple mutants were completed within 35–70 min, whereas the reaction with the parental enzyme (4-OT FYA) showed no conversion due to rapid protein precipitation (Table 1).

The best mutant, 4-OT A33I/FYA, catalyzed the Michael-type addition reaction practically as efficient in 40% ethanol as in 10% ethanol (Figure 3B). Moreover, pre-incubation of 4-OT A33I/FYA in 50% ethanol for 10 h resulted in only 25% loss of activity (Figure 3A). On the contrary, the parental enzyme (4-OT FYA) rapidly lost its activity upon incubation in 10% ethanol (Figure 3A,B). Interestingly, an increase in the T<sub>60</sub> of approximately 6°C was observed for 4-OT A33I/FYA compared to 4-OT FYA, indicating that 4-OT A33I/FYA is also somewhat more thermostable than 4-OT FYA (Figure 3C). Finally, to further demonstrate the synthetic usefulness of 4-OT A33I/FYA, a semi-preparative scale reaction was performed, using 40% ethanol as cosolvent, which allowed for the solubilization of 15 m<sup>4</sup>a. Using a 6.7-fold excess of 3 over 4a, the reaction was finished within 200 min. Product (R)-5a was obtained in excellent isolated yield (89%) and enantiopurity (ee = 98%; Figures S3 and S4). Taken together, these results demonstrate that 4-OT can be engineered to efficiently catalyze enantioselective Michael-type reactions in ethanol concentrations up to 40%.

**Discussion**

Enzymes are highly attractive catalysts for organic synthesis because of their unparalleled enantio-, regio- and chemoselectivity. Given that enzymes have evolved to operate in the mild aqueous environment of the cell, they are usually not fit for preparative biocatalysis in the presence of high concentrations of organic cosolvents required for substrate solubilization. A solution to this problem is the engineering of enzymes to improve their cosolvent tolerance. Rational enzyme engineering towards increased cosolvent tolerance is still very challenging due to our relatively poor understanding of the interactions between enzymes and solvent molecules. Currently employed rational engineering strategies include stabilization of flexible regions, introduction of new cysteine bridges and modification of access tunnels. An important strategy to guide enzyme-engineering efforts is to make use of mutability landscapes. By screening a large collection of nearly all single mutants of an enzyme, important information is obtained on single mutations or residue positions that influence a desired characteristic of the enzyme. Here we have applied mutability-landscape-guided enzyme engineering to improve the ethanol tolerance of 4-OT. Screening of a collection of nearly all single-mutant variants of 4-OT revealed that mutations at particularly positions Ser30 and Ala33 resulted in improved ethanol tolerance. Interestingly, a previously reported 4-OT variant with 3.5-fold increased "Michael-
We used the information from the ethanol-tolerance mutability landscape to further engineer a previously constructed highly enantioselective 4-OT variant (FYA) that exhibits poor ethanol stability. All 19 possible variants at position Ala33 in the context of 4-OT FYA were constructed, expressed and purified. Remarkably, from this small, focused set of quadruple mutants, six mutants showed strongly improved ethanol tolerance. It is interesting to note that all six variants, including 4-OT A33D/FYA, are highly enantioselective towards the synthe-

![Figure 3. Characterization of 4-OT A33I/FYA (FYA). A) Enzyme activity for the Michael-type addition of 3 to 4a after pre-incubation (1, 2, 4, 6, 8 or 10 h) in the presence of 10, 30 or 50% ethanol. The data is normalized to the activity of the enzyme without pre-incubation. B) Progress curves of 4-OT A33I/FYA- and 4-OT FYA-catalyzed Michael-type additions in the presence of different ethanol concentrations. C) Temperature-induced inactivation profiles of 4-OT A33I/FYA and 4-OT FYA. The enzyme activity after incubation for 60 min at 30°C was set as 100%. D) Photograph of cuvettes in which 4-OT FYA (190 µg mL⁻¹, top five cuvettes) and 4-OT A33I/FYA (190 µg mL⁻¹, bottom five cuvettes) were incubated for 1 h in 20 mM NaH₂PO₄ buffer (pH 7.3) containing 10, 20, 30, 40 or 50% (v/v) ethanol (cuvette 1 to 5, respectively, from left to right). 4-OT FYA shows rapid precipitation in buffer containing >10% ethanol.

