Combinatorial Saturation Mutagenesis by *in vivo* Overlap Extension for the Engineering of Fungal Laccases.

M Alcalde[1]*, M Zumárraga[1], J Polaina[2], A Ballesteros[1] and FJ Plou[1].

[1]Departamento de Biocatálisis, Instituto de Catálisis y Petroleoquímica, CSIC, 28049 Madrid, Spain.

[2]Instituto de Agroquímica y Tecnología de Alimentos, (CSIC), Paterna, 46980 Valencia, Spain.

*Corresponding author: malcalde@icp.csic.es, phone: +34915855479, fax: +34 915854760

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ABSTRACT

Combinatorial saturation mutagenesis -CSM- is a valuable tool for improving enzymatic properties from *hot-spot* residues discovered by directed enzyme evolution or performing semi-rational studies.

CSM coupled to a reliable high-throughput screening assay -CV below 10 %- has been used to enhance turnover rates in the fungal laccase variant T2 from *Myceliophthora thermophila*. The influence of the highly conserved pentapeptide 509-513 on the redox potential of blue-copper containing enzymes is well described. We focused combinatorial saturation mutagenesis in residues Ser510 and Leu513. Libraries were constructed in *Saccharomyces cerevisiae* by *in vivo* overlap extension -IVOE- of the PCR products. This methodology provides a simple manner to build CSM libraries avoiding extra PCR reactions, by-products formation and *in vitro* ligation steps. After exploring more than 1,700 clones, mutant (7E1) with ~3-fold better kinetics than parent type was found. 7E1 showed one synonymous mutation (L513L, CGT/TTG) and beneficial mutation S510G (TCG/GGG) which can not be achieved by conventional error-prone PCR techniques. Mutation S510G seems to affect the C-terminal plug, which modulates the transit of water and oxygen to the trinuclear copper cluster.

INTRODUCTION

In the last decade, biocatalysis has undergone a spectacular renovation in the way of engineering enzymes towards biotechnological applications. To this end, laboratory enzyme evolution and semi-rational approaches have been successfully used as the main molecular tools to get biocatalysts more robust, stable, efficient and in general with many improved features [1-4].

Mimicking the darwinist algorithm of natural selection, through several rounds of random mutagenesis and/or DNA recombination coupled to massive high-throughput screenings[5], directed molecular evolution allows to tailor enzymes which can be applied from remediation to novel green processes[6]. In this evolutionary scenario, semi-rational analysis -where researches are taking advantage from protein structural information to create and explore libraries constructed by saturation mutagenesis- constitutes also a powerful methodology[7].

Both *Escherichia coli* and *Saccharomyces cerevisiae* are the most successfully used host organisms for laboratory evolution experiments. Unlike the limited *E. coli* physiology -which posses a low DNA homologous recombination frequency-, *S. cerevisiae* eukaryotic machinery confers a broad array of possibilities for the construction of mutant libraries or for recombining –shuffling- DNA fragments[8;9].

Exchange of genetic material by recombination occurs in all living organisms and is the major contributor to high-quality diversity generation in the evolution of the species. In the particular case of *S. cerevisiae* the high frequency of homologous recombination encourages *evolvers scientists* to use it as host to clone not only mutant libraries with linearized vectors, but also to try new *in vivo* alternatives to the standard *in vitro* protocols of generation of diversity[10-12]. Keeping this in mind, we are focusing part of our research in exploring and validating new high-throughput methods based on yeast

heterologous expression, which can successfully be employed to engineer enzymatic functions by *forced* evolution [12-15].

Saturation random mutagenesis has long been used to investigate protein-DNA interactions and protein function. With this technique, one single amino acid codon is mutated to all other codons encoding the 20 naturally occurring amino acids. This method is used in directed evolution experiments to expand the number of amino acid substitutions accessible by random mutagenesis[16]. Furthermore, this technique can be employed to simultaneously mutagenize several codons -combinatorial saturation mutagenesis-, either in contiguous blocks or in separate positions, allowing the exploration of all possible combinations and permutations of interesting amino acid residues, in order to identify their optimal interactions and geometry for desirable protein or even create new functions or redesign active site for new substrates [17;18]. Combinatorial saturation mutagenesis is typically carried out by laborious *in vitro* experiments based on the well-reported sequence overlap extension (SOE)[19]. Gene splicing by SOE recombines DNA sequences containing the mutated DNA fragments by several consecutives PCR reactions, which also requires an additional *in vitro* ligation step with the vector for cloning the whole fragment. Here, we are presenting a modification of the standard protocol of combinatorial saturation mutagenesis, using *S. cerevisiae* apparatus for recombining and splicing mutated libraries and at the same time creating a circular autonomously replicating vector, which can be easily coupled to high-throughput enzymatic schemes. To validate this protocol we have chosen as scaffold a laccase gene from the fungi *Myceliophthora thermophila*, which was previously evolved towards high expression levels in *S. cerevisiae*[12]. Residues Ser510 and Leu513 were targeted because of their relevance in the modulation of redox potential of this enzyme[20]. Libraries were constructed and explored by high-throughput methodology discovering a variant (7E1) which can provide new insights in the

parameters that are implicated in the modulation of the activity and redox potential of ascomycete laccases.

EXPERIMENTAL PROCEDURES

All chemicals were of reagent-grade purity. ABTS (2,2´-azino-bis(3-ethybenzthizoline-6-sulfonic acid)) and *S. cerevisiae* transformation kit were purchased from Sigma-Aldrich (Madrid, Spain). Parent laccase gene (MtLT2) was engineered as reported elsewhere[12]. *E. coli* XL2-blue competent cells and high-fidelity polymerase *Pfu*-Ultra were from Stratagene (La Jolla, CA). Protease deficient *S. cerevisiae* strain BJ5465 was from LGCPromochem (Barcelona, Spain). Shuttle vector pJRoC30 with auxotrophy for uracil and with ampicillin resistance gene was from Novozymes (Davis, CA). Zymoprep yeast plasmid miniprep kit, zymoclean gel DNA recovery kit, and DNA clean and concentrator TM-5 Kit were from Zymo Research (Orange, CA). QIAprep spin miniprep kit was from QIAGEN (West Sussex, UK). Restriction enzymes BamHI and XhoI were from New England Biolabs (Hertfordshire, UK).

