Universidad Europea MADRID

BACHELOR'S DEGREE THESIS

Degree in Biotechnology

PEROXYGENASE ENGINEERING FOR STUDIES ON DEGRADATION OF COMPLEX EPOXY MATERIALS

Sara Andrés Campos

Tutors
Javier Viña-Gonzalez (EvoEnzyme)
Miguel Alcalde Galeote (CSIC)

Academic Tutor
Paloma Santos Moriano

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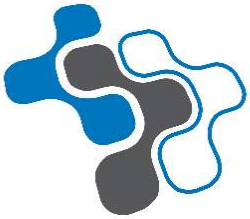
ANNEX IX

Essay title: PEROXYGENASE ENGINEERING FOR STUDIES ON DEGRADATION OF COMPLEX EPOXY MATERIALS

This thesis has been carried out at:



EvoEnzyme
Customized Biocatalysts

Tutors:

Javier Viña González (EvoEnzyme), Miguel Alcalde Galeote (CSIC)

Universidad Europea de Madrid: Paloma Carmen Santos Moriano

Research center path and credentials

EvoEnzyme, S.L. is a biotechnological company that started activities in 2019 and is located in Comunidad de Madrid. It was created as a spin-off from ICP-CSIC's (Institute of Catalysis and Petrochemistry - Consejo Superior de Investigaciones Científicas) research team on Directed Enzyme Evolution led by Professor Miguel Alcalde. EvoEnzyme is the first Spanish company specialized on protein engineering by laboratory evolution, mastering ligninolytic enzymes, and thus far have made advanced proteins as solution for diverse problems posed by their various clients, from the pharmaceutical to the chemistry/energy sectors. (https://evoenzyme.com/)

The astounding credentials of founders and counsels motivated and boosted the enterprise's growth, starting from Dr. Miguel Alcalde himself, who has over 20 years of experience in the field of directed enzyme evolution, being introduced to the in CALTECH during his postdoctoral studies in 2001. There, he worked hand in hand with Professor, doctor and Nobel laureate Frances Arnold for 2 years. It was then, in 2003, when he established his own research group. Around 13 years later, a team of scientists from the group gathered the accumulated experience and founded EvoEnzyme based on the patent WO/2017/081355 and the directed evolution techniques employed and developed during two decades. EvoEnzyme is located at the Scientific Park of Madrid (https://fpcm.es/) with a full equipped laboratory for biocatalyst research and development: from molecular biology, robotic platforms for high-throughput screening of mutant libraries, equipment for protein purification and a lab-bench bioreactor.

Index

Acronyms

1Phe	1-phenoxy-2-propanolol
4-AAP	4-aminoantipyrine
AAO	Aryl alcohol oxidase
Abs	Absorbance
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
Amp	Ampicillin
BPA	Bisphenol A
CV	Coefficient of variation
***dd*H_2O**	Ultra-pure and sterile water
DMP	2,6-Dimethylphenol, also known as 2,6-Xylenol
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotide triphosphates
GC-MS	Gas chromatography-mass spectrometry
GM-PCR	PCR using GeneMorph II polymerase
GOI	Gene of interest
HDMs	Human drug metabolites
HDW	Half-deep-well 96-well plate
HTS	High-throughput screening
KPi	10 mM Potassium Phosphate and 100 mM NaCl buffer
LacRo	Laccase from *Moniliophtora roreri*

LB	Luria Bertani medium
m	Mutations
nm	Non-measured data
OD_{600}	Optical density at 600 nm
PSK	Potassium persulfate
Sat174PCR	Site Saturation Mutagenesis of position 174 PCR
Sat184PCR	Site Saturation Mutagenesis of position 184 PCR
SC	Synthetic complete medium
SDR	Site Directed Recombination
SSM	Site Saturation Mutagenesis
TAI	Total Activity Improvement
***Taq*PCR**	PCR using Taq polymerase
TLC	Thin-Layer Chromatography
UPO	Unspecific fungal peroxygenase
VA	Veratryl alcohol
YNB	Yeast nitrogen base
YP	Yeast peptone medium
YPD	Yeast peptone dextrose medium

PEROXYGENASE ENGINEERING FOR STUDIES ON DEGRADATION OF COMPLEX EPOXY MATERIALS

Abstract

Due to the current end-of-life of thermoset composites, which mainly comprises incineration and landfill storage, is of utmost necessity to find sustainable biodegradation methods. In the present work, we approach enzymatic degradation of epoxy resins by exploiting fungal peroxygenases' (UPOs) versatility in terms of C-H oxyfunctionalization reactions. Aiming at engineering UPOs through directed evolution towards epoxy resins biodegradation, a colorimetric high-throughput screening assay was developed based on the hydroxylation of the model molecule 1-phenoxy-2-propanol; the main structural scaffold of Araldite/Aradur epoxy resin. Employing the evolved UPO variant GroGu as parental type, we were able to identify and characterized several mutants with improved activity in the degradation of the model compound. Further directed evolution rounds will be needed to obtain one final variant that will be subjected to lab-scale degradation studies with the real material, setting out the bases for a more sustainable end-of-life management of thermoset plastics.

Resumen

Actualmente, la eliminación de composites termoestables se limita a incineración y acumulación en vertederos, poniendo en evidencia la necesidad de encontrar una solución más sostenible. En este trabajo, abordamos la degradación enzimática de resinas de tipo epoxy aprovechando la versatilidad de las peroxigenasas fúngicas (UPOs) en cuanto a la amplia variedad de oxifuncionalizaciones sobre los enlaces C-H que son capaces de catalizar. Con objeto de realizar ingeniería sobre las UPOs a través de evolución dirigida hacia la biodegradación de resinas epoxi, se desarrolló un método de cribado colorimétrico basado en la detección de la hidroxilación sobre la molécula modelo 1-fenoxi-2-propanol; que engloba los principales motivos estructurales de la resina epoxi Araldite/Aradur. Utilizando la variante de UPO larga GroGu evolucionada como parental, fuimos capaces de identificar y caracterizar varios mutantes con actividad mejorada en la degradación del compuesto modelo. En el futuro, se realizarán campañas de evolución dirigida adicionales para obtener una variante final que se empleará en estudios de degradación enzimática a escala de laboratorio con el material real, sentando las bases para una gestión más sostenible de los composites termoestables.

1. Introduction

Thermoset composites are three-dimensional or linearly-linked polymers obtained from the reaction of precursors, such as epoxy, vinyl esters and polyesters; with curing agents or hardeners (1). These materials are widely used in many industries because of their mechanical, chemical and physical properties, which provide a strong, durable and versatile platform to build on. However, given their specific characteristics as thermoset polymers, their corresponding usage and further disposal pose serious issues in the realm of sustainability and global pollution, considering their main final destinations involve incineration and landfill accumulation. Accordingly, mass-scale production for aerospacial and construction industries is more damaging to the environment by the minute, and even though perspectives in material engineering are extensively growing, epoxy-based scaffolds seem to be increasing in demand unceasingly (2). Therefore, new environmental and sustainable responses are indeed needed to sustain the use of these materials in the near future.

Within this context, BIZENTE Project (Applying ligninases to resolve end-of-life issues of thermoset composite plastics; BBI-JU grant agreement Nº 886567; https://bizente.eu/) aims to apply green technologies for the end-of-life management by reducing non-biodegradable waste, specifically thermostable composites as epoxy resins in a Circular Economy approach. Thus, the main objective of this BBI Project is to degrade these complex polymers so they can be reused, as a means to reduce their environmental impact. This collaboration extends to 10 partners across Europe, which include companies like EvoEnzyme (SPA), Acciona (SPA), BioSphere (ITA) or Specific Polymers (FRA) (3).