Table 1. Biocatalytic addition of 3 to 4b catalyzed by 4-OT mutants in different ethanol concentrations.[a]

| Enzyme          | 5% v/v ethanol ee | Reaction time [min][b] | 30% v/v ethanol ee | Reaction time [min][b] | 50% v/v ethanol ee | Reaction time [min][b] |
|-----------------|--------------------|------------------------|--------------------|------------------------|--------------------|------------------------|
| FYA             | 98 (S)             | 40                     | 98 (S)             | 70                     | 98 (S)             | 92 (S)                 |
| A33D/FYA        | 98 (S)             | 50                     | 98 (S)             | 70                     | 98 (S)             | > 360[f]               |
| A33E/FYA        | 98 (S)             | 30                     | 98 (S)             | 35                     | 98 (S)             | 90                     |
| A33C/FYA        | 98 (S)             | 35                     | 98 (S)             | 50                     | 98 (S)             | 180                    |
| A33V/FYA        | 98 (S)             | 35                     | 98 (S)             | 45                     | 98 (S)             | 120                    |

[a] Assay conditions: 3 mM 4b, 100 mM 3, 75 µM 4-OT, 20 mM NaH₂PO₄ (pH 7.3), 0.3 mL reaction volume. [b] Determined by GC using a chiral stationary phase; the absolute configuration was determined by literature comparison.[8,9] [c] Reaction progress was monitored by following the depletion in absorbance at 249 nm. [d] No data due to protein precipitation. [e] Reaction was not finished after 360 min.

lase activity, 4-OT A33D, also showed improved ethanol tolerance.[6] We used the information from the ethanol-tolerance mutability landscape to further engineer a previously constructed highly enantioselective 4-OT variant (FYA) that exhibits poor ethanol stability. All 19 possible variants at position Ala33 in the context of 4-OT FYA were constructed, expressed and purified. Remarkably, from this small, focused set of quadruple mutants, six mutants showed strongly improved ethanol tolerance. It is interesting to note that all six variants, including 4-OT A33D/FYA, are highly enantioselective towards the synte-
sis of (S)-5b, similar to the parental mutant 4-OT FYA.\textsuperscript{[9]} Conversely, the single mutant 4-OT A33D markedly improved the enantioselectivity towards the opposite enantiomer (R)-5b.\textsuperscript{[9]}

Incubation of the best mutant, 4-OT A33I/FYA, for 10 h in the presence of 50% ethanol resulted in only 25% loss of activity, whereas the parental enzyme 4-OT FYA lost all its activity upon incubation for 1 h in 10% ethanol. 4-OT A33I/FYA also showed an increase in thermostability compared to 4-OT FYA, an effect that has also been observed for other enzymes that have been engineered towards increased solvent tolerance.\textsuperscript{[9,23]} We further show that 4-OT A33I/FYA can be used to efficiently catalyze the Michael-type addition of 3 to 4a in the presence of 40% ethanol, which permitted the use of a higher substrate loading (up to 15 mM 4a). Product (R)-5a could be obtained in good isolated yield (89%) and with excellent enantiopurity (ee = 98%).

Conclusion

In summary, our results demonstrate the power of mutability landscapes to guide engineering efforts to improve the cosolvent tolerance of enzymes. By specifically targeting the identified “hotspot” position Ala33, we could engineer an ethanol-sensitive mutant, 4-OT FYA, into a highly ethanol-resistant mutant 4-OT A33I/FYA. Further tuning of 4-OT A33I/FYA might lead to new synthetic opportunities in almost neat organic solvents.

