Stabilization and activity improvement of a Baeyer-Villiger monooxygenase by mutagenesis of the susceptible to oxidation residues around flavin

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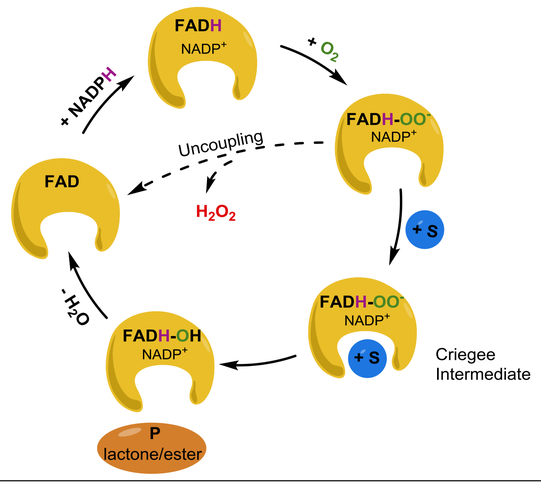
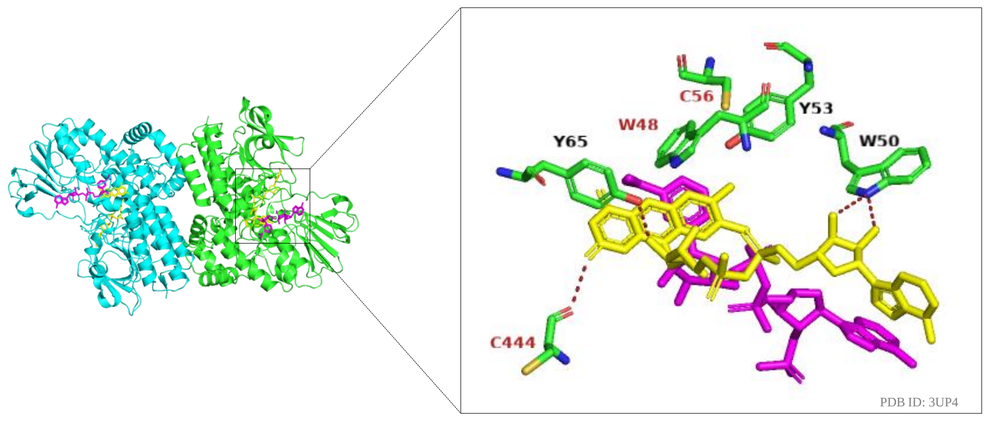
Introduction

Baeyer-Villiger-Monooxygenases (BVMOs) belong to the large family of Flavoprotein-Monooxygenases (FPMOs), and can catalyze the Baeyer-Villiger oxidation reaction under mild conditions, leading to a sustainable chemical process.[1] BVMOs type I, have FAD as a prosthetic group in their active site, and use NADPH as a coenzyme. They utilize molecular oxygen from air to perform the insertion of an oxygen atom next to a carbonyl group, leading to the formation of an ester or a lactone, often with high enantio- and regioselectivity. Despite that, there are many limitations in the application of BVMOs on an industrial scale, due to their low thermo-, pH-, solvent- and oxidative stability.[2] **In this study, 2-oxo-D3-4,5,5-trimethylcyclopentenylacetyl-CoA monooxygenase from *Pseudomonas putida* (OTEMO), was biochemically characterized and its oxidative stability was studied using rational design mutagenesis, in order to expand the reaction portfolio of the enzyme.**

Rational design for the optimization of oxidative stability

Because of the uncoupling reaction that occurs in the active site of BVMOs, **H_2O_2 is formed very close to the isoalloxazine ring of FAD**, and can lead to loss of activity due to oxidation of the enzyme.[2] Trp, Tyr, His, Met and Cys residues are susceptible to oxidation.[3] We identified the respective residues, 5 Å from FAD, and we designed three variants **(C56A, C444S, W48L).** The wild type, and the mutants were overexpressed in *E. coli* BL21[DE3] strain, and were purified through affinity chromatography using a Ni-NTA column, as the proteins carry a N-terminal 6-His-Tag sequence.





