Shifting the optimal pH of activity for a laccase from the fungus Trametes versicolor by structure-based mutagenesis

Claude Jolivalt, Catherine Madzak, Maria-Chiara Mimmi, Pierre Briozzo, Christian Mougin

Shifting the optimal pH of activity for a laccase by structure-based mutagenesis at the reducing substrate Cu binding site

Claude JOLIVALT[1], Catherine MADZAK[2], Maria-Chiara MIMMI[3], Pierre BRIOZZO[3], Christian MOUGIN[4]

[1] UMR Synthèse Sélective Organique et Produits Naturels, CNRS / ENSCP, Paris.

[2] UMR Microbiologie et Génétique Moléculaire,INRA / CNRS / INA-PG, Thiverval-Grignon.

[3] UMR INRA-INAPG 206 de Chimie Biologique, 78850 Thiverval-Grignon

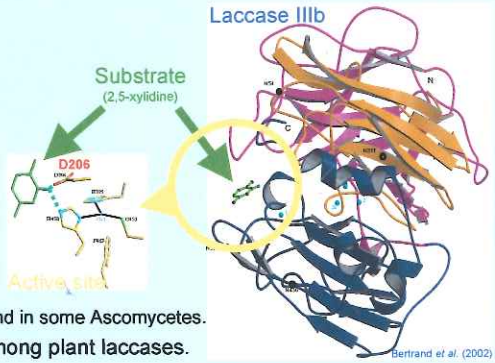
[4] Unité Phytopharmacie et Médiateurs Chimiques, INRA, Versailles.

Directed Mutagenesis of Laccase IIIb from the white rot fungus *Trametes versicolor*

Laccases are blue multicopper oxidases that catalyze the oxidation of a large range of phenols and arylamines.

Fungal laccases are used in industrial oxidative processes, and have many other potential applications, such as depollution.

Genetic engineering could allow obtaining modified recombinant laccases with a wider substrate range, a higher oxidation potential, or a neutral optimum pH.



The analysis of crystals from *T. versicolor* laccase IIIb, complexed with a substrate, allowed determining the first structure of an active laccase, and enlighted the interaction of one amino acid, **Aspartate 206**, with the substrate. This amino acid is highly conserved among laccases from Basidiomycetes, and is also found in some Ascomycetes.

We tested the effect of the following mutations at amino acid **206 position**:

- **D206E**: replacement of Aspartate by **Glutamate**, another residue with a negative chain, found in some Ascomycetes.
- **D206N**: replacement by **Asparagine**, a residue with a polar uncharged chain, conserved among plant laccases.
- **D206A**: replacement by **Alanine**, a residue with an uncharged chain.

The activity of recombinant wild-type or mutant laccases, produced in *Yarrowia lipolytica* was assessed on different substrates:

ABTS
2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic)acid

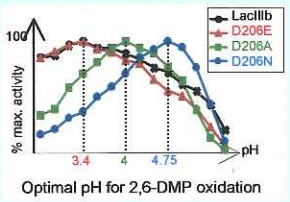
2,6-dimethoxyphenol
2,6-DMP

2,5-xylidine

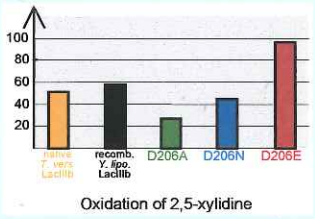
monolignol: coniferyl alcohol

	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/mM)
LacIIIb	50	1.29	26
D206E	25.8	1.58	61
D206N	16	3.35	209
D206A	10.2	4.35	426

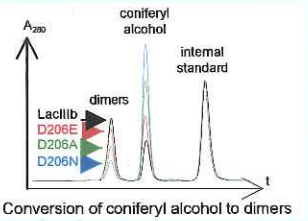
Kinetics parameters for ABTS oxidation



Optimal pH for 2,6-DMP oxidation



Oxidation of 2,5-xylidine

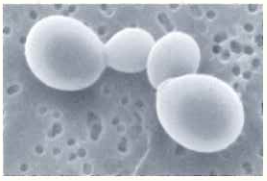


Conversion of coniferyl alcohol to dimers

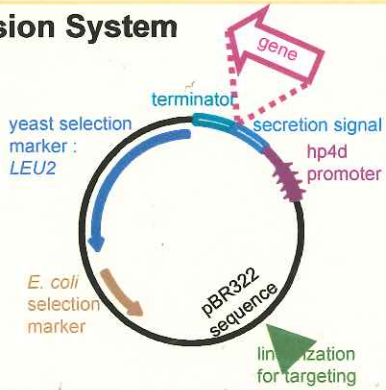
The pH dependence of the activity profile with 2,6-DMP shows that D206N led to a significant shift (ΔpH = 1.4) of the optimum towards higher pH. The same tendency is expected for other phenolic substrates known as environmental pollutants, and could contribute to an optimized degradation of these molecules *in situ*, in soils at neutral pH.

Relative transformation efficiencies of wild type and mutated laccases varies with the substrate and can be explained by the interaction of each residue at position 206 with the substrate, at the catalytic site.

Yarrowia lipolytica Expression System



- optimized vectors and strains
- high transformation efficiency
- targeted monocopy integration
- high secretion levels of active proteins



We used an expression/secretion vector based on the recombinant promoter hp4d, driving a strong growth-phase-dependent expression.This vector allows the precise targeting of monocopy integration events at an integrated pBR322 docking platform into the genome of the recipient *Yarrowia lipolytica* strain. This system reproducibly provides transformants carrying a unique expression cassette, integrated at a precise known site in the genome of the producing strain.

The obtention of transformants comparable in terms of copy number and integration locus enables to analyze precisely the consequences of each mutation in the gene of interest.

on *Trametes versicolor* laccase:
Structure: Bertrand *et al.*, Biochemistry (2002) 41, 7325- 7333.
Applications: Mougin *et al.*, Environ. Chem. Lett. (2003) 1, 145-148.
Production: Jolivalt *et al.*, App. Microbiol. Biotechnol. (2005) 66, 450-456.

Contacts: **Dr Claude Jolivalt** (Claude-Jolivalt@enscp.fr)
Dr Christian Mougin (mougin@versailles.inra.fr)

on *Yarrowia lipolytica* expression system:
Review: Madzak *et al.*, J. Biotechnol. (2004) 109, 63-81.

Contact: Dr Catherine Madzak (madzak@grignon.inra.fr)