



Aim of the project

Members of the ene reductases enzyme class (EREDs) and more specifically of the Old Yellow Enzyme (OYE) family are effective biocatalysts for stereo-selective *trans*-hydrogenation of activated alkenes^[1] complementing the respective chemical *cis*-hydrogenations via chiral rhodium or ruthenium phosphines (Knowles and Noyori, Nobel prize in chemistry 2001). However, the industrial application of EREDs remains limited due to their restricted availability in form of commercial enzyme libraries but also due to enzyme stability issues (pH, temperature, organic solvent).

Thus, to facilitate the industrial use of biocatalysts for the asymmetric reduction of activated C=C bonds, we set out to expand the known library of ene reductases with novel enzymes having complementary activities and improved stabilities.

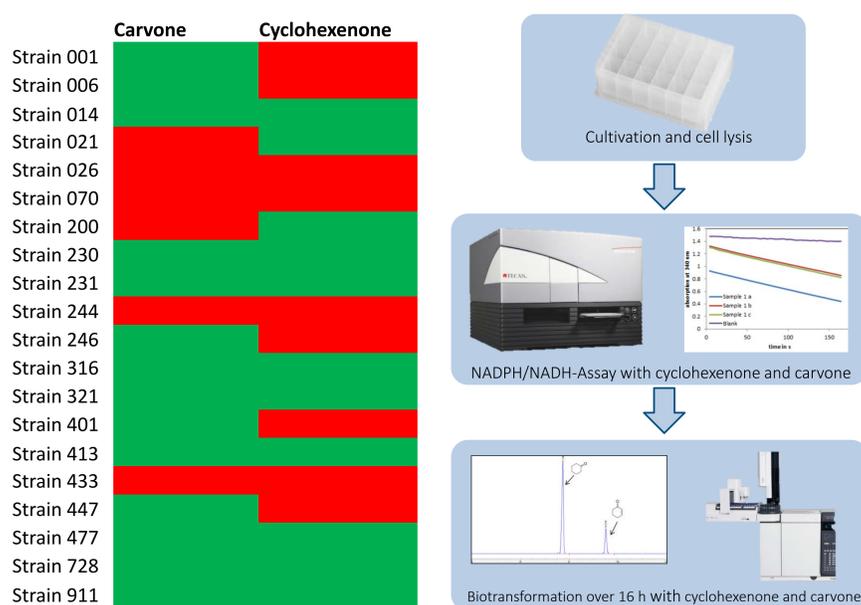
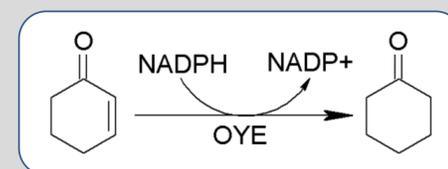


Figure 1. Screening results and schema of the screening process.

Screening

20 bacterial wild type strains were screened to identify new EREDs. Strains with ERED suggesting properties but no described EREDs to date were chosen from the Culture Collection of Switzerland (Wädenswil). As a positive control *Bacillus subtilis* and *Gluconobacter* were used containing the known Old Yellow Enzymes YqjM^[2] and GOX^[3]. For the activity screening the NADPH-Assay and biocatalysis reaction were investigated with the substrates cyclohexenone and carvone. 16 out of the 20 screened strains converted at least one of the substrates.



Phylogenetic analysis

To identify putative ene reductases in active strains we used a motif search in the genomes and registered 48 interesting genes. Based on sequence motifs 24 exhibit sequences of the putative EREDs can be classified to belong into the well-known Old Yellow Enzyme class. The phylogenetic analysis of the 24 new genes indicates that 9 genes belong to the classic OYE subclass and 6 are member of the thermophilic-like subclass. Interestingly, 9 putative OYE genes seem to build up two new subclasses in the OYE family. In all classes the important glutamine (Gln102 in YqjM) and arginine (Arg215 in YqjM) were strictly conserved for interacting with the pyrimidine ring of the cofactor FMN.^[2] The two new subclasses we propose here contain conserved sequence motifs from the classic and the thermophilic-like subclasses, but also have unique new motifs.^[6]

