	Experiment title: Laboratory-evolved vanillyl-alcohol oxidase produces natural vanillin	**Experiment number**: MX-129
Beamline: ID14 1	**Date of experiment**: from: 15 May 2003 to 17 May 2003	**Date of report**: 22 June 2004
Shifts: 3	**Local contact(s)**: Cecile JAMIN	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

Stefano Rovida* and Robert van den Heuvel*

University of Pavia

Report:

The flavoenzyme vanillyl-alcohol oxidase (VAO) was subjected to random mutagenesis to generate mutants with enhanced reactivity towards creosol (2 methoxy-4-methylphenol). The VAO-mediated conversion of creosol proceeds via a two-step process in which the initially formed vanillyl alcohol (4 hydroxy-3-methoxybenzyl alcohol) is oxidized to the widely used flavor compound vanillin (4-hydroxy-3-methoxybenzaldehyde). The first step of this reaction is extremely slow due to the formation of a covalent FAD (N5)-creosol adduct. After a single round of error-prone polymerase chain reaction, seven mutants were generated with an increased reactivity towards creosol. The single-point mutants Ile238Thr, Phe454Tyr, Glu502Gly and Thr505Ser showed an up to 40-fold increase in catalytic efficiency (kcat/Km) for creosol compared to wild-type enzyme. This enhanced reactivity was due to a lower stability of the covalent flavin-substrate adduct, thereby promoting vanillin formation. The catalytic efficiencies of the mutants were also enhanced for other ortho-substituted 4 methylphenols, but not for p cresol (4 methylphenol). The replaced amino acid residues are not located within a distance of direct interaction with the substrate and the determined three-dimensional structures of the mutant enzymes are highly similar to wild-type enzyme. The

present results clearly show the importance of remote residues, not readily be predicted by rational design, for the substrate specificity of enzymes.

Published in:
Van Den Heuvel RH, Van Den Berg WA, Rovida S, Van Berkel WJ. Related Articles, Laboratory-evolved vanillyl-alcohol oxidase produces natural vanillin. J Biol Chem. 2004, in press