Subsequentially, enzymatic biodegradation through oxidative enzymes emerges as a compelling solution for the recycling and revalorization of these challenging materials, due to their ability to degrade and depolymerize as recalcitrant structures as lignin (4). In particular, owing to their high versatility and high redox potential, fungal Unspecific Peroxygenases (UPOs, EC 1.11.2.1) were on the limelight as a starting point. There are two main phylogenetic families of peroxygenases: short-type UPOs presented in most

of the cases as a homodimeric structure comprised by monomers of around 25 kDa; and long-type UPOs, which solely consist of a 40-kDa monomer (5). Also, structurally, they vary in heme-channel geometry, which largely affects substrate affinity and activity, as well as product selectivity. UPOs catalyze a wide range of reactions, as they present both peroxygenase (insertion of oxygen into non-activated C-H bonds, especially aromatic and aliphatic alkene hydroxylation), and peroxidase (phenolic oxidation) activity, using H_2O_2 which serves as both the oxygen donor and electron acceptor (5,6). The dossier of oxyfunctionalization reactivities of this superfamily of enzymes includes aromatic and aliphatic hydroxylations, O-dealkylations and N-dealkylations – among others – which are of high interest for epoxy resin biodegradation. Additionally, their kinetic properties as well as extracellular nature, are of industrial and environmental significance (7). A very interesting long UPO variant, GroGu mutant, was selected as departure point for engineering in this project. GroGu is an evolved mutant with increased secretion in yeast that originates from the long UPO of *Candolleomyces (Psathyrella) aberdarensis* (*Pab*UPO-I). This novel enzyme displays a huge versatility with a broad pH profile for reactivity, while containing a hybrid catalytic active site among long-UPOs (8).

Despite the high level of biotechnological interest in this superfamily, no protein engineering experiments on UPOs have been conducted to accommodate their aforementioned enzymatic traits to the in-vogue plastic biodegradation field. In this regard, directed evolution has proved to be a very effective approach to overcome different bottlenecks as heterologous expression or find tailored catalysts with novel activities (9). Wide interesting research has been presented on the improvement of peroxygenases by directed evolution, from functional expression and the production of Human Drug Metabolites (HDMs), to the design of enzymatic chimeras for chemical synthesis (10-13).

Thereby, this study centers around the development of a directed evolution platform for the engineering of future epoxy resin degrading UPOs, that led to the identification of several mutants with enhanced activity to provide a cleaner alternative to thermoset composites after-life management.

2. Objectives

The main objective for the present original research is the establishment of an engineering platform for the directed evolution of UPOs to be used in modification/degradation processes of epoxy resin materials (*i.e.* Araldite/Aradur systems). The project aims to construct and analyze mutant libraries to detect and study long UPO variants with interesting properties on the frame of an industrial degradation process.

3. Methodology

1. Strains and chemicals

Araldite LY 1568 structure was provided by Aernnova Aerospace, S.A; 1-phenoxy-2-propanol and Taq polymerase were purchased from Sigma-Aldrich (Madrid, Spain). Restriction enzymes BamHI and XhoI were acquired from New England BioLabs (Hertfordshire, United Kingdom). *Escherichia coli* strain XL1-Blue competent cells, GeneMorph II mutazyme polymerase and iProof high-fidelity DNA polymerase were purchased from Agilent (Santa Clara, CA, United States). Oligonucleotide primers were acquired from Integrated DNA Technologies (Coralville, IA, United States). NucleoSpin plasmid, NucleoSpin gel and PCR clean-up kits were obtained from Macherey-Nagel (Düren, Germany). The episomal shuttle vector pJRoC30 came from the California Institute of Technology (Caltech) and protease-deficient *Saccharomyces cerevisiae* strain BJ5465 (MATa ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prbΔ1.6R can 1 GAL) was purchased from LGC Prochem (Barcelona, Spain). ABTS [2,29-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] was purchased from Panreac AppliChem (Darmstadt, Germany). DMP (2,6-dimethoxyphenol) was purchased from TCI Europe (Zwijndrecht, Belgium). Veratryl alcohol and H_2O_2 were purchased from Merck Life Science (Madrid, Spain). Evolved UPO mutant GroGu was prepared as reported in Gomez de Santos et al., 2021. All chemicals and medium components were reagent-grade purity.

2. Culture Media

Table 1. *Synthetic Complete (SC) medium composition for yeast growth.*

Component	For 1L
Raffinose (20%)	100 mL
Yeast Synthetic Drop-out Medium Supplements (without uracil)	100 mL
Yeast Nitrogen Base (YNB) (67 g/L)	100 mL
***dd*H_2O (sterile)**	700 mL
Chloramphenicol (25 g/L)	1 mL

- For SC agar, first 20 g of agar were added to 700 mL *dd*H_2O and autoclaved. Then, the remaining components were combined (previously sterilized by filtration).

Table 2. *YP (yeast peptone) (2x) medium composition.*

Component	For 1 L
Bactopeptone	40 g
Bacto Yeast Extract	20 g
***dd*H_2O**	1 L

- The medium was then sterilized in an autoclave.

Table 3. *Yeast expression medium composition for UPOs.*

Component	For 1 L
YP (2x)	500 mL
Galactose (20%)	111 mL
1 M KH_2PO_4 pH 6.0)	67 mL
$MgSO_4$	22 mL
Ethanol (100%)	31.6 mL
***dd*H_2O (sterile)**	Bring to 1L
Chloramphenicol (25 g/L)	1 mL

Table 4. *YPD (yeast peptone dextrose) medium composition.*

Component	For 1 L
YP (2x)	500 mL
Glucose (20%)	100 mL
***dd*H_2O (sterile)**	400 mL
Chloramphenicol (25 g/L)	1 mL

- For YPD agar, YP and 20 g of agar were added to 400 mL *dd*H_2O and autoclaved. The remaining components were then added in sterile conditions.

Table 5. *LB (Luria Bertani) medium composition.*

Component	For 1 L
LB Broth	25 g
***dd*H_2O (sterile)**	1 L
Chloramphenicol (25 g/L)	1 mL

- For LB agar, 20 g of agar were autoclaved in water and then chloramphenicol was added to avoid degradation.

* For further information on culture media see **Link S1**.

3. Directed evolution

Every directed evolution campaign follows the fashion depicted in **Figure 1**. As mentioned before, in this study GroGu variant was used as parental type. Initially, error-prone PCR was used as the mutagenic method to promote random mutagenesis. The resulting mutant library was cloned alongside episomal shuttle vector pJRoC30 in the host organism *S. cerevisiae* for in vivo reassembly and DNA shuffling. Individual colonies (clones) were then picked and expressed in a microtiter scale. Finally, the different variants were screened towards the property of interest, in our case, hydroxylation of 1-phenoxy-2-propanol (1Phe), and winner variants were selected. Furthermore, mutant winners can be subjected to successive directed evolution cycles, accumulating and combining beneficial mutations generation after generation.