Experimental Section

Production of cell-free extract: Cell-free extracts (CFE) of 4-OT single mutants were prepared according to a reported procedure.\textsuperscript{[9]}

Construction of the ethanol-tolerance mutability landscape: The CFEs prepared from cells producing 4-OT single-mutant variants were used in two reactions, containing either 5% or 25% v/v ethanol. The following reaction conditions were used: CFE (20% v/v), 3 (50 mM), 4a (0.5 mM) in 20 mM NaH$_2$PO$_4$ buffer (pH 7.3), 100 µL final volume. The reactions were performed in 96-well microtiter plates (MTP; UV-star, Amsterdam, the Netherlands) until all of the 19 quadruplet mutants in different organic solvents could be obtained. We further show that 4-OT A33I/FYA can be used to efficiently catalyze the Michael-type addition of 3 to 4a in the presence of 40% ethanol, which permitted the use of a higher substrate loading (up to 15 mM 4a). Product (R)-5a could be obtained in good isolated yield (89%) and with excellent enantiopurity (ee = 98%).

UV-spectroscopic assay for the enzymatic activity of 4-OT single mutants in different organic solvents: The enzymatic activities of the 4-OT mutants and wild-type 4-OT was monitored by following the decrease in absorbance at 320 nm, which corresponds to the depletion of 4a. Purified enzyme (150 µg, 73 µM) was incubated in a 1 mm cuvette with 3 (50 mM) and 4a (2 mM) in 20 mM NaH$_2$PO$_4$ (pH 7.3; 0.3 mL final volume).

Construction of 19 Ala33 mutants of 4-OT L8F/M45Y/F50A: Ala33 was randomized by Quikchange technology using the gene encoding 4-OT L8F/M45Y/F50A cloned in the pET20b vector as the template. The following two primers were used: 5'-GCTCCCTGGAT-NNKCCCGTGACCAG-3' and 5'-CTGGTACGGGNNATCAGGGAGC-3'. After transformation of the DNA into Escherichia coli cells, random colonies were picked from an agar plate, and the mutant 4-OT genes were sequenced by Macrogen Europe (Meibergdreef 31, 1105AZ, Amsterdam, the Netherlands) until all of the 19 quadruplet mutants were obtained.

Activity assays of the six best quadruple mutants: The enzymatic activities of the 4-OT quadruple mutants and 4-OT L8F/M45Y/F50A were monitored by following the decrease in absorbance at 249 nm, which corresponds to the depletion of 4b. Purified enzyme (150 µg, 73 µM) was incubated in a 1 mm cuvette with 3 (100 mM) and 4b (3 mM) in 20 mM NaH$_2$PO$_4$ buffer (pH 7.3; 0.3 mL final volume). After the reactions were completed, product 5b was extracted with ethyl acetate (400 µL) and analyzed by gas chromatography using an Astec CHIRALDEX G-TA column, isocratic 125°C. Retention time (S)-5b: 25.6 min, retention time (R)-5b: 26.9. The assignment of the absolute configuration was based on earlier reported data.\textsuperscript{[9]}

Determination of $T_D$: 4-OT L8F/M45Y/F50A and 4-OT L8F/A33I/M45Y/F50A (50 µL of 2 mg mL$^{-1}$ in 20 mM NaH$_2$PO$_4$, pH 7.3) were incubated in 0.2 mL PCR tubes at temperatures ranging from 30 to 90°C for 60 min in a thermal cycler. After incubation, the enzymes were cooled on ice for 10 min followed by equilibration at 25°C for 10 min. Samples were centrifuged to remove any precipitated protein. The residual “Michaelase” activity for the addition of 3 to 4a was tested in a plate reader. Following conditions were used: 25 µL of enzyme supernatant, 50 mM 3, 0.5 mM 4a, 5% v/v ethanol in 20 mM NaH$_2$PO$_4$ buffer (pH 7.3), 100 µL final volume. The “Michaelase” activity was normalized to that obtained after 60 min incubation at 30°C.