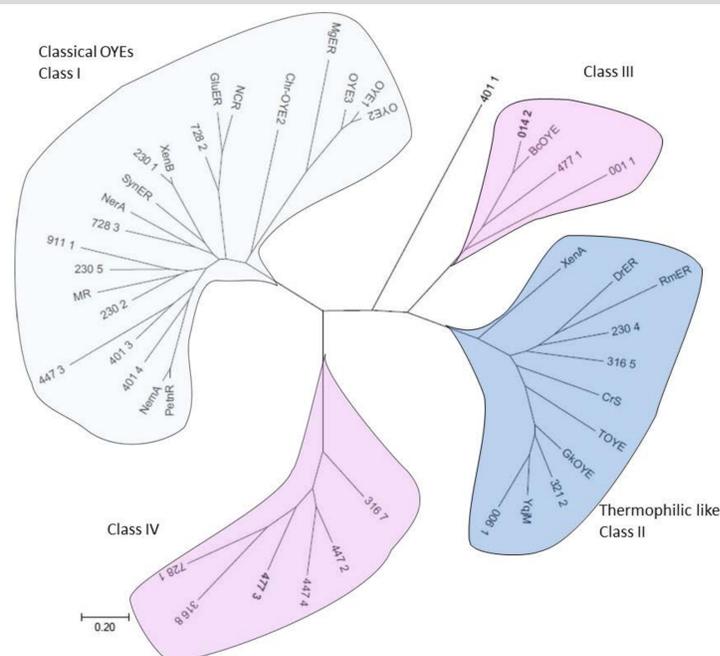
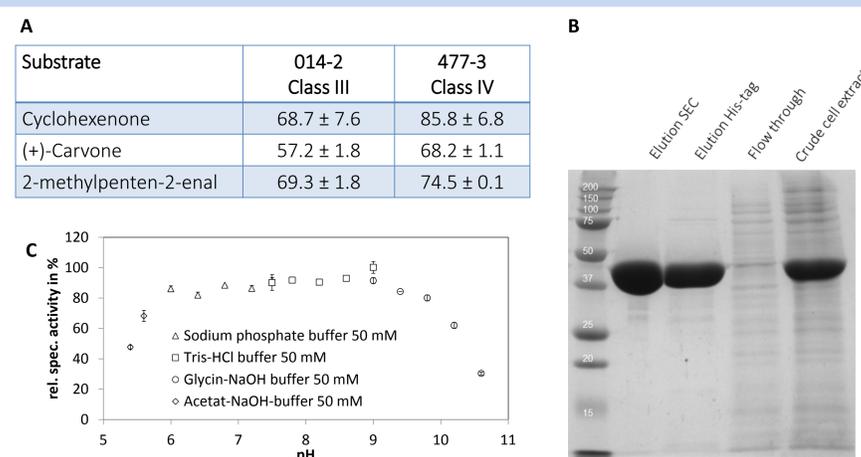
Figure 2. Phylogenetic tree that illustrates the relationship of well-known and new enzymes in the OYE family. The tree was constructed by using Clustal W and edited with Mega 7.^[4]

Figure 3. A Conversion in % of Biotransformation with 5mM substrate, NADPH and 100 µg/ml enzyme over 1h. B SDS-Page with samples of the 014-2 purification. C pH-Optimum of 014-2 at 25 °C with 2 mM Cyclohexenone

Enzymes from the two novel subclasses

We cloned and recombinantly expressed enzymes stemming from new subclasses in *E. coli* BL21(DE3). As an example, we present the characterization of ERED 014-2^[5] from Class III and ERED 477-3 from Class IV. Both enzymes can be purified via Ni-affinity chromatography and were subsequently characterized biochemically. The enzymes have a preference for the cofactor NADPH over NADH. 014-2 has a broad pH-optimum and good stability at 20 °C and 30 °C (data not shown). The substrate spectrum of both enzymes include cyclic ketones, small aliphatic aldehydes and aldehydes with an aromatic residue.^[6]

With the initial strain screening and genome analysis we identified 44 putative completely new ene reductases genes. The phylogenetic analysis of the 24 genes belonging to the OYE family leads us to suggest the introduction of two new subclasses besides the classical and the thermophilic like OYE subclasses.

References

- [1] H. S. Toogood, J. M. Gardiner, N. S. Scrutton, *ChemCatChem* **2010**, *2*, 892-914. [2] K. Kitzing, T. B. Fitzpatrick, C. Wilken, J. Sawa, G. P. Bourenkov, P. Macheroux, T. Clausen, *Journal of Biological Chemistry* **2005**, *280*, 27904-27913. [3] N. Richter, H. Gröger, W. Hummel, *Applied Microbiology and Biotechnology* **2011**, *89*, 79-89. [4] S. Kumar, G. Stecher, K. Tamura, *Molecular Biology and Evolution* **2016**, *33*, 1870-1874. [5] X. Sheng, M. Yan, L. Xu, M. Wei, *Journal of Molecular Catalysis B: Enzymatic* **2016**, *130*, 18-24. [6] C. Peters, D. Frasson, M. Sievers, R. Buller Manuscript in preparation