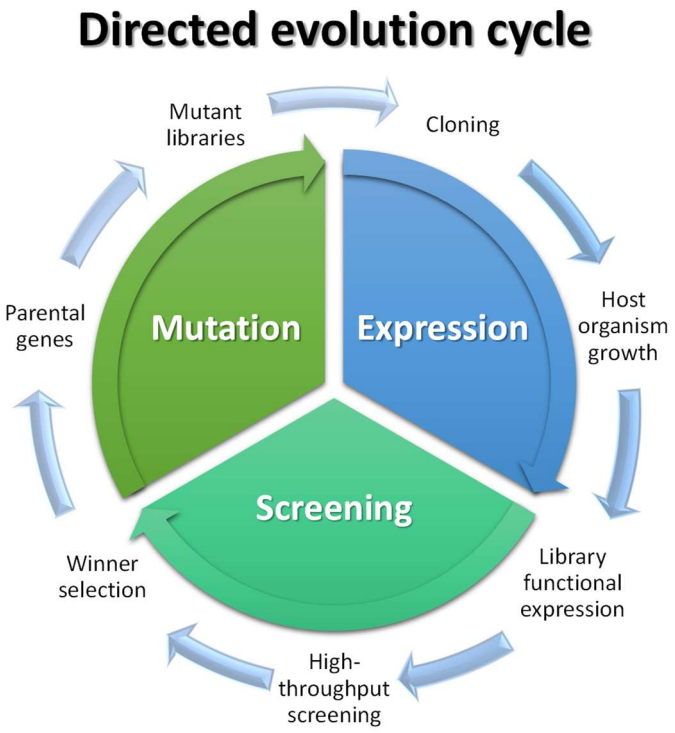


Figure 1. *Directed evolution workflow.*

4. pJRoC30 plasmid linearization

For plasmid linearization, digestion with restriction enzymes BamHI HF (20 U/µL) and XhoI (20 U/µL) was carried out for 1 h 30 minutes at 37 ^{o}C (**Table A1**), followed by isolation using agarose gel electrophoresis for 1 h at 70 V. For reference, 1Kb ladder was also charged along with samples. Subsequent gel cutting on UV light transilluminator (Benchtop UV Transilluminator, UVP LLC, CA) and DNA extraction rendered functional transformation plasmid (Nucleospin Gel and PCR Clean-Up Kit by Macherey-Nagel used for gel DNA purification, protocol in **Document S2**).

5. Mutant library construction

- Mutagenic landscapes generation

Six independent mutant libraries were built by error-prone PCR and further recombined *in vivo* by DNA shuffling. Library creation was performed by error prone PCR using GeneMorph II kit (Stratagene, 2003) (GM-PCR) (**Table A2**) and *Taq* Polymerase (*Taq*PCR). Diverse mutational rates were achieved modifying the amount of PCR cycles and the concentration of $MnCl_2$ respectively, employing these methods as mutagenesis inducers on parental GroGu sequence.

Regarding GM-PCR, the PCR mixture was prepared in 50 µL final volume using dimethyl sulfoxide (DMSO) (3%), deoxynucleotide triphosphates (dNTPs) (0.08 mM; 0.2mM each), GeneMorph II polymerase (0.05 U/µL), GroGu template DNA (56.12 ng/µL) and corresponding primers (**Table 6**). Two different conditions were applied: 20 cycles and 30 cycles, increasing mutagenesis for higher cycle procedures (20-cycle estimate mutation frequency: 3 – 6 m/Kb; 30-cycle estimate mutation frequency: 4.5 – 9 m/Kb).

In relation to *Taq*PCR, the PCR mixture contained in a 50 µL final volume: DMSO (3%), $MgCl_2$ (1.5 mM), deoxynucleotide triphosphates (dNTPs) (0.3 mM; 0.075 mM each), $MnCl_2$ (0.005, 0.010, 0.015, 0.10, 0.15 mM; from lower to higher mutational rates) Taq polymerase (0.05 U/µL), the template *GroGu* (10 ng) and corresponding primers (**Table 6**).

Table 6. *Sequences of primers employed for the different mutant libraries.*[1]

Library	Construction	Primer	Primer Sequences (5'→3')[1]
GM-30 & *Taq*PCR	**A**	**RMLN**	5' – CCTCTATACTTTAACGTCAAGG – 3'
		RMLC	5' – GGGAGGGCGTGAATGTAAGC – 3'
Sat184	**B**	**RMLN**	5' – CCTCTATACTTTAACGTCAAGG – 3'
		Sat184Rv	5'–GTGTTTGATTGAAGTTGTGMNNATCGCCGAAAAAAAAGTCAG– 3'
	B'	**Sat184Fw**	5'–CTGACTTTTTTTTCGGCGATNNKCACAACTTCAATCAAACACT–3'
		RMLC	5' – GGGAGGGCGTGAATGTAAGC – 3'
Sat174	**C**	**RMLN**	5' – CCTCTATACTTTAACGTCAAGG – 3'
		Sat174Rv	5'– GAAAAAAAAGTCAGCTCTTGTMNNGCTAGCGTCACCCTCGAAGAC –3'
	C'	**Sat174Fw**	5'–GTCTTCGAGGGTGACGCTAGCNNKACAAGAGCTGACTTTTTTTTC–3'
		RMLC	5' – GGGAGGGCGTGAATGTAAGC – 3'

[1]Overlapping areas for *in vivo* cloning gap repair (*S. cerevisiae*) shown in blue. Degenerated codons coding for all residues shown in green.

Both PCRs were performed on a gradient thermocycler (MyCycler, Bio-Rad, United States) using their corresponding conditions (**Table 7** & **Table 8**).

Table 7. *PCR conditions for GM-PCR.*

Process	Temperature	Time	
Initial	95°C	2 min	
Denaturalization	95°C	30 sec	20/30 cycles
Annealing	50°C	30 sec	
Extension	72°C	1 min	
Final extension	72°C	10 min	
End	4°C	∞	

Table 8. *PCR conditions for* Taq*PCR.*

Process	Temperature	Time	
Initial	95˚C	2 min	
Denaturalization	95˚C	45 sec	30 cycles
Annealing	50˚C	45 sec	
Extension	74˚C	1 min	
Final extension	74˚C	10 min	
End	4˚C	∞	

- 31F12 Site saturation mutagenesis (Sat184PCR)

Site saturation mutagenesis was performed on residue Ile184 of the first generation mutant 31F12 using degenerated forward NNK (N = A/T/C/G; K= G/T) and reversed MNN codons as seen in **Table 6**. PCR mixture included: the PCR blend contained in a 50 µL final volume, DMSO (3%), deoxynucleotide triphosphates (dNTPs) (0.8 mM; 0.2 mM each), iProof high-fidelity DNA polymerase (0.02 U/µL), the template 31F12 (10 ng) and the following primers depending on the PCR (**Table 6**). PCRs were performed on a gradient thermocycler using the parameters in **Table 9.**

- 4AC Site saturation mutagenesis (Sat174PCR)

Site saturation mutagenesis was performed on residue Val174 of the first generation mutant 4AC using degenerated forward NNK (N = A/T/C/G; K= G/T) and reversed MNN codons as seen in **Table 6**. PCR mixture (50 µL) included: DMSO (3%), deoxynucleotide triphosphates (dNTPs) (0.8 mM; 0.2 mM each), iProof high-fidelity DNA polymerase (0.02 U/µL), the template 4AC (10 ng) and the following primers depending on the PCR (**Table 6**). PCRs were performed on a gradient thermocycler using the parameters in **Table 9.**

Table 9. *PCR conditions for Sat184PCR & Sat174PCR.*

Process	Temperature	Time	
Initial	98°C	30 sec	
Denaturalization	98°C	10 sec	30 cycles
Annealing	54°C	30 sec	
Extension	72°C	1 min	
Final extension	72°C	10 min	
End	4°C	∞	

PCR products were then isolated by electrophoresis and purified in same conditions as pJRoC30 linearization.