Culture media

Minimal medium contained 100 ml 6.7% sterile yeast nitrogen base, 100 ml 19.2 g/L sterile yeast synthetic drop-out medium supplement without uracil, 100 ml sterile 20% raffinose, 700 ml *sdd* H_2O, 1 ml 25 g/L chloramphenicol. YP medium contained 10 g yeast extract, 20 g peptone, and *dd* H_2O to 650 ml. Expression medium contained 720 ml YP, 67 ml 1M KH_2PO_4 pH 6.0 buffer, 10 μl 1M $CuSO_4$, 111 ml 20% galactose, 1 ml 25 g/L chloramphenicol and *dd* H_2O to 1000 ml. YPAD solution contained 10 g yeast extract, 20 g peptone, 100 ml 20% sterile glucose, 100 mg adenine hemisulfate, 1 ml 25 g/L chloramphenicol and *dd* H_2O to 1000 ml. SC drop-out plates contained 6.7 g yeast nitrogen base, 100 ml 19.2 g/L sterile yeast synthetic drop-out medium

supplement without uracil, 20 g bacto agar, 100 ml 20% sterile glucose, 1 ml 25 g/L chloramphenicol, and *dd* H_2O to 1000 ml.

Production of MtLT2 in *S. cerevisiae*

One single colony from *S. cerevisiae* clone containing MtLT2 parent-type or mutant laccase genes was picked from a SC drop-out plate, inoculated in 3 ml of minimal medium and incubated for 48 h at 30ºC and 225 rpm (Micromagmix shaker, Ovan, Spain). An aliquot of cells was removed and inoculated into a final volume of 50 ml of minimal medium (optical density, OD_{600}=0.25) in a 500 ml flask. Incubation proceeded until two growth phases were completed (OD_{600}=1.0; 6 to 8 h). Thereafter, 450 ml of expression medium containing 5.6mM $CuSO_4$ were inoculated with the 50 ml preculture in a 2.0 -litter baffled flask (OD_{600}=0.1). Incubation was stopped after 38-42 h at 30ºC and 225 rpm (laccase activity was maximal reaching a plateau; OD_{600}=28-30). The cells were separated by centrifugation for 20 min at 3000 *g* (4ºC). Supernatant was double-filtered (by both glass membrane and a nitrocellulose membrane of 0.45 μm pore size) and concentrated to 20 ml in an ultrafiltration cell (Amicon/Millipore, Barcelona, Spain) equipped with a 10 kDa cutoff membrane.

Purification of laccases

Native and mutant laccases were purified using fast protein liquid chromatography (FPLC) equipment (LCC-500CI, Amersham Bioscience, Barcelona, Spain). Concentrated crude extract was first submitted to fractional precipitation with ammonium sulfate to 50% saturation (w/v) at 0ºC and centrifuged at 17,000 *g* for 20 min. The supernatant was filtered and loaded into a hydrophobic interaction column (HIC, Hiload 16/10 Phenyl Sepharose High Performance, Amersham Pharmacia) equilibrated with 1.8 M $(NH_4)_2SO_4$ in 10 mM sodium phosphate buffer pH 6.1. Proteins were eluted with a linear gradient from 1.8 to 0 M $(NH_4)_2SO_4$. Fractions with laccase activity were pooled, concentrated and dialyzed against 10 mM sodium phosphate buffer pH 6.1. Semi-

purified laccase was applied to an anion exchange column (DEAE Sepharose CL-6B) preequilibrated with 10 mM sodium phosphate buffer pH 6.1. Proteins were eluted with a linear gradient from 0 to 0.4 M of NaCl. Fractions with laccase activity were pooled, concentrated and dialyzed against 10 mM sodium phosphate buffer pH 6.1. Fractions throughout the purification protocol were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed with 8 % polyacrilamide using a Miniprotean 3 Cell (Biorad, US). Proteins were stained with both Coomassie brilliant blue and silver. Protein concentrations were determined by the method of Bradford[21] using the Bio-Rad protein reagent and bovine serum albumin as a standard. Purified laccases were stored at –20ºC.

***In vivo* overlap extension (IVOE) for the construction of combinatorial saturation mutagenesis libraries.**

1.- PCR amplification

Two separate PCR reactions (thermocycler Mycycler, Biorad, US) were simultaneously carried out to amplify the two DNA fragments which overlapped at specific positions corresponding to aminoacids 510 and 513 in MtLT2 sequence. PCR reactions were performed in a final volume of 50 μl contained 0.25 μM of each primer, 100 ng of template (MtLT2), dNTPs (0.25 mM each), 3% dimethyl sulfoxide (DMSO) and 2.5 Units of Pfu-Ultra DNA polymerase.

PCR 1 was done using the following oligonucleotides: RMLNsense (5'-CCTCTATACTTTAACGTCAAGG-3', binds at bp 5'-160-180-3' of pJRoC30) and 3CPOantisense (5´-GTAGACGACGCC**SNN**GCCGCC**SNN**GACGTGCCAGGCGAT-3´, binds at bp 5´-1859-1899- 3´ of MtLT2). Primers for PCR 2 were: 3CPOsense (5´-ATCGCCTGGCACGTC**NNS**GGCGGC**NNS**GGCGTCGTCTACC-3´, binds at bp 5´-1859-1899-3´ of MtLT2) and RMLCantisense (5' GGGAGGGCGTGAATGTAAGC 3', binds at bp 5'-2139-2158-3' of pJRoC30). Underlined and in bold are shown the codons submitted

to saturation mutagenesis, where **N** is (A+T+C+G) and **S** is (G+C). PCR conditions were as follows: 95ºC for 2 min (1 cycle), 94ºC for 0.45 min, 55ºC for 0.45 min, 74ºC for 2 min (28 cycles), and 74ºC for 10 min (1 cycle). PCR fragments where cleaned and concentrated (DNA clean and concentrator TM-5 Kit, Zymo Research), loaded into a low-melting-point preparative agarose, and purified using the Zymoclean gel DNA recovery kit (Zymo Research).

