Expression of laccase IIIb from the white-rot fungus Trametes versicolor in the yeast Yarrowia lipolytica for environmental applications

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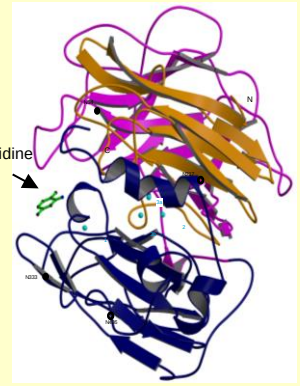
INTRODUCTION

- Fungal laccases (EC 1.10.3.2) are involved in lignin transformation,morphogenesis, pathogenesis and fungal virulence, as well as in the oxidation of numerous xenobiotics [1-3]
- A wide range of their substrates are of environmental interest, and are recognized pollutants of waters and soils
- Laccases are potential useful tools to improve the chemical quality of polluted media through bioremediation[1,3]

Our objective is the design of laccases with:

- a high efficiency in catalysis (high redox potential)
- a wider variety of substrates
- a pH of activity optimized for natural media

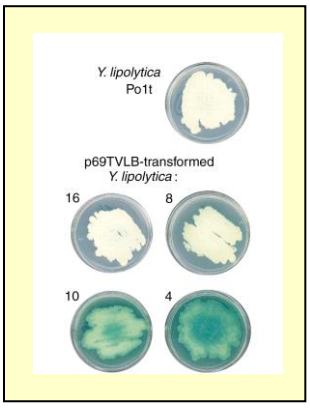
Our approach is based on the knowledge of the cavity enclosing a weak reducing substrate of the enzyme, the arylamine 2,5-xylidine, and on the replacement of neighboring interacting residues



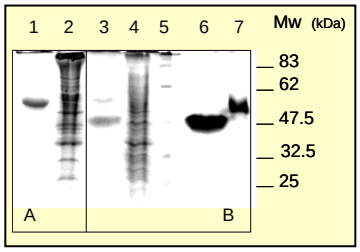
Structure of LacIIIb [4]
(atomic coordinates: pdb accession number 1KYA)
●, copper atoms; ● and o, glycosylation sites

RESULTS: Expression of LacIIIb by *Yarrowia lipolytica*

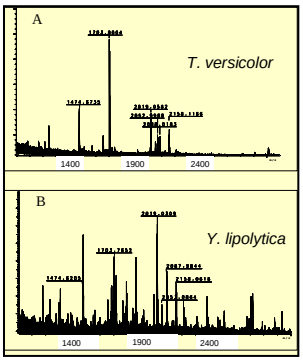
- The gene encoding LacIIIb has been cloned in *Escherichia coli* using the pGEM®-T plasmid
- It has been sequenced and comprises 1563 bases (sequence accession number AF414109), including a peptide signal
- It was then expressed in the yeast *Yarrowia lipolytica* using the pINA1269 vector (Patent INRA/INA-PG), thus allowing the production of an active form of the recombinant enzyme (transformation of the substrate ABTS to a green oxidized form on solid culture media and oxidation of guaïcol after electrophoretic separation). After tryptic digestion of the transformed *Y. lipolytica* extract, the mass spectrum contains the most significative ions detected and assigned to *T. versicolor* laccase peptides.



Laccase activity detection on agar plates assay.
Numbers refer to different transformant clones of *Y. lipolytica* Po1g strain having received p69TVLB plasmid.



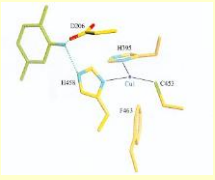
Electrophoresis analysis of recombinant laccase IIIb.
[A] Samples were boiled before loading on the gel. Lane 1 - Laccase IIIb from *T. versicolor*. Lane 2 - Concentrated crude cell free extract from a transformant (clone 4) of *Yarrowia lipolytica* expressing laccase IIIb.
[B] Samples were not boiled before loading on the gel. Lanes 3 and 6 - Laccase IIIb from *T. versicolor*. Lanes 4 and 7 - Concentrated crude cell free extract from a transformant (clone 4) of *Yarrowia lipolytica* expressing laccase IIIb. Lane 5 - Molecular weight markers.
Lanes 1 to 5 were stained with Coomassie bleu (R250). Laccase activity was detected in lanes 6 and 7 by coloration with guaïacol.



MALDI-TOF spectra
[A] Laccase IIIb from *T. versicolor*.
[B] Analysis of the band corresponding to the activity detected in electrophoresis pattern of crude supernatant from a p69TVLB-derived transformant (clone 4) of *Yarrowia lipolytica* expressing laccase IIIb.

APPLICATION: Towards the engineering of fungal laccases

1- T1 site and neighboring 2,5-xylidine ligand



Two charged or polar residue interact with the amino group of the xylidine:
- His (H458) coordinates the copper that functions as the primary electron acceptor
- Asp (D206) possesses acido-basic properties that could be responsible for the pH-dependent activity towards substrates

2 – Sequence alignment of laccases from fungi and higher plants

- Biological roles of laccases from fungi and higher plants appear to be opposite: fungal laccases are involved in ligninolysis whereas enzymes from plants catalyze the initial polymerization of monolignols during lignification
- Fungal laccases have a negatively charged residue at the 206 position (Asp, D or Glu, E), whilst those of higher plants have an asparagine (Asn, N)

Fungi: Tversicolor = *Trametes versicolor*; Ccinereus = *Coprinus cinereus*; Pcinnabarinus = *Pycnoporus cinnabarinus*; Malbomyces = *Melanocarpus albomyces*
Higher plants: Athaliana = *Arabidopsis thaliana*; Rvernicifera = *Rhus vernicifera*; Apeudoplatanus = *Acer pseudoplatanus*

3- Strategy of directed mutagenesis

Three mutants have been obtained comprising:
- a residue with another negative chain: D206E
- a residue with a polar uncharged chain: D206N
- a residue with an uncharged chain: D206A

-Their catalytic properties are currently studied.

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