6. *S. cerevisiae* Transformation

Following Sigma-Aldrich's protocol, first *S. cerevisiae* strain BJ5465 was made competent by inoculating yeast from YPD plates (**Table 4**) into YPD in a 100 mL flask. After *ON* (overnight) growth and OD_{600} check, it was diluted so OD_{600} was around 0.3 and grown until it reached 1.2. Following its harvest, wash and resuspension in Transformation Buffer (Product Code T0809), *S. cerevisiae* cells were ready to use (see **Table A4** for concrete protocol). Then, for transformation, 10 mg/mL salmon testes DNA (Product Code D9156) (see also **Link S3**), 100 ng yeast plasmid DNA and mutated GroGu, competent cells, PLATE Buffer (Product Code P8966) (see also **Link S4**) and DMSO to 10% were mixed together in a microcentrifuge tube for heat shock in a water bath or heat block. Afterwards, transformed cells were plated on SC agar plates (**Table 1**) and incubated at 30 ^{o}C for 2-3 days until colonies appeared (see **Table A5** for specific protocol).

7. Microplate expression of Unspecific Peroxygenases (UPOs)

For recombinant clone analysis, individual clones from SC agar plates were picked into microplate following the pattern shown on **Figure 2**. First, 50 µL of SC medium were added to each well. Then, using a toothpick, a colony was picked and placed on a well. In each plate, column 6 was inoculated with GroGu as parental control, and well H1 was inoculated with negative control (*S. cerevisiae* cells not expressing peroxygenase). Plates were sealed to prevent evaporation and incubated at 30 ^{o}C and 220 rpm with 80% relative humidity in a humidity shaker (Minitron-Infors, Biogen, Spain). After 48 h, 150 µL of UPO expression medium (**Table 3**) were added to each well, followed by culture for an additional 48 h at 25 ^{o}C.

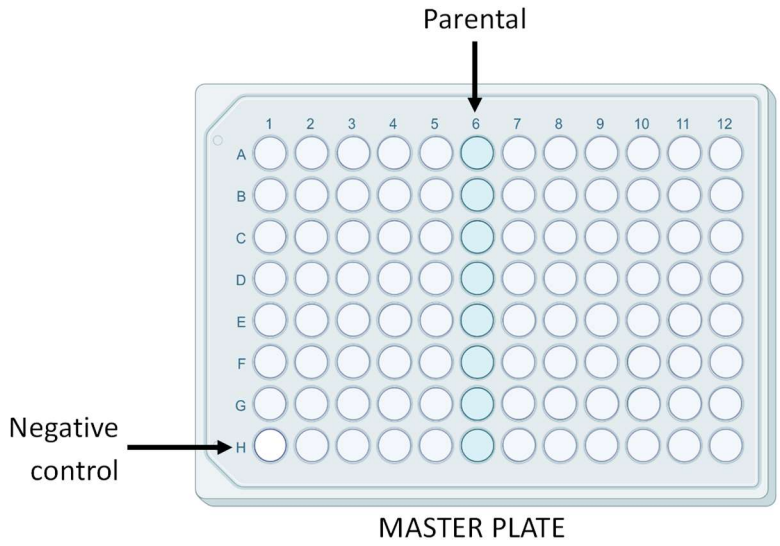


Figure 2. *Plate-picking pattern on a Half-Deep-Well plate (HDW).*

8. High-Throughput Screening (HTS) Assay

Before analysis, plates were centrifuged for 10 min at 4 ^{o}C and 2500 rpm. 20 µL of supernatant was then removed with the help of a liquid handler (Freedom Evoware 150 MCA, Tecan, Switzerland) and transferred to new standard 96 well plates (activity plate) where they were screened for the activity of interest.

- 1-phenoxy-2-propanol (1Phe) activity measurements

For enzymatic reaction, reaction mix is prepared following the formula described on **Table 10**.

Table 10. *1Phe reaction mix.*

Component	Volume per well (µL)
1 M KH_2PO_4 buffer pH 7.0	3.5 µL
20 mM H_2O_2	7 µL
500 mM 1-phenoxy-2-propanol	0.7 µL
***dd*H_2O**	38.8 µL

For colorimetric reaction (**Figure 3**), 50 µL of sodium hydroxide (NaOH), 4-aminoantipyrine (4-AAP) and potassium persulfate (PSK) were added to each well.

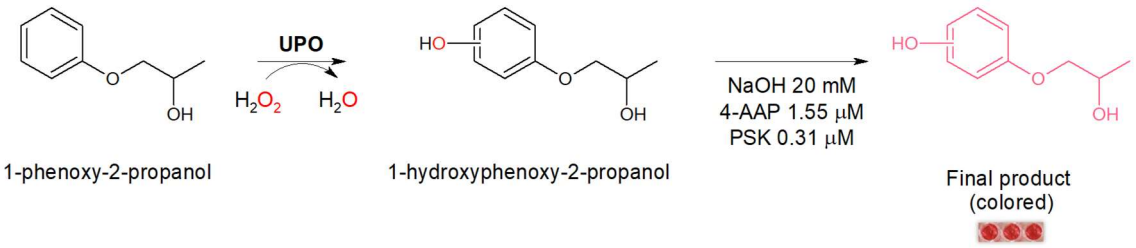


Figure 3. *Colorimetric screening assay to detect 1Phe model molecule. UPO used was GroGu, which hydroxylates 1Phe, allowing 4-aminoantipyrine (4-AAP) to bind, providing a colored compound measurable by endpoint at 500 nm. Developing process consists on adding 50 µL of NaOH 20 mM, 4-AAP 1.55 µM and PSK 0.31 µM sequentially, waiting 2 minutes after adding 4-AAP and 10 minutes after PSK.*

For screening, the absorbance was measured in endpoint at 500 nm. Increase in activity is directly proportional to absorbance.

- Veratryl alcohol (VA) activity measurements

Reaction mix was prepared following the formula described on **Table 11**.

Table 11. *VA reaction mix.*

Component	Volume per well (µL)
1 M KH_2PO_4 buffer pH 6.0	20 µL
20 mM H_2O_2	20 µL
50 mM Veratryl alcohol	20 µL
***dd*H_2O**	120 µL

For screening (**Figure 4**), the absorbance was measured in endpoint or kinetic mode at 310 nm. Activity is directly proportional to absorbance increment.

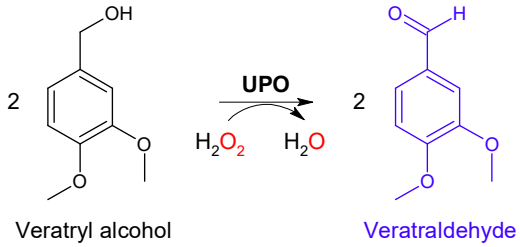


Figure 4. *Colorimetric screening assay for VA.*

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

Reaction mix was prepared following the formula described on **Table 12.**

Table 122. *ABTS reaction mix.*

Component	Volume per well (µL)
500 mM KH_2PO_4 buffer pH	40 µL
20 mM H_2O_2	10 µL
40 mM ABTS	10 µL
***dd*H_2O**	120 µL

For screening (**Figure 5**), the absorbance was measured in kinetic mode at 418 nm. Increase in activity is directly proportional to absorbance.