2.- Plasmid linearization

Plasmid pJRoC30 containing MtLT2 gene was linearized by cutting with restriction enzymes XhoI and BamHI (New England Biolabs, UK). Linearized vector (without parent gene) was cleaned, concentrated and purified as described above for PCR fragments.

3.- *In vivo* overlap extension and cloning in *Saccharomyces cerevisiae*

PCR fragments (200 ng each) were mixed with linearized vector (100 ng, ratio PCR-product:vector 4:1) and transformed into competent yeast cells using the yeast transformation kit (Sigma, Madrid, Spain). Right before thermal shock, 10% DMSO was added in the transformation mixture to improve transformation efficiency (close to 20,000 clones per transformation reaction). Transformed cells were plated in SC drop-out plates and incubated for 2 days at 30ºC. Colonies containing the whole autonomously replicating vector were submitted to high-throughput screening (HTPS) assay.

Coupling IVOE to HTPS assays: end-point colorimetric assay.

Individual clones were picked into 96-well plates (Sero-well, Staffordshire, UK) containing 50 μl of minimal medium per well. In every single plate the column number 6 was inoculated with standard (parent type, MtLT2), and 1 well (H1) was not inoculated (control). Plates were wrapped with parafilm (to prevent evaporation) and incubated at 30ºC and 225 rpm in a humidity shaker (Minitron-INFORS, Biogen, Spain).

After 48 h, 160 μl of expression medium were added to each well, and plates were again incubated. After 24 h, OD_{600} was recorded to determine the cell density in each well using a microplate reader (Versa Max, Molecular Devices, Sunnyvale, CA).

Plates (master plates) were centrifuged (Eppendorf 5810R centrifuge, Germany) for 5 min at 3000 *g* at 4ºC. 20 μl of supernatant were transferred from master plate (Liquid Handler Quadra96-320, Tomtec, Hamden, CT, US) to a replica plate. 180 μl of assay solution (final concentration in the well 3 mM ABTS, 100 mM acetate buffer pH 4.5) was added to each well of replica plate containing supernatant. Plates were stirred and the absorption at 418 nm ($\varepsilon_{ABTS}^{\bullet+}$=36,000 $M^{-1}cm^{-1}$) was recorded in the plate reader.

Plates were incubated at room temperature until green color was developed, and the absorption was measured again. Relative activities were calculated from the difference between initial absorption and that after the incubation divided by the OD_{600} of each well. Relative activities were normalized against the parent type in the corresponding plate.

Rescreening procedures

First rescreening: aliquots of 5 μl of the best clones were used from master plates to inoculate 50 μl of minimal media in new 96-well plates. Columns 1 and 12 (rows A and H) were not used to prevent the appearance of false positives. Five wells on the microplates were inoculated with the same clone. Five wells on each plate were used for the standard. The screening procedure then was the same as above, but including not only a endpoint assay but also a kinetic assay[22].

Second rescreening: an aliquot from the wells with the most active clones of first rescreening was inoculated in 3 ml of YPAD and incubated at 30ºC and 225 rpm for 24 h. Plasmids from these cultures were extracted (Zymoprep yest plasmid miniprep kit, Zymo Research). As the product of the zymoprep was very impure and the concentration of extracted DNA was very low, the shuttle vectors were transformed

into super-competent *E. coli* cells (XL2-Blue, Stratagene) and plated onto LB-amp plates. Single colonies were picked and used to inoculate 5 ml LB-amp media and were grown overnight at 37 ºC and 225 rpm. Plasmids were then extracted (QIAprep spin miniprep kit, QIAGEN). *S. cerevisiae* was transformed with plasmids from the best mutants and also with parent type. Five colonies of every single mutant were picked and rescreened as described above (using both end-point and kinetic assays).

DNA sequencing.

Plasmid-containing variants laccase genes were sequenced at the Sequencing Core Facility of the Instituto de Investigaciones Biomedicas, CSIC, Madrid, using an Applied Biosystems 377 automated fluorescent DNA sequencer. The primers used were as follows: forward: RMLN and mtlsq2 (5´-GAAGGGCACCAACCTGC-3´, binds at bp 5´-643-659-3´ of pJRoC30); reverse: mtlsq3 (5´-CGCACGTAAAAGTCGTGG-3´, binds at bp 5´-1657-1673-3´of pJRoC30) and RMLC.

Protein modeling

We carried out a search in the Protein Data Bank for proteins of known structure homologous to MtLT2. The most similar protein to MtLT2, showing 75% identity in sequence, was a laccase from *Melanocarpus albomyces* [23] (PDB id: 1GW0). A structure-based alignment of the sequences of both proteins was performed with GenTHREADER[24]. This alignment was used to obtain a model from the Swiss-Model protein modelling server (http://swissmodel.expasy.org/) which was analyzed with DeepView/Swiss-Pdb Viewer.[25;26]

RESULTS

Construction of libraries by IVOE.

IVOE methodology (Fig. 1) is based on the high frequency of homologous recombination of eukaryotic machinery to splice saturated mutagenized DNA fragments and at the same time use yeast gap repair mechanism in order to substitute *in vitro* ligation. First, two PCR reactions were carried out using degenerated primers to produce two PCR fragments which shared homologous sequences at the 3´and 5´ends respectively. These products already contained the saturated mutagenized codons, and were used directly by *S. cerevisiae* to *in vivo* shuffle each other through their recombination areas giving rise to a whole gene. Likewise, recombination not only spliced the two fragments in a complete gene but also shuffled the mutagenized codons. The whole mutagenized gene possessed big overhangs which recombined with the ends of the linearized vector forming an autonomously replicating plasmid. It is not straightforward to find out which event take place first (the splicing of the PCR fragments between themselves or their linkage to the linearized plasmid); in fact it is likely that even both phenomena happen simultaneously. With this approach, reliable high transformation frequency and short protocols for library expression can be derived, with a readily coupling to a HTPS assay.