Stability of 4-OT L8F/M45Y/F50A and 4-OT L8F/A33I/M45Y/F50A upon incubation with increasing ethanol concentrations: 4-OT L8F/M45Y/F50A and 4-OT L8F/A33I/M45Y/F50A (1 mL of 1.5 mg mL$^{-1}$ in 20 mM NaH$_2$PO$_4$, pH 7.3) were incubated in 20 mM NaH$_2$PO$_4$ buffer (pH 7.3) containing 10, 30 or 50% v/v ethanol in a water bath of 25°C. Aliquots of enzyme (80 µL) were taken at different time intervals and centrifuged to remove any aggregated protein. 50 µL of the supernatant was used to test the residual enzymatic activity. The reaction mixture consisted of the following: 3 (50 mM), 4a (2 mM, from a 40 mM stock solution in 100% (v/v) ethanol) in 20 mM NaH$_2$PO$_4$ buffer (pH 7.3), 0.3 mL final volume. Depletion of 4a was monitored by following the decrease in absorbance at 320 nm in time. The activities were normalized to the activity measured without incubation of the enzyme.

Semipreparative-scale synthesis: To a 50 mL round bottom flask was added: 6 mL ethanol, 112 µL 3, 12 mL buffer (20 mM NaH$_2$PO$_4$, pH 6.5) containing 4-OT L8F/A33I/M45Y/F50A. The reaction was initiated by the addition of 2 mL ethanol containing 150 mM 4a. The final concentrations were: 3 (100 mM), 4a (15 mM), 4-OT L8F/A33I/M45Y/F50A (75 µM, based on monomer concentration), and 40%
(v/v) ethanol in 20 mM NaH₂PO₄ buffer (pH 6.5). The reaction progress was monitored using UV-spectrophotometric analysis. At timely intervals, a sample of 30 µL was collected from the reaction mixture and diluted to 300 µL with 20 mM NaH₂PO₄ buffer and a full spectrum from 200 nm to 500 nm was recorded. After 200 min, the reaction was finished. The reaction mixture was extracted 3 × with ethyl acetate. The combined organic layers were washed with brine and dried over anhydrous Na₂SO₄. The organic layer was concentrated in vacuo, yielding 5a without any further purification (51.5 mg, 89% yield). The aldehyde functionality of 5a was derivatized to a cyclic acetal according to a reported procedure.[5] The enantiopurity of derivatized 5a was determined by reverse phase HPLC using a Chiralpak AD-RH column (150 mm × 4.6 mm, Daicel) MeCN/water 70:30. Retention time (R)-5a: 7.8 min, (S)-5a: 10.8 min.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: biocatalysis · enzyme engineering · Michael addition · mutability landscape · solvents