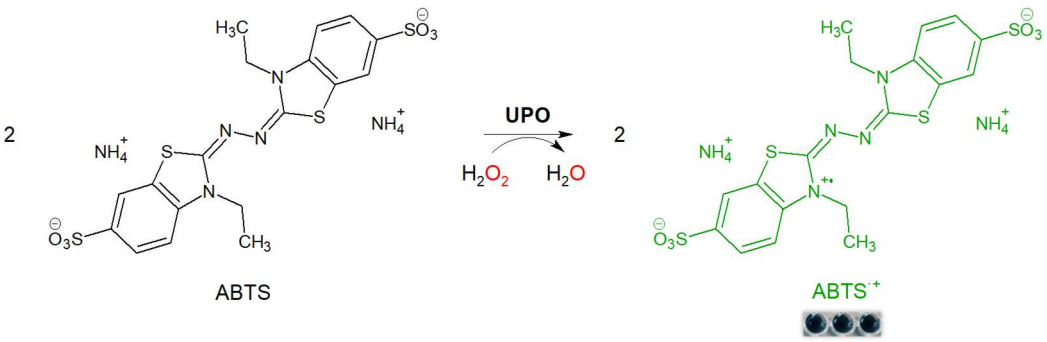


Figure 5. *Colorimetric assay for ABTS.*

- 2,6-dimethoxyphenol (DMP) activity measurements

Reaction mix was prepared following the formula described on **Table 13**.

Table 133. *DMP reaction mix.*

Component	Per well (50 µL)
1 M KH_2PO_4 buffer pH 6.0	20 µL
20 mM H_2O_2	10 µL
40 mM DMP	10 µL
***dd*H_2O**	140 µL

For screening (**Figure 6**), absorbance was measured in kinetic mode at 469 nm. Increase in activity is directly proportional to absorbance.

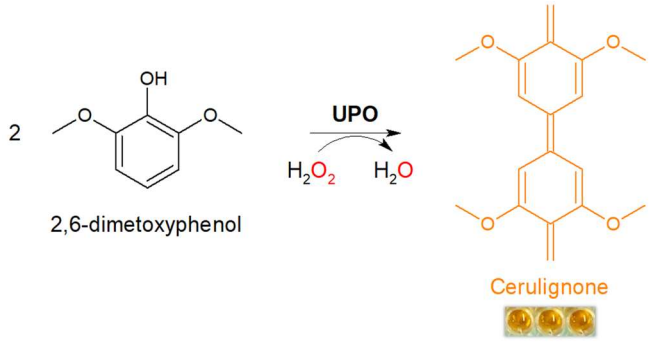


Figure 6. *Colorimetric screening assay of DMP substrate.*

In order to rule out the selection of false positives, two consecutive re-screenings were carried out:

- **1st re-screening**: Aliquots of 5 µL of the best clones of the screening were transferred to new sterile 96 half-deep-well plates with 50 µL of SC medium per well. Columns 1 and 12 plus rows A and H were not used to prevent the appearance of false positives (**Figure 7**). After 24 h of incubation at 30 ºC and 220 rpm, 5 µL were transferred to the adjacent wells and further incubated for 24 h. Finally, 150 µL of expression medium were added and plates were incubated for 48 h at 25 ºC. Parental type went through the same process. Finally, microplates were screened according to the HTS described above.

- **2nd re-screening:** An aliquot from the best clones from the first re-screening was inoculated in 3 ml YPD medium and incubated at 30 ^{o}C for 24 h at 220 rpm. The plasmids from these cultures were isolated and purified with Zymoprep yeast plasmid miniprep kit I. For further purification and higher plasmid yields, the ZymoPrep product was transformed into *E. coli* XL1-Blue cells following Agilent transformation protocol (**Document S7**) and plated onto LB-ampicillin (LB-amp) plates which were incubated at 37 ^{o}C *ON*. Single colonies were selected to inoculate 5 ml of LB-amp medium, and incubated overnight at 37 ^{o}C and 220 rpm. For plasmid isolation and purification in *E. coli*, Nucleospin Plasmid kit was used (protocol in **Document S6**) and transformed into *S. cerevisiae*. Finally, ten colonies of each mutant are picked according to **Figure 7** and screened as described above.

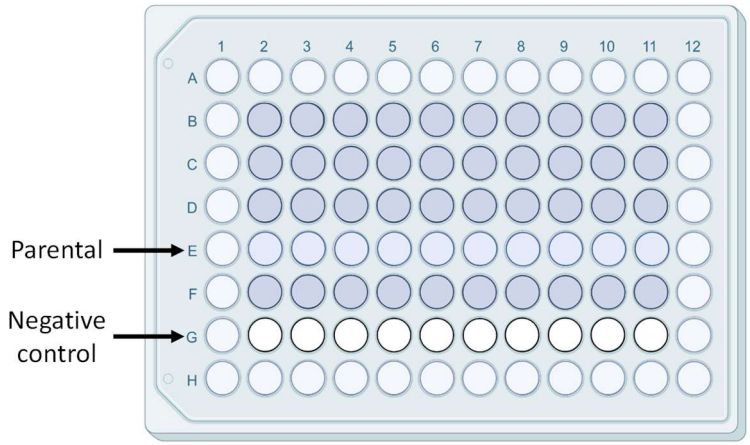


Figure 7. *First and second rescreening picking pattern on HDW.*

9. Linearity and Variation coefficient

To determine the linearity of the assay, 0 µL, 5 µL, 10 µL, 15 µL & 20 µL of fresh GroGu supernatants were incubated with 5 mM 1Phe and 2 mM H_2O_2 in 50 mM KPi buffer (10 mM Potassium Phosphate and 100 mM NaCl) (pH 7.0), completing with *dd*H_2O to a final volume of 70 µL. Screening proceeded as described earlier. Each point and standard deviation came from three independent measurements. The CV (coefficient of variation)

was determined in *S. cerevisiae* cells transformed with pJRoC30-GroGu and plated on SC dropout plates. Individual colonies were picked and inoculated in a 96 well plate and the activity of the clones was evaluated in fresh supernatant preparations.

10. Mutant characterization

- pH activity profile:

For 31F12 pH profiles with 1Phe, VA, ABTS and DMP, substrate mixes were prepared as described previously except for buffer addition. KPi and citrate/phosphate buffers were prepared ranging from pH 6-10 and 2-6 respectively, and 11 reactions were carried out at increasing pH. GroGu was used as positive control, and aryl alcohol oxidase (AAO) and laccase from *Moniliophtora roreri* (LacRo) were used as negative controls.

- Flask expression:

Flask expression of 31F12 was performed on 100 mL glass Erlenmeyer flasks with and without 0.03% ethanol in order to detect potential ethanol inhibition or contribution to 31F12 expression. First, a colony with 31F12 plasmid was grown on SC medium for 2 days at 30 ^{o}C with agitation, following by 25 ^{o}C expression on Yeast Expression Medium for two additional days. OD_{600} measures were taken along the process and final screening with 1Phe was performed following previously-mentioned directions.