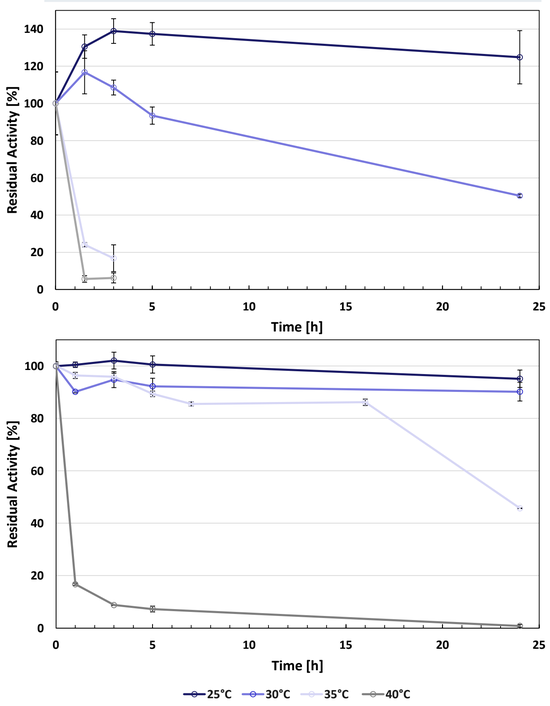
	Expression in *E. coli*	µmol FAD/ µmol protein [color]	Specific Activity [U/mg]
$OTEMO_{WT}$	**	1,01 [yellow]	0,72 ± 0,01
$OTEMO_{C444S}$	**	1,09 [yellow]	2,80 ± 0,03
$OTEMO_{W48L}$	*	0,30 [colorless]	0,037 ± 0,003
$OTEMO_{C56A}$	Work in progress		

$OTEMO_{C444S}$ maintains good expression levels in *E. coli* and FAD coordination in the active site. W48L affects negatively the expression, while it seems to lead to mostly apoprotein. Proteins expressed as holoenzymes exhibit activity, **with the $OTEMO_{C444S}$ having 4-fold increased, compare to the wild type**.

➢ The activity measurements for the oxidation of bicyclo[3.2.0]hept-2-en-6-one were performed spectrophotometrically by monitoring the NADPH consumption at λ=340 nm.
➢ To determine the concentration of FAD, standard curve was made in the range of 0,014 to 0,140 mg mL^{-1} FAD, and absorbance was taken at λ=448 nm [4] .

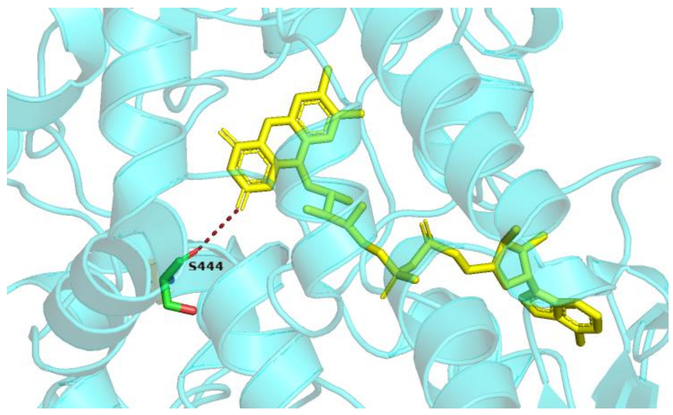
Biochemical characterization of $OTEMO_{WT}$ and $OTEMO_{C444S}$

Thermal Stability



From the determination of **pH profile, temperature profile, pH, temperature** and **cosolvent stability**, $OTEMO_{C444S}$ shows an improvement compared to the WT.
The mutant and the wild type are more stable in phosphate buffers and in presence of 5% DMSO. Optimum pH was found to be 9,0 in Tris-HCl 50 mM buffer, and optimum temperature 40°C.
The $OTEMO_{C444S}$ variant appeared to be stable for 24h at 30°C, a temperature at which $OTEMO_{wt}$ loses 50% of its catalytic activity. Also, an improved stability was observed at 35°C.

➢For the biochemical characterization of the $OTEMO_{WT}$ and $OTEMO_{C444S}$, the NADPH-dependent activity towards bicyclo[3.2.0]hept-2-en-6-one, was measured spectrophotometrically, by monitoring NADPH consumption at 340 nm.



Phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* is the only protein showing remarkable stability.[5]
[PDB ID: 1W4X]

Conclusions

➢ $OTEMO_{C444S}$, shows good expression in *E. coli* and keeps FAD coordination in the active site
➢The specific activity of $OTEMO_{C444S}$ towards bicyclo[3.2.0]hept-2-en-6-one, is 4-fold increased compared to $OTEMO_{WT}$
➢The mutation C444S, increased the thermostability of the $OTEMO_{wt,}$ witch comes to correlation with the tertiary structure of PAMO

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References

[1] S. Schmidt, et al., *The Enzymes,* P. Chaiyen, F. Tamanoi (Eds.), Academic Press (2020), 231-281.
[2] K. Balke, et al., *Biotechnol. Adv*., 36 (2017), 247-263.
[3] M. J. Davies, *Biochim. Biophys. Acta* 1703, (2005), 93-109.
[4] D. M. Shafiqul Islam, et al., *Chem. Phys.,* 295, (2003), 137-149.
[5] H. M. Dudek, et al., *AEM,* (2011), 5730-5738.