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# New Enzymes for Biotechnological Applications

## Project 1 – Improving thermostability of PpAzoR, an azoredutase from *Pseudomonas putida* MET94, by directed evolution

Over 100,000 commercially dyes exist and more than $7x10^5$ tons of dyestuff is produced annually of which $1\text{-}1.5x10^5$ is released to the environment in wastewaters. These dyes are hardly removed from effluents by conventional wastewater treatment representing anthropogenic pollutants causing deterioration of water quality and becoming a health threat due to their mutagenic or carcinogenic properties. Enzymatic processes are particularly sought for the treatment of dyeing effluents since due to their specificity, enzymes only attack the dye molecules, while valuable dyeing additives or fibers are kept intact and can potentially be re-used. Recently we show that PpAzoR of *P. putida* MET94 is a FMN dependent NADPH:dye oxidoreductase with a broad substrate specificity towards the degradation of a range of structurally diverse azo dyes. However, its thermal stability is rather low (half life of 30 min at 40°C) which impairs its application in biological treatment technologies. Therefore, evolution of PpAzroR towards a higher stability will be addressed through random mutagenesis followed by robotic high-throughput screening. The most thermostable variants identified in the first generation will be further optimized in subsequent cycles. We aim at improving the robustness of this enzymatic system in order to maximize its biotransformation potential through protein engineering strategies.

## Project 2 – Unraveling the catalytic mechanism of bacterial DyP-peroxidases

Peroxidases are heme-containing enzymes that use hydrogen peroxide as the electron acceptor to catalyze numerous oxidative reactions with potential application in diagnostic and bioindustrial applications. Recently, a novel family of heme peroxidases has been identified in numerous fungal strains and in a few bacteria. These enzymes oxidise synthetic high redox-potential dyes of the anthraquinone type, which are not converted by any other peroxidase so far described and have been proposed to belong to the novel dye-decolourising peroxidases (DyP) family. DyP shows a structural divergence from classical plant and animal peroxidases lacking for example the typical conserved active distal histidine. They show broad substrate specificity, low pH optima, and they are also relevant enzymes in the field of lignocellulosics degradation. We have screened microbial genome

databases for the presence of DyP and have recently cloned and expressed in *E.coli* two genes coding for DyP-type enzymes from *Pseudomonas putida* MET94 and from *Bacillus subtilis*. The full kinetic characterization of these enzymes will allow for the understanding of molecular determinants of substrate specificity, probing structure-function relationships in this new class of peroxidases, with key importance in defining their biotechnological potential.

**Project 3 – Role of metionine rich-motifs as determinants of substrate specificity in multicopper oxidases**

Multicopper oxidases (MCOs) are a large family of enzymes that couple the one-electron oxidation of substrates with the four-electron reduction of molecular oxygen to water. This family is unique among copper proteins since its members contain one of each of the three types of biologic copper sites, type 1 (T1), type 2 (T2) and the binuclear type 3 (T3). T1 Cu is the site of substrate oxidation and, in this respect, the MCO family can be separated into two classes; enzymes that oxidize aromatic substrates with high efficiency, i.e. laccases, and those that oxidize metal ion substrates, such as Cu(I) or Fe(II) or metallo-oxidases. Laccases have a high potential for biotechnological applications, mainly due to their wide range of oxidizing substrates, the use of readily available oxygen as final electron acceptor and the lack of requirement for expensive cofactors. Physiologically, the distinction among laccases and metalo-oxidase remains the most critical question about structure-function relationships in this family of enzymes. This project will focus primarily on research involving recombinant metallo-oxidase McoA from *Aquifex aeolicus* with a special focus on the characterization of the molecular features of the substrate binding site, close to the T1 copper centre. A comparative model structure of McoA has been constructed and a striking structural feature is the presence of a methionine-rich region (42 amino acids segment containing 12 Met residues residues) reminiscent of those found in copper homeostasis proteins. This region was shown to modulate the enzyme activity most probably through copper binding and release. In this project synthetic model peptides based on the 42 amino acids McoA Met-rich region will be used to determine copper affinity to this segment and to identify Met residues putatively involved in copper coordination. This will allows for the delineation of a protein mutagenesis strategy that will permit identifying the structural determinants of multicopper oxidases efficiency and selectivity.

**Areas:**
Microbiology/Molecular Biology/Biochemistry/Biotechnology

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