4. Results and Discussion

Amongst the many resins studied in BIZENTE project, this thesis targets degradation of epoxy resin Araldite LY 1568 particularly. This resin is one of the most commonly used ones, due to its properties and accessibility. It is Bisphenol A (BPA)-based, forming a web of linear structures when exposed to amine hardener Aradur 3489 (2, 14). Among its many advantages, the ones that stand out are low viscosity, high temperature resistance, and good stiffness and strength-to-weight ratios (15). The complexity and high hydrophobicity of the epoxy resin hinders its direct use in a directed evolution experiment. Consequently, it was decided to employ a smaller water-soluble scaffold that exhibits the key structural motifs of the resin. After a thoughtful study, 1-phenoxy-2-propanol (1Phe) was appointed as a model molecule to be applied in the development of a HTS assay (**Figure 8**). Preliminary studies conducted by GC-MS revealed the hydroxylation of 1Phe by UPO (*manuscript in preparation*). Even though the scaffold substrate was transformed by hydroxylation instead of O-dealkylation, this modification will increase the hydrophilicity of the epoxy resin and therefore its degradability in combination with other approaches, such as laccase combined reaction. This feedback benefits from the formation of phenolic motifs which increases laccase and/or laccase mediator system attacks, as occurs in natural laccase degradation of lignin's phenolic fraction (16). Accordingly, the colorimetric HTS assay was developed in order to detect aromatic hydroxylation based on the 4-AAP method: a specific reagent that reacts with ortho- and para- hydroxylated phenols to develop a stable colored complex that can be monitored at 500 nm (**Figure 3**) (17).

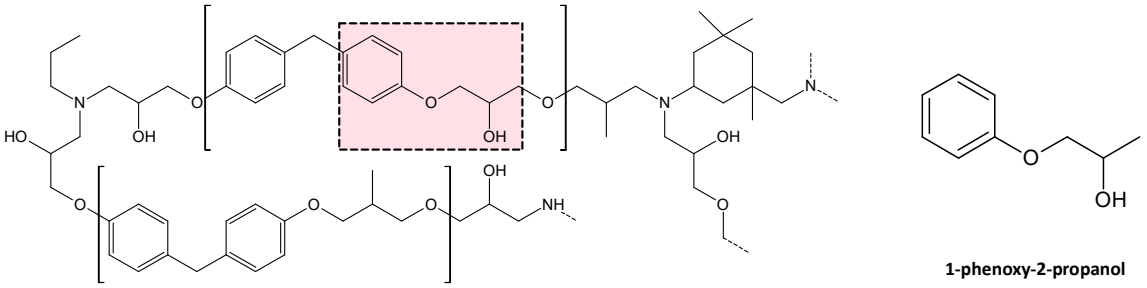


Figure 8. *Structural composition of Araldite LY 1568 and the model molecule 1-phenoxy-2-propanol (1Phe). On the left, the chemical structure of Araldite LY 1568 is represented, and the structural motif of interest is highlighted in pink. On the right the structure of model 1Phe compound is illustrated.*

1. High-throughput Screening assay validation

The identification of improve variants through directed evolution relies on the robustness and accuracy of the HTS assay. To validate the assay, the linearity and coefficient of variation (CV) was determined using fresh transformants from microtiter fermentations (**Figure 9**).

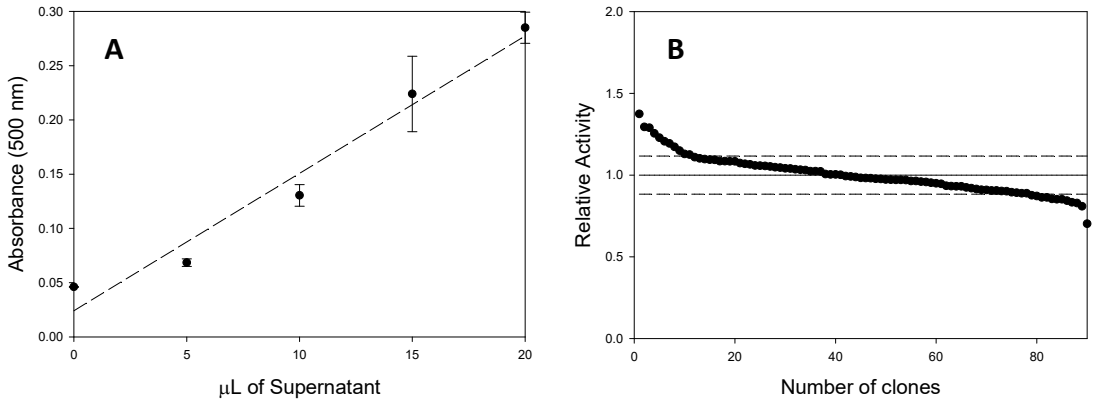


Figure 9. *Validation of the colorimetric assay. The activities are plotted in descending order, dashed lines depict the CV of the assay. Each point and standard deviation came from three independent measurements.*

The CV of the assay (12%) was an acceptable value to explore mutant libraries and linearity was established successfully up to 20 µL of supernatant as detected by absorbance. Once the HTS assay was established, it was used to analyze a small subset of mutant libraries (300 clones each). For the creation of mutant library constructions, two routes were explored to determine best library rendering. On one hand, 20-cycle (low mutagenic rate) and 30-cycle (high mutagenic rate) libraries were made with GeneMorph II Kit. On the other hand, Taq Polymerase libraries with increasing concentrations of $MnCl_2$ were prepared: low mutagenic rates (0.005 mM $MnCl_2$), medium mutagenic rates (0.01 mM $MnCl_2$), high mutagenic rates (0.05 mM $MnCl_2$) and very high mutagenic rates (0.1, 0.15 mM $MnCl_2$) (**Figure 10** & **Table 14**).

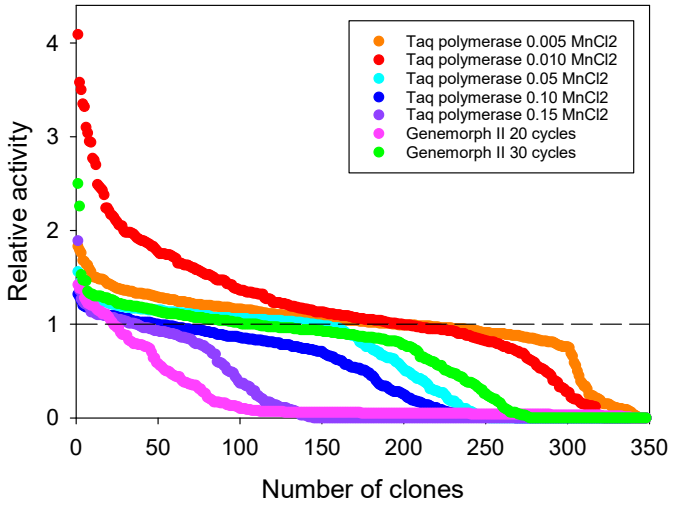


Figure 10. *GroGu mutagenic landscapes for the aromatic hydroxylation of the 1Phe model scaffold. Parental GroGu activity in the experiment is represented by the dashed horizontal line. Individual clones' activities are plotted from highest to lowest, according to 7 different mutagenesis conditions: orange circles correspond to Condition I (Taq polymerase, 0.005 mM $MnCl_2$); red circles, Condition II (Taq polymerase, 0.010 mM $MnCl_2$); light blue circles, Condition III (Taq polymerase, 0.05 mM $MnCl_2$); dark blue circles, Condition IV (Taq polymerase, 0.010 mM $MnCl_2$); purple circles, Condition V (Taq polymerase, 0.15 mM $MnCl_2$); pink circles, Condition VI (GeneMorph II, 30 cycles); and green circles, Condition VII (GeneMorph II, 20 cycles).*

Table 144. *Mutagenic conditions for mutant library construction.*

Library	Polymerase	$MnCl_2$ Concentration (mM)	No. of cycles	Inactive Clones (%)[1]
1	Taq polymerase	0.005	30	4.31
2	Taq polymerase	0.010	30	8.91
3	Taq polymerase	0.05	30	31.61
4	Taq polymerase	0.10	30	36.78
5	Taq polymerase	0.15	30	62.93
6	Mutazyme II	---	20	70.40
7	Mutazyme II	---	30	24.71

[1]Inactive clones are those that have less than 10% of the parental type's activity.