Library size and design of the recombination area: the size of the combinatorial saturation mutagenesis library is strictly depending on the genetic code, type of mutagenic codon and the number of sites chosen for mutagenesis[27]. In our case, two positions (510 and 513) were selected and submitted to combinatorial saturation mutagenesis using NNG/C randomization strategy instead of NNN randomization. NNG/C reduces the total number of variants, while all amino acids remain accessible and the complexity of the library can still be maintained. Positions 510 and 513 have a distance between them of only 6 bp, which enable them to recombine within one

primer. Degenerated primers were designed in such a manner that the mismatches lay on the middle of the primer. To get accurate annealing during PCR, 15 and 22 nucleotides flanked the mismatches. Cloning efficiency did not change much if the homologous sequences were longer than 50 bp (data not shown; in our case the two PCR products to be *in vivo* recombined had as homology region 40 bp which comes from the own length of the degenerated primers). To recombine with linearized plasmid, overhangs design was ruled under the same principles, however if overhangs were smaller than 20 bp efficiency would be compromised.

Library analysis

A total of 1,740 clones from the library constructed by IVOE/combinatorial saturation mutagenesis at the codons for residues 510 and 513 were picked and screened for activity. Only 2.98 % showed improvements and 93.1 % displayed less than 10 % of the parent enzyme´s activity, MtLT2 (Fig. 2). The coefficient of variance (CV) of the end-point assay was found to be below 10 % (sensitivity limit of ABTS assay 5 nU/ml, Fig. 3). Figure 4 summarizes the HTPS used to explore the library constructed by IVOE. It is worth noting that the second rescreening using freshly transformed cells was necessary to discard false-positives, getting a correct comparison of the clones. This second rescreening was highly reliable since included mutants which were all in the same metabolic stage. After the 2 consecutives rescreens, only one mutant (7E1) was found to show significantly better kinetics than parent type. Variant 7E1 was purified to homogeneity and further characterized, displaying 3.1 fold better activity (K_{cat} 4440 min^{-1} and 1420 min^{-1} for 7E1 and MtLT2, respectively). 7E1 variant was sequenced showing one synonymous mutation L513L (CTG/TTG) and one beneficial mutation S510G (TCG/GGG), which is responsible for the activity enhancement.

DISCUSSION

SOE (sequence overlap extension) is a PCR-based method of recombining DNA sequences without reliance on restriction sites and of directly generating mutated DNA fragments *in vitro*. This method has been widely used to carry out the construction of libraries by combinatorial saturation mutagenesis[27]. Based on gene splicing, degenerate oligonucleotides are designed so that the ends of the resultant PCR products contain complementary sequences. Each primer pair is synthesized with a mismatched random nucleotide in the middle (such as –NNN-), flanked on both sides by nucleotides that specifically anneal to the target region. Therefore, first the DNA fragments must be amplified by two separate PCR reactions giving rise to 2 DNA fragments that overlap at a specific region. Afterwards, a third PCR reaction must be performed, where the two PCR products are mixed and their complementary sequences at their 3´ends anneal and then act as primers for each other. This step allows to reassemble a new version of the original full-length sequence, where the target codons are effectively randomized. Finally, the whole amplified and specifically/randomly mutagenized in one/several codon(s) must be *in-vitro*-ligated to a linearized vector to guarantee protein expression.

Apart from the indispensable *in vitro* ligation, main bottlenecks of combinatorial saturation mutagenesis based on SOE stem from the consecutives PCR reactions which are associated with poor reaction yields and the formation of by-products. To overcome all these shortcomings we have developed an *in vivo* methodology which is faster, more efficient and non-mutagenic (the proofreading apparatus of the yeast cell prevents the appearance of additional mutations that are common with *in vitro* methods). In a simple and elegant experiment IVOE proceeds recombining PCR fragments, shuffling the mutagenized codons and repairing linearized vector with the help of specifically engineered overhangs. In SOE, an additional PCR reaction and the

in vitro ligation are fundamental requisites. IVOE saves those steps straightforwardly, just taking advantage of the eukaryotic machinery (since *S. cerevisiae* displays an exquisite frequency of homologous recombination). This technique was tuned using as parent type the laccase from *Myceliophthora thermophila* variant T2, (MtLT2) which is highly expressed in *S. cerevisiae*[12]. In spite of the fact that fungal laccases posses the highest redox potential –Eº- of this enzymatic group (close to +800 mV), their practical application in the oxidation of high Eº substrates and non-phenolic compounds (such as polycyclic aromatic hydrocarbons or in the pulp-kraft bleaching) are still under investigation[28-35]. Indeed, over the years, the broad range in the fungal laccases Eº (from +465 mV of *Myceliophthora thermophila* laccase –MtL-, to +790 mV of *Trametes –Polyporus* or *Coriolus- versicolor* laccase) has been subject of study trying to figure out which parameters are behind the differences in the Eº[20;36-38]. It is well known that a hydrophobic residue (either Phe or Leu) at the axial position of the T1 Cu site is implicated in the elevated Eº of fungal laccases[39]. However, there are broad differences even amongst the fungal laccases –see table 1-; thus it is clear that the non-coordinating Leu or Phe residue at the T1 site cannot be the sole contributor to this effect[40]. In this sense, it has been described the significance of a pentapeptide 509-513 segment highly conserved located near the Cu T1, which also contains the mentioned axial ligand of the T1 site at position 513[20]. In MtLT2, Ser510 forms part of the tripeptide VSG which is common to the low redox potential laccases. Unlike VSG, the tripeptide LEA appears in high redox potential laccases. VSG/LEA tripeptide is located in the vicinity of substrate binding and the T1 Cu. Because of their relevance and their proximity in their sequence space (just 6 bp of distance in between), we decided to study residues 510 and axial ligand at 513 by IVOE. As expected, the activity landscape displayed a large fraction of the library losing performance (i.e. the region was quite sensitive to amino acid replacement, Fig. 2). Variant 7E1 was the only

