

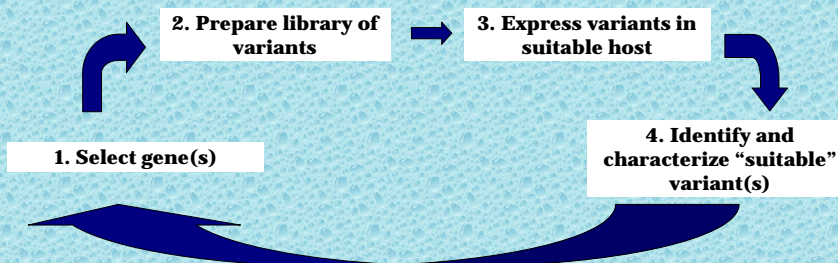
Directed evolution of *Pleurotus ostreatus* laccases

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During the last few years, directed evolution has emerged as method of choice for engineering enzymes. This approach (Fig.1) mimics *in vitro* the natural process of molecular evolution that is able to generate a potentially infinite plethora of proteins with new function and properties, such as stability to temperature and solvents, improved catalytic performance and substrate specificity [1]. cDNAs encoding *Pleurotus ostreatus* laccases, POXC [2] and POXA1b [3], were selected as “parent molecules” to guide the evolution of laccases with higher specific activity.

Fig.1

Key steps of a typical directed enzyme evolution experiment



Genetic variants were created by Error-Prone PCR (EP-PCR) and DNA shuffling and then were expressed in *Saccharomyces cerevisiae*. To screen yeast colonies for the ability to express high levels of laccase activity, three sequential selections were performed (Table 1): only one clone (named 1M9B) expressed activity higher than that of wild type.

Table 1

Libraries		Screening procedure			
		First screening in multiwell plates	Second screening in multiwell plates on three days	Investigation in shaken flasks	Clones expressing laccase activity higher than wild-type
EP-PCR on <i>poxa1b</i> cDNA	200 elements mutated at low mutation rate (0-3 mut/ kbase)	200 mutants	29 mutants	1 mutant	0
	200 elements mutated at medium mutation rate (3-7 mut/ kbase)	200 mutants	3 mutants	1 mutant	1 mutant named 1M9B
	300 elements mutated at high mutation rate (more than mut/ kbase)	300 mutants	0	0	0
EP-PCR on <i>poxc</i> cDNA	100 elements mutated at low mutation rate (0-3 mut/ kbase)	100 mutants	0	0	0
DNA shuffling on <i>poxa1b</i> and <i>poxc</i> cDNAs	300 elements	300 mutants	6 mutants	5 mutants	0

1M9b mutant was used as template for producing a new collection of genetic variants: 500 “low range” and 700 “medium range” mutants were generated by error-prone PCR on *1m9b* cDNA. The screening procedure on these colonies allowed the identification of three more interesting elements (named 1L2B, 1M10B, 3M7C). These mutants, and 1M9B mutant, were characterised from a structural point of view. Nucleotidic sequence revealed point mutations which resulted in the aminoacidic substitutions reported in Table 2.

Table 2

Mutants	Codon substitution	Aminoacid substitution
1M9B	CTT→TTT ACA→ACG	L112F conservative
1L2B	AAC→TAC AAC→AAG GTT→ATT	N248Y N261K V350I
1M10B	AAG→CAG AAG→AAT GGA→GGT	K37Q K51N conservative
3M7C	ATC→ATT CCA→ACA	conservative P494T

Kinetic parameters towards three different substrates were determined for mutated and wild-type recombinant proteins (Table 3).

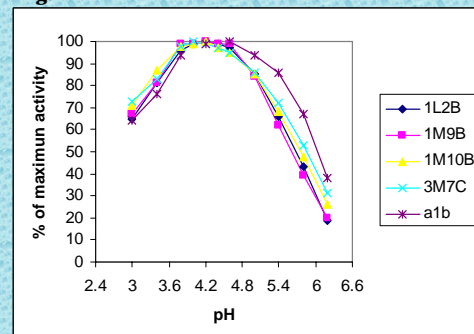
Table 3

Laccases	SUBSTRATES								
	ABTS			DMP			SGZ		
	K_m (mM)	Specific activity (U/mg)	Catalytic efficiency (U/mg mM)	K_m (mM)	Specific activity (U/mg)	Catalytic efficiency (U/mg mM)	K_m (mM)	Specific activity (U/mg)	Catalytic efficiency (U/mg mM)
wt	0.085	183	2153	0.54	187	346	0.048	23.2	483
1M9B	0.067	315	4701	0.36	168	467	0.038	9.30	245
1L2B	0.107	420	3925	0.31	295	952	0.025	24.3	972
1M10B	0.100	421	4210	0.35	281	803	0.040	24.3	607
3M7C	0.077	454	5896	0.24	337	1404	0.055	26.9	489

Michaelis constant (K_M) values of mutants are similar to that of wild type. The mutants with specific activity higher than wild type are highlighted in Table 3.

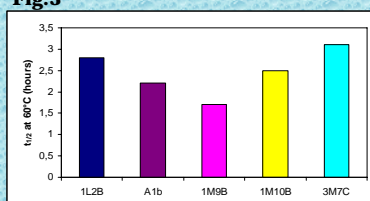
The effect of pH on the activity of laccases towards DMP was studied in the pH range 3.0÷6.2. The wild type protein and the mutants show maximum activity at pH value of ~4.2. The only observed slight difference was that, at pH above 4.4, the activity loss of the wt laccase is less rapid than that of the mutants (Fig.2). Laccase activity was also studied as function of temperature. All the recombinant proteins turned out to be almost fully active in the temperature range 40-70°C.

Fig.2



The stability of the enzymes with respect to temperature and pH was also studied.

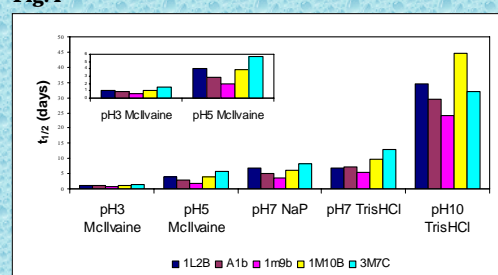
Fig.3



No significant difference in $t_{1/2}$ values at 60°C is observed (Fig.3).

At pH5 one mutant, 3M7C, is more stable (~2 fold) than the wt protein. At alkaline pH one mutant, 1M10B, displays higher stability (~1.5 fold) compared with that of wt protein (Fig. 4).

Fig.4



References:

- [1] Farinas ET, et al., 2001, *Curr. Opin. Biotechnol.*, 12, 545-55.
- [2] Palmieri G., et al., 1993, *Appl. Microbiol. Biotechnol.*, 39,632-636
- [3] Giardina P. et al., 1999, *Biochem. J.*, 34,655-663