[1] S. Harayama, M. Rekik, K. L. Ngai, L. N. Ormston, J. Bacteriol. 1989, 171, 6251–6258.
[2] C. P. Whitman, B. A. Aird, W. R. Gillespie, N. J. Stolovich, J. Am. Chem. Soc. 1991, 113, 3154–3162.
[3] M. Rahimi, J. Y. van der Meer, E. M. Geertsema, H. Poddar, B. J. Baas, G. J. Poelarends, ChemBioChem 2016, 17, 1225–1228.
[4] M. Rahimi, J. Y. van der Meer, E. M. Geertsema, G. J. Poelarends, ChemBioChem 2017, 18, 1435–1441.
[5] E. M. Geertsema, Y. Miao, P. G. Tepper, P. de Haan, E. Zandvoort, G. J. Poelarends, Chem. Eur. J. 2013, 19, 14407–14410.
[6] E. Zandvoort, E. M. Geertsema, B. J. Baas, W. J. Quax, G. J. Poelarends, Angew. Chem. Int. Ed. 2012, 51, 1240–1243; Angew. Chem. 2012, 124, 1266–1269.
[7] C. Guo, M. Safiuddin, T. Saravanan, M. Sharifi, G. J. Poelarends, ACS Catal. 2019, 9, 4369–4373.
[8] J. Y. van der Meer, H. Poddar, B. J. Baas, Y. Miao, M. Rahimi, A. Kunzendorf, R. van Merkerk, P. G. Tepper, E. M. Geertsema, A. M. W. H. Thunnissen, W. J. Quax, G. J. Poelarends, Nat. Commun. 2016, 7, 10911.
[9] L. Biewenga, T. Saravanan, A. Kunzendorf, J. Y. van der Meer, T. Pijning, P. G. Tepper, R. van Merkerk, S. J. Charnock, A. M. W. H. Thunnissen, G. J. Poelarends, ACS Catal. 2019, 9, 1503–1513.
[10] V. Stepankova, S. Bidmanova, T. Koudelakova, Z. Prokop, R. Chaloupkova, J. Damborsky, ACS Catal. 2013, 3, 2823–2836.
[11] D. Prat, O. Paradigm, H. W. Fleming, S. Letestu, V. Ducandas, P. Isnard, E. Guentz, T. Senac, D. Ruscic, P. Hovey, Org. Process Res. Dev. 2013, 17, 1517–1525.
[12] R. J. Kazlauskas, U. T. Borsncheuer, Nat. Chem. Biol. 2009, 5, 526–529.
[13] F. H. Arnold, FASEB J. 1993, 7, 744–749.
[14] V. G. H. Ejsing, A. Bjork, S. Gaseidnes, R. Sirevåg, B. Synstad, B. van den Burg, G. Friend, J. Biotechnol. 2004, 113, 105–120.
[15] M. T. Reetz, J. D. Carballeira, A. Vogel, Angew. Chem. Int. Ed. 2006, 45, 7745–7751; Angew. Chem. 2006, 118, 7909–7915.
[16] T. Koudelakova, R. Chaloupkova, J. Brezovsky, Z. Prokop, E. Sebestova, M. Hessel, M. Khabir, M. Plevaka, D. Kulik, I. Kuta Smatanova, P. Rezacova, R. Ettrich, U. T. Borsncheuer, J. Damborsky, Angew. Chem. Int. Ed. 2013, 52, 1959–1963; Angew. Chem. 2013, 125, 2013–2017.
[17] M. Karpusas, W. A. Baase, M. Matsumura, B. W. Matthews, Proc. Natl. Acad. Sci. USA 1989, 86, 8237–8241.
[18] A. Fulton, V. J. Frauenkron-Machedjou, P. Skoczynski, S. Wilhelim, L. Zhu, U. Schwaneberg, K. E. Jaeger, ChemBioChem 2015, 16, 930–936.
[19] P. A. Romero, T. M. Tran, A. R. Abate, Proc. Natl. Acad. Sci. USA 2015, 112, 7159–7164.
[20] N. Palackal, Y. Brennan, W. N. Callen, P. Dupree, G. Frey, F. Goubet, G. P. Hazlewood, S. Healey, E. J. Mathur, J. M. Short, D. E. Robertson, B. A. Steer, Protein Sci. 2004, 13, 494–503.
[21] J. Y. van der Meer, L. Biewenga, G. J. Poelarends, ChemBioChem 2016, 17, 1792–1799.
[22] B. Rasekh, K. Khajeh, B. Ranjbar, N. Mollania, H. Tendaraz, Eng. Life Sci. 2014, 14, 442–448.
[23] T. Börm, S. Rämisch, S. Bartsch, A. Vogel, P. Adlercreutz, C. Grey, ChemBioChem 2017, 18, 1482–1486.
[24] E. Zandvoort, B. J. Baas, W. J. Quax, G. J. Poelarends, ChemBioChem 2011, 12, 602–609.

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