ENGINEERING ALCOHOL OXIDASES FOR SUBSTRATE SCOPE AND THEIR APPLICATION IN FLOW AND CASCADE BIOCATALYSIS

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Alcohol oxidases have significant advantages over alcohol dehydrogenases (ADHs) for biocatalytic oxidation of alcohols: they don't require addition of (expensive) nicotinamide cofactors (or a recycling system for cofactor regeneration) and the catalytic reaction is irreversible. Although alcohol oxidases generate hydrogen peroxide when they turn over, this issue can be alleviated by addition of catalase, which, not only removes the peroxide, but also creates more oxygen for cofactor regeneration. Alcohol oxidases are perceived to have a limited substrate scope preventing their wider use in synthesis. Thus, we present the engineering of two alcohol oxidases for increased substrate scope, one for the selective oxidation of primary alcohols and one for secondary alcohol oxidation.

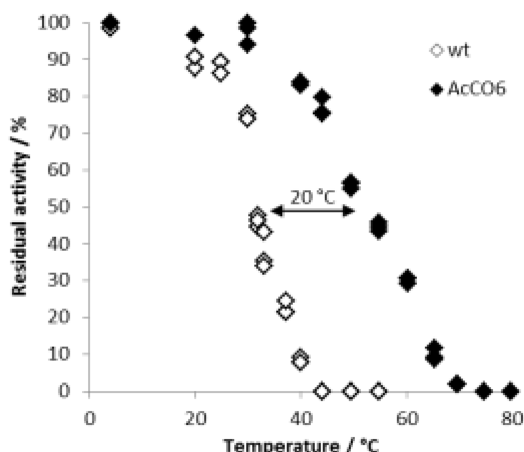

Figure 1. Improved thermostability of choline oxidase six-point variant compared to the wildtype.

Arthrobacter chlorophenolicus choline oxidase and *Streptomyces hygroscopicus* cholesterol oxidase were selected as the primary and secondary alcohol oxidase, respectively. Examination of crystal structures of homologous alcohol oxidases revealed positions in the active site and entrance channel to target for saturation mutagenesis. Libraries were screened using a high-throughput assay based on the detection of hydrogen peroxide using hexanol as the substrate for evolution of choline oxidase and cyclohexanol for evolution of cholesterol oxidase. Mutations from positive hits were combined and, in these cases, resulted in variants with further improvements in both k_{cat} and conversion to the carbonyl in biotransformations. The enzyme variants maintained selectivity for primary or secondary alcohols consistent with the source alcohol oxidase. Choline oxidase variants also showed increased activity towards a variety of other primary alcohols such as terminal diols and benzylic alcohols such as vanillyl alcohol and cinnamyl alcohol. Mutation of residues with high B-factors led to an enzyme with increased thermostability, with a T_{50} 20 °C above that of the wildtype (Figure 1) as well as improved solvent tolerance. For cholesterol oxidase we also gained activity with linear secondary alcohols, and secondary benzylic alcohols such as indanol.

These evolved alcohol oxidases have been immobilized and applied in a flow system. Due to the solvent tolerance of the primary alcohol oxidase we were able to run the reaction in pure cyclohexane.[2] However, modelling showed that, despite the increased solubility of oxygen in cyclohexane compared to water, the reaction was still oxygen limited. Thus, we have applied it in a microreactor with catalase and hydrogen peroxide for increased oxygen supply. The oxidases have also been successfully applied in cascade reactions, in particular with reductive aminases for conversion of alcohols to secondary amines.[3]

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