

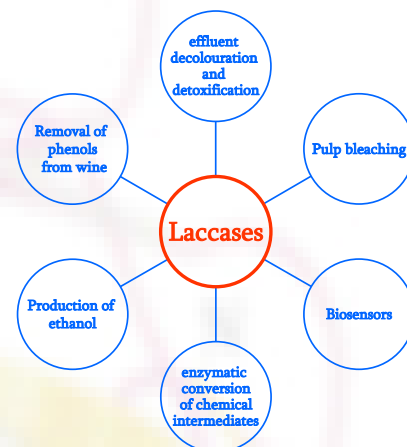
# DEVELOPMENT OF NEW OXIDASES

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Laccases are multicopper enzymes catalysing the oxidation of aromatic compounds, primarily phenols and anilines, with the concomitant reduction of molecular oxygen to water. These enzymes have been isolated from plants, fungi and insects.

The importance of laccases in various biotechnological areas, due to the very broad substrate range they can oxidise, underlines the need for enhancing their properties.



The white-rot fungus *Pleurotus ostreatus* is able to express multiple laccase genes encoding isoenzymes with different and particularly interesting physico-chemical and catalytic properties.



POX1	protein	cDNA	gene
POXC	protein	cDNA	gene
POXA1w	protein	cDNA	gene
POXA1b	protein	cDNA	gene
POXA3	protein	cDNA	gene

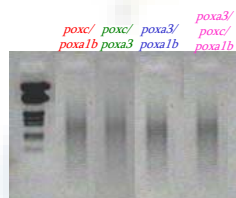
Localisation	<i>K. lactis</i>		<i>S. cerevisiae</i>	
	POXA1b	POXC	POXA1b	POXC
Intra:Extra (1:1)	Intra:Extra (1:1)	Intra:Extra (1:1)	Intra:Extra (3:1)	Intra:Extra (3:1)
Extra-cellular activity	4.2 U ml <sup>-1</sup>	0.1 U ml <sup>-1</sup>	0.2 U ml <sup>-1</sup>	Very low
Extra-cellular production	1.1 mg ml <sup>-1</sup>	1.4 mg ml <sup>-1</sup>	Low	n. d.

*P. ostreatus* laccases POXC and POXA1b were successfully expressed in the two yeasts *Kluyveromyces lactis* and *Saccharomyces cerevisiae*.

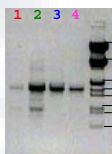
*S. cerevisiae* was used to engineer laccases, taking advantage of the high level of homologous recombination in yeast, which makes *in vivo* approaches fast and efficient.

## DNA shuffling

*poxc*, *poxa1b* and *poxa3* cDNAs have been broken into 50+250 bp fragments by restriction enzymes hydrolysis and then reassembled by self-priming PCR followed by a conventional PCR.



PCR self priming



**Conventional PCR:**  
 1: primers *poxc/poxa1b*  
 2: primers *poxc/poxa3*  
 3: primers *poxa3/poxa1b*  
 4: primers *poxc/poxa1b/poxa3*

A library from *poxc* and *poxa1b* shuffling has been cloned in expression vector, expressed in *S. cerevisiae* and analysed by preliminary plate test.



## Errore-prone PCR

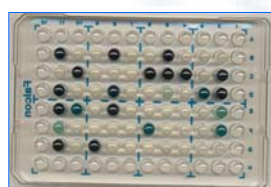
Random mutagenesis experiments have been performed at controlled mutation rate on *poxa1b* and *poxc* cDNAs. Libraries of mutated laccases, at controlled mutation rate, have been cloned in expression vector by homologous recombination in *S. cerevisiae*.

Preliminary analyses were conducted spreading yeast cells on selective medium containing a chromogenic laccase substrate



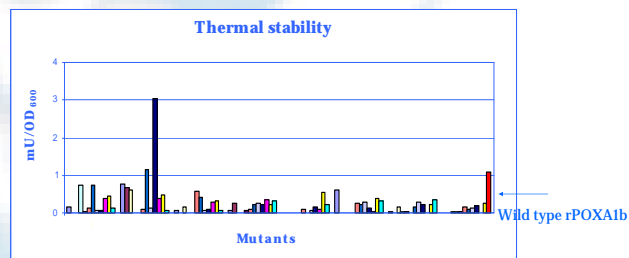
Wild type rPOXA1b

Preliminary screenings of mutants were performed looking at mutants with enhanced activity assaying laccase activity and comparing with wild-type enzyme.



Laccase activity test with ABTS as substrate

Thermo-resistance was also evaluated measuring laccase activity after incubation at 70 °C for 30 min.



## Perspectives

- ✓ Increase libraries representatively
- ✓ Screening for:
  - \*changed substrate specificity
  - \*enhanced stability.