positive mutant with significant improvements *vs* parent type, showing only one amino acid change in position 510 and one synonymous mutation at position 513. It is worth noting that although other positives variants were sequenced none displayed changes at position 513. To our knowledge, this is the first time that the position corresponding to the axial ligand is submitted to saturation mutagenesis. Previously Xu and coworkers[20;38] replaced the axial ligand by site-directed mutagenesis without improving the enzymological features of fungal laccases. In a recent work the axial ligand of the T1 site was site-mutated in the bacterial laccase CotA from *Bacillus subtilis* and, although redox potential was enhanced, the modification affected negatively the thermodynamic stability of the enzyme[41]. According to our experimental data, axial ligand of the T1 site in MtLT2 behaves as a strictly conserved amino acid (or in other words, has already achieved the highest level of fitness or evolution) and any change by another residue would not give a significant increase in the activity of the biocatalyst. On the other hand, S510G was the only mutation that conferred better performance to variant 7E1. Interestingly, substitution S510G of 7E1 can not be achieved from a single base substitution (i.e. by random mutagenesis experiment, see table 2). Taking into account that mutation rates must be low for whole-gene evolution and that error-prone PCR methodologies are limited to single point mutation and specific bias, a large fraction of protein sequence space would remain still unexplored (in fact, with single base mutation, only 5.7 amino acid substitutions on average are accessible from any given amino acid residue). Unlike error-prone PCR, IVOE enabled us to access to the overall array of possibilities at the targeted residues giving us a whole picture about the interactions, incompatibilities and synergy of studied positions.

We used the structure of *Melanocarpus albomyces* laccase[23] (75 % of sequence identity to MtLT2) to build a model for 7E1. In parent type Ser510 was connected with the terminal Asp556 through a hydrogen bond (Fig. 5). Asp556 is part of the C-

terminal plug, a structure highly conserved in ascomycete laccases[42]. This plug is supposed to be involved in modulating redox potential of these enzymes regulating both the entrance and exit of oxygen and water from the active pocket through a broad tunnel. In variant 7E1 the interaction between Ser510 and Asp556 no longer exists, which may have a double effect:

i) the removal of lateral chain of Ser510 enhances the tunnel which leads to the trinuclear copper cluster (Fig. 6).

ii) After breaking the hydrogen bond between Ser510 and Asp556 the grade of tightness is lowered.

Our research is currently focused in the electrochemical characterization of 7E1 and in the use of this variant as parent type for further studies of combinatorial saturation mutagenesis of the C-terminal plug by IVOE.

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LEGENDS

Fig.1. *In vivo* overlap extensión –IVOE- strategy for combinatorial saturation mutagenesis. N-terminal overhang was created in PCR 1 generating 35 bp of homology to the BamHI end site of linearized vector. C-terminal overhang was created in PCR 2 generating 62 bp of homology to the XhoI end site of linearized vector. Combinatorial saturation mutagenesis at amino acid positions 510 and 513 was carried out using a set of degenerate synthetic oligonucleotides (NNG/C for each targeted residue) with 22 bp and 15 bp flanking mutation sites.

Fig.2. Activity landscape of library constructed by IVOE. Activities of clones are plotted in descending order. The library size for two mutated sites was calculated by a binomial probability approximation[27]. Solid horizontal line shows the activity of the parent (MtLT2) in the assay. Dashed lines indicate the coefficient of variation of the assay.

Fig.3. Validation of the screening method with a plate of 96 parent type samples. Enzymes activities are plotted versus well position (columns) and in descending order (circles). The CV for this screen was 9.0 %.

Fig.4. High-throughput screening methodology.

Fig. 5. Local interactions in position 510.

Fig. 6. Relative position of Ser510 (yellow) and Asp556 (red). In variant 7E1 it is apparent the enhancement of the tunnel which leads to the trinuclear copper cluster.

Table 1. E° of several blue multicopper enzymes[28].

Species	Organism	Enzyme	CuT1 E°(V)	Potential axial ligand
Trametes versicolor	Basidiomycete	Laccase	+0.79	Phe
Trametes villosa	Basidiomycete	Laccase	+0.79	Phe
Neurospora crassa	Ascomycete	Laccase	+0.78	Leu
Rhizoctonia solani	Deuteromycete	Laccase	+0.71	Leu
Coprinus cinereus	Basidiomycete	Laccase	+0.55	Leu
Scytalidium thermophilum	Basidiomycete	Laccase	+0.51	Leu
Homo sapiens	Mammalian	Ceruloplasmin	+0.49	Met
Myrothecium verrucaria	Fungi mitosporic	Bilirubin oxidase	+0.48	Met
Myceliophthora thermophila	Ascomycete	Laccase	+0.47	Leu
Rhus vernicifera	Plant	Laccase	+0.43	Met
Zucchini (Cuburbita pepo)	Plant	Ascorbate oxidase	+0.34	Met

Table 2. Comparison between mutation achieved by IVOE at position 510 and accessible mutations by conventional error-prone PCR.

BENEFICIAL MUTATION	ACCESIBLE AMINO ACID BY SINGLE BASE SUBSTITUTION
S510G ($TCG_{8.6}$/$GGG_{6.0}$)	L($TTG_{27.2}$), P($CCG_{5.3}$), T($ACG_{8.0}$), A($GCG_{6.2}$), W($TGG_{10.4}$)

*Subscript numbers indicate codon usage in *S. cerevisiae*.