2. Mutant library analysis

- First generation: error-prone PCR

During the revision of the different libraries, two mutants from 30-cycle GeneMorph II (31F12 (N184I) and 4AC (M174V)) library displayed outstanding activity levels and therefore were selected for analysis. However, typically best libraries are characterized by a 30-50% dead clone ratio, considering dead clones those that have less than 10% parental activity. These conditions mostly ensure that the gene of interest (GOI) is being significantly mutated, and thus, avoiding to an extent silent or mild mutations to achieve best activity yields (**Figure 11**).

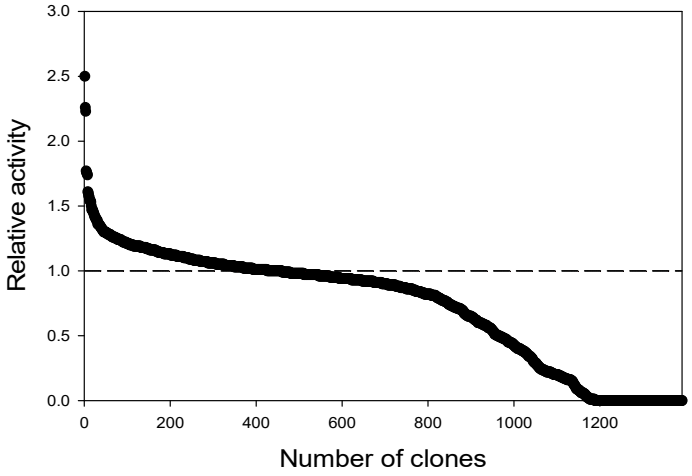


Figure 11. *Mutagenic landscape of 30-cycle GeneMorph II condition (GM-30) library. The activity of the clones is plotted in descending order; horizontal dashed line indicates the activity of parental type GroGu.*

After 1st and 2nd re-screenings were performed, it was possible to confirm that the two found variants with highest activity in relation to the parental type GroGu were indeed 31F12 and 4AC, which displayed a total activity improvement (TAI) of 1.7 & 1.6-fold, respectively (**Table 15**). In addition, to evaluate if activity boost is due to better enzyme expression or actual mutagenic effect, additional DMP and ABTS (peroxidase substrates) assays were performed, discovering an absence of activity for both substrates (**Figure 12**); proving that the identified variants were not expression mutants. Further assays described next were conducted to further study this activity shift. The variants were also tested with veratryl alcohol (VA), a very characteristic peroxygenase-activity substrate, and 31F12 was still active with detectable measurements of absorbance, although

showing activity decrease compared to parental GroGu. On the other hand, 4AC mutant still showed activity with ABTS and DMP, even though it was still much lesser than the parental's. VA activity levels were similar to 31F12's.

Table 15. *Winner selection in 1st screening of 30-cycle library.*

Variant	Library	Mutation	TAI
31F12	GM-30	$_{AAC}$N184I$_{ATC}$	1.7
4AC	GM-30	$_{ATG}$M174V$_{GTG}$	1.6
Parental GroGu			1

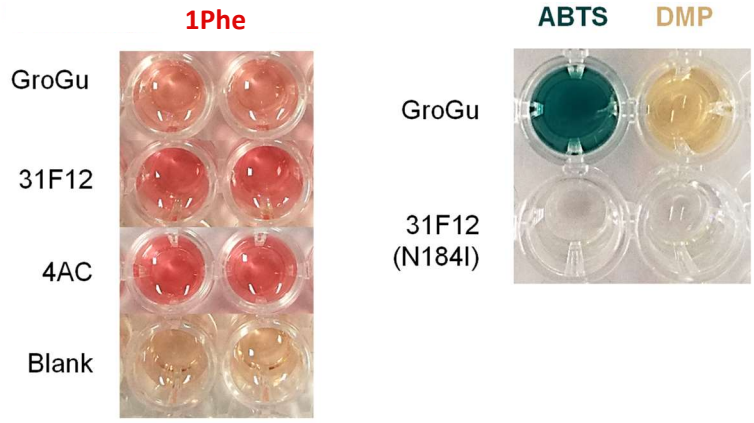


Figure 12. *Winner 31F12 and 4AC 1Phe activity enhancements (left). 31F12 ABTS and DMP activity loss compared to parental (right).*

- <u>31F12 variant characterization</u>

As stated previously, during the screening of the 31F12 variant an apparent loss of peroxidase activity was observed, at least regarding DMP and ABTS assays – both peroxidase substrates – which did not render any colorimetric response. However, 31F12 mutant shows a considerable increase in 1Phe peroxygenase activity in relation to GroGu, overcoming even 4AC's apparent peroxygenase activity. It was this peculiar characteristic, coupled with peroxygenase peak activity, that led to the further characterization of the mutant. Therefore, in order to determine what changes could provoke that sudden shift, ABTS and DMP pH activity profiles were determined to discern whether or not peroxidase activity still prevailed but was displaced to a higher or lower pH. Concurrently, to describe its peroxygenase activity, these experiments

were also performed employing the model compound, 1Phe, and a typical peroxygenase substrate as VA. As depicted in **Figure 13**, 31F12 shows a wider pH range for 1Phe compared to GroGu; although for VA and DMP, 31F12 displays a higher pH sensitivity (8, 18). On 1Phe, highest activity ranges between pH 7.0 and 10.0, with optimal pH switching from 7.0 to 8.0. With DMP, optimal pH is set at pH 8.0, varying the most from GroGu in pH 6.0 conditions, which cements the observed lack of peroxidase activity at evaluated pH 6.0 in DMP assays. Regarding VA, optimum pH activity is reported at pH 8, varying greatly from GroGu's, positioning optimal pH at 8.0 instead of the evaluated pH 6.0. Lastly, verification of ABTS lack of activity was confirmed, reporting no activity on the whole pH range.