Fig.1

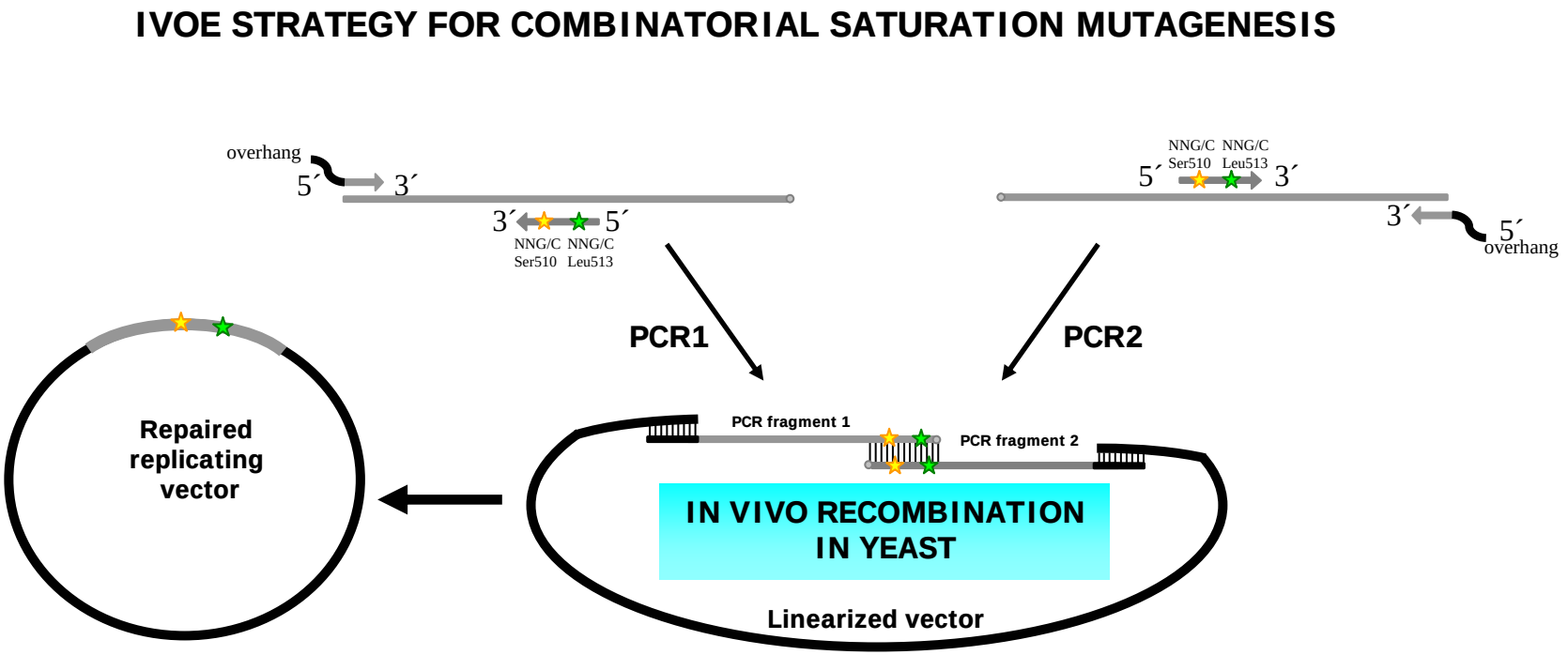

IVOE STRATEGY FOR COMBINATORIAL SATURATION MUTAGENESIS
overhang
5´
3´
3´
5´
NNG/C Ser510
NNG/C Leu513
NNG/C Ser510
NNG/C Leu513
5´
3´
3´
5´
overhang
PCR1
PCR2
PCR fragment 1
PCR fragment 2
IN VIVO RECOMBINATION IN YEAST
Linearized vector
Repaired replicating vector

Fig.2

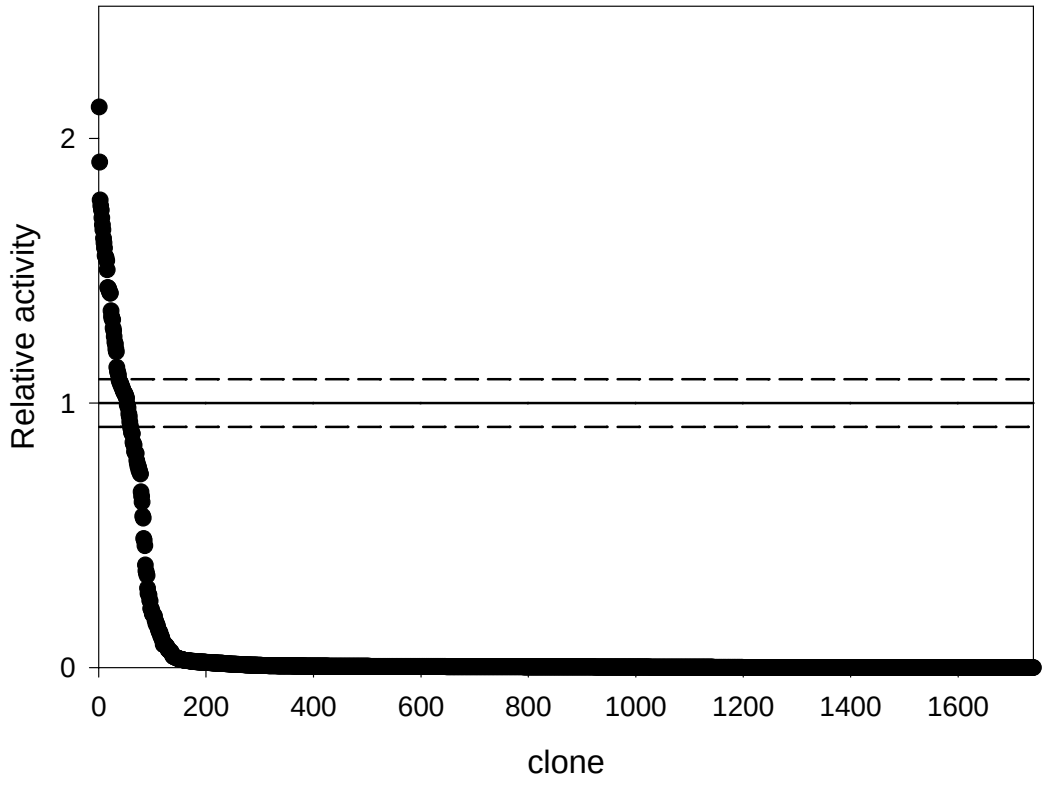

Relative activity
2
1
0
0
200
400
600
800
1000
1200
1400
1600
clone

Fig.3

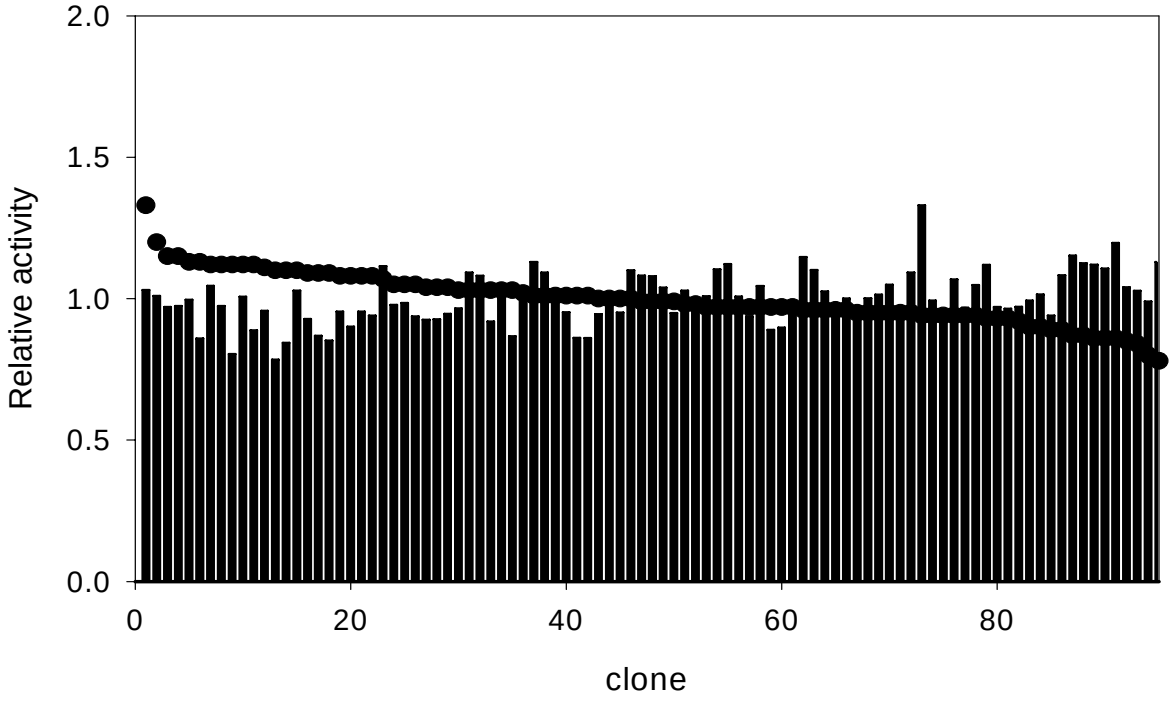


Fig.4

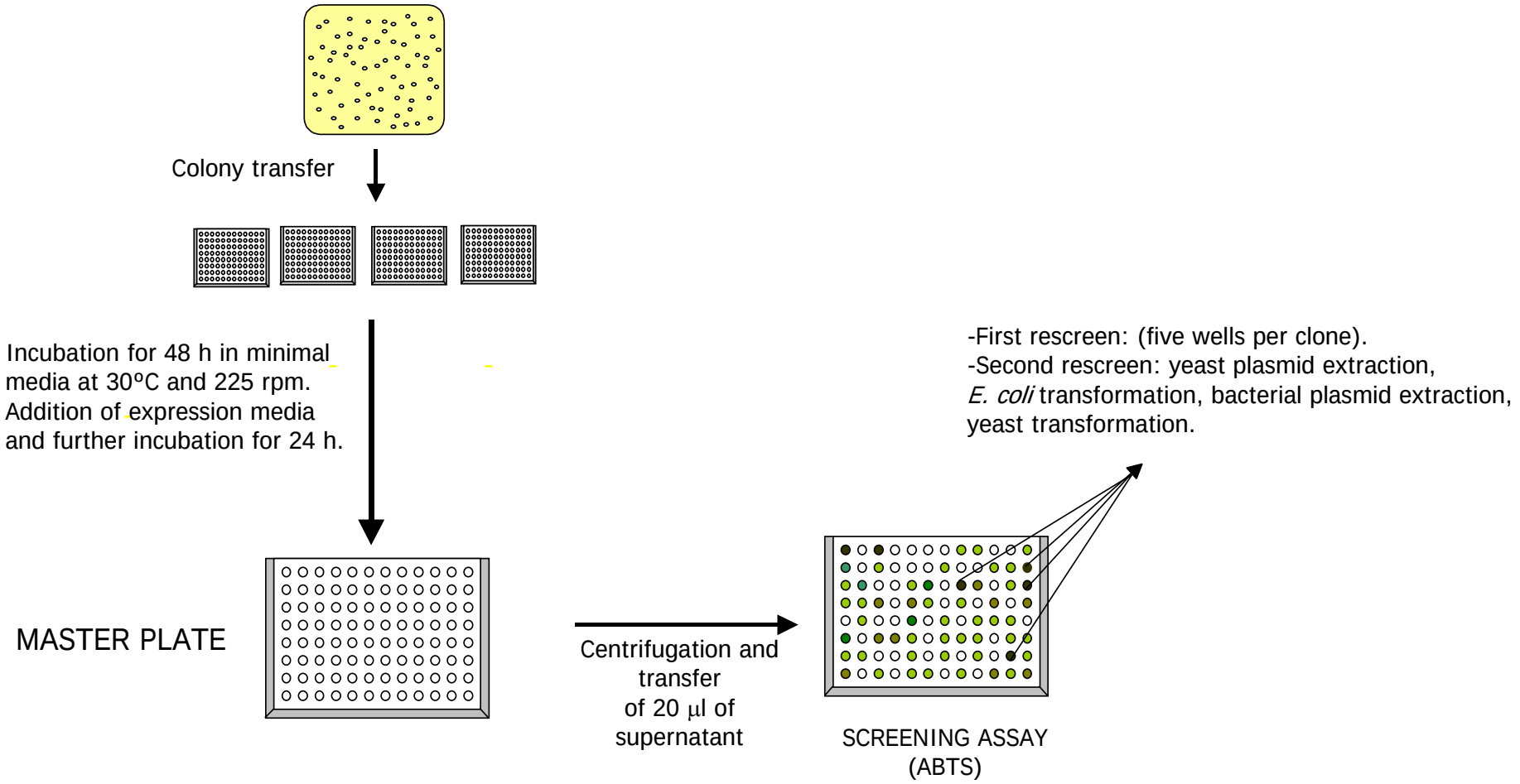
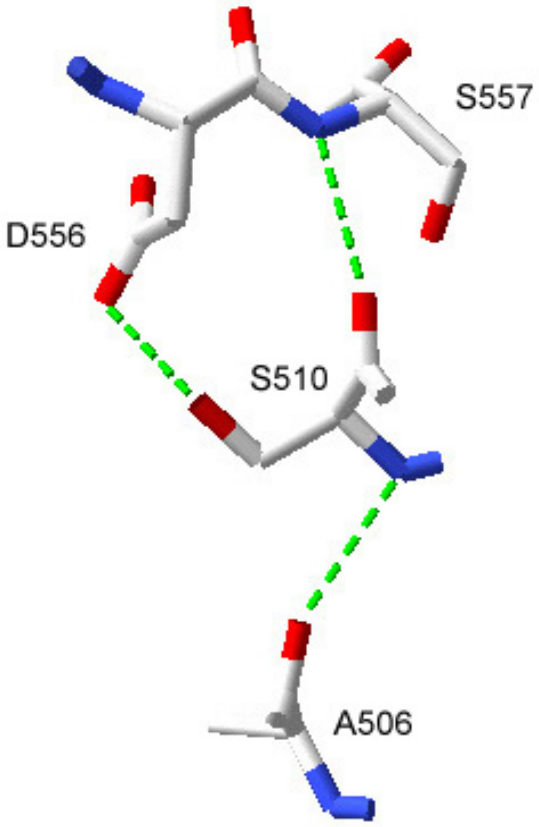

Colony transfer
Incubation for 48 h in minimal media at 30ºC and 225 rpm. Addition of expression media and further incubation for 24 h.
MASTER PLATE
Centrifugation and transfer of 20 µl of supernatant
SCREENING ASSAY (ABTS)
-First rescreen: (five wells per clone).
-Second rescreen: yeast plasmid extraction, *E. coli* transformation, bacterial plasmid extraction, yeast transformation.

Fig.5

Parent type MtLT2



7E1 variant

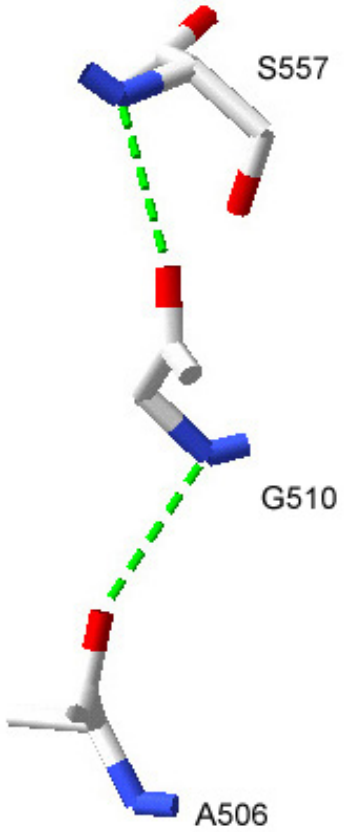
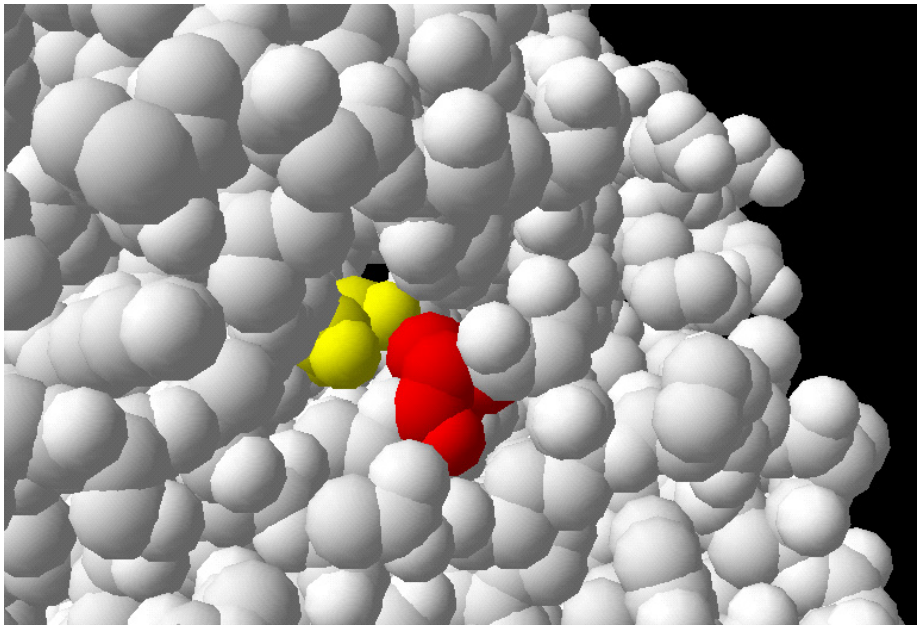


Fig.6

Parent type MtLT2



7E1 variant