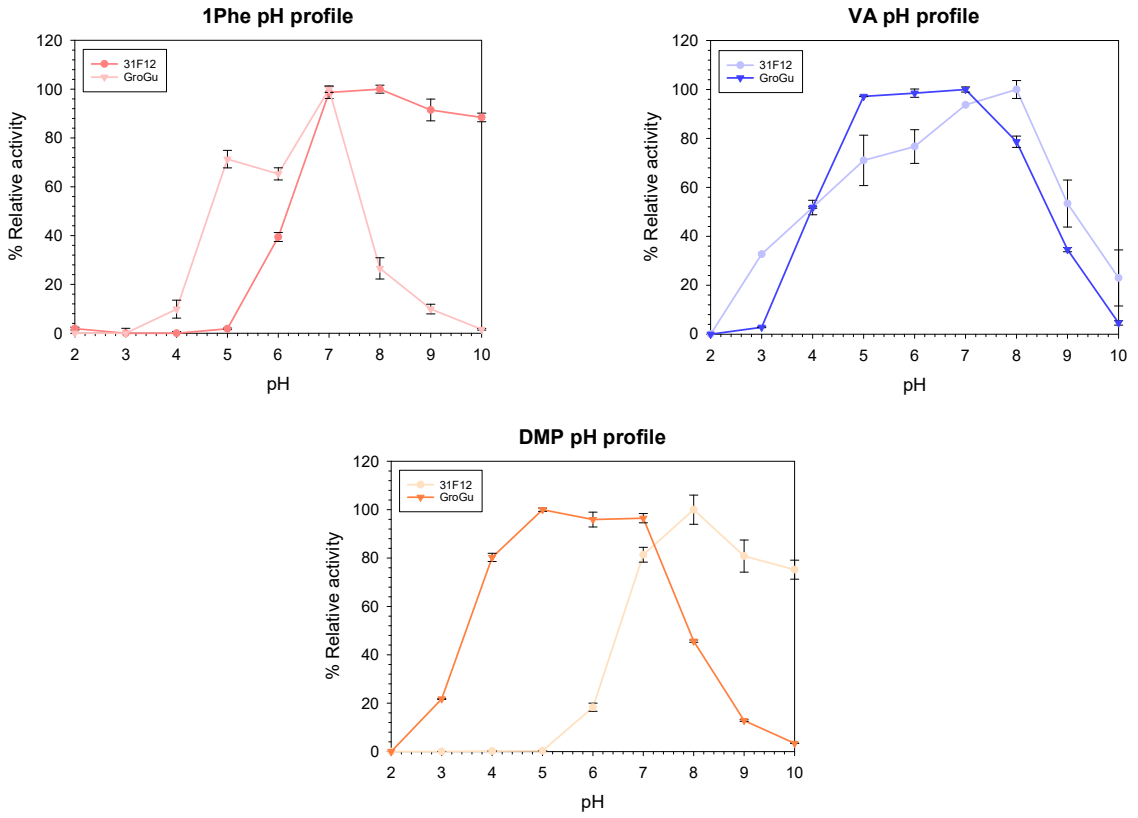


Figure 13. *pH activity profiles for 3 different substrates: DMP (orange); VA (violet) and 1Phe (pink). Citrate/phosphate (C/P) and KPi buffer were employed; AAO and LacRo were used as negative control and GroGu as positive control. Higher color intensity represents higher substrate activity. The mean and standard deviation of three independent experiments is calculated for each point.*

Additionally, 31F12 fermentation was evaluated in larger volumes (100 mL) to assess the best conditions for enzyme expression (**Figure 14**).

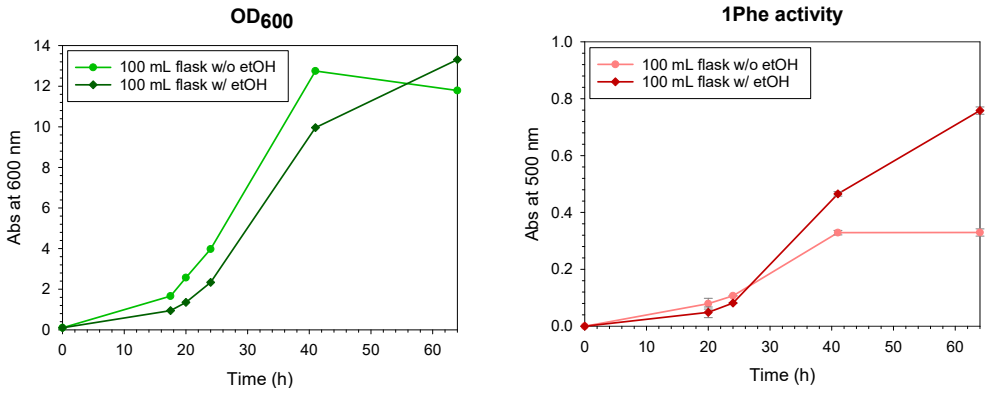


Figure 14. *Expression characterization. Cellular growth measured by optical density (OD) at 600 nm of* S. cerevisiae *heterologous system with and without ethanol (left). Enzymatic activity measured for 1Phe, measured by absorbance (Abs) observed at 500 nm (right).*

As was detected in the activity measurements, the optimal secretion time for the peroxygenase was set after 60 hours of fermentation. In addition, ethanol proved to contribute to enzymatic production with an increase of 2x activity.

- <u>Second generation: Site Saturation Mutagenesis (SSM)</u>

31F12 and 4AC were subjected to SSM to exhaust all possible residue options on both mutated positions, in order to seek highest viable activities and further understand their catalytic behavior. This approach proved to be interesting to both evaluate the importance of that position for activity improvement, and at the same time, to pursue the residues that yield the best conformation for that position.

In the case of the SSM for 31F12, two new mutants were spotted, 2A8 (Val184) and 1C9 (His184), with a TAI of 2.4 and 2.3, respectively (**Table 16**). These mutants, differed considerably from 31F12 parental, as described in detail next.

Table 16. *Winner selection in 1st screening of Sat184 (Sat31F12) library.*

Variant	Library	Residue	TAI
2A8	Sat184	$_{GTG}$Val184	2.4
1C9	Sat184	$_{CAT}$His184	2.3
31F12	GM-30	$_{ATC}$Ile184	1.7
Parental GroGu		$_{AAC}$Asn184	1

In relation to 4AC SSM, another mutant was identified with improvement in activity, 1E8 (Thr174), with a TAI of 1.7 and similar performance to the parental 4AC. (**Table 17**).

Table 17. *Winner selection in 1st screening of 30-cycle library.*

Variant	Library	Residue	TAI
1E8	Sat174	$_{ACT}$Thr174	1.7
4AC	GM-30	$_{GTG}$Val174	1.6
Parental GroGu		$_{ATG}$Met174	1

- <u>Final variants characterization</u>

The role of both UPO peroxygenase/peroxidase activities has been a key factor in the evolution experiments. To see a general picture, activities with ABTS and DMP were compared throughout variants. It is worth mentioning that 2nd generation mutants 2A8 and 1C9 recovered some DMP activity, although still none of the mutants showed activity with ABTS (**Figure 15**). This indicates that position 184 plays a key role in the peroxidase activity of the enzyme and depending on the nature of the residue this reactivity is indeed altered (**Table 18**).

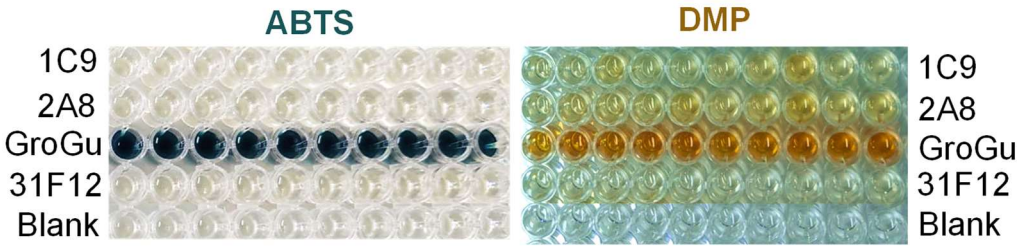


Figure 15. *Sat184PCR mutant winners (1C9 & 2A8), 31F12 and GroGu screened with ABTS (left) and DMP (right).*

Table 158. *Final mutants total activity improvement (TAI) respective to GroGu for each substrate. nm refers to non-measured data.*

Variant	Library	Residue	1Phe (TAI)	VA (TAI)	ABTS (TAI)	DMP (TAI)
2A8	Sat184	$_{GTG}$Val184	2.4	0.24	0.00	0.06
1C9	Sat184	$_{CAT}$His184	2.3	0.25	0.00	0.03
1E8	Sat174	$_{ACT}$Thr174	1.7	nm	nm	nm
31F12	GM-30	$_{ATC}$Ile184	1.7	0.10	0.00	0.00
4AC	GM-30	$_{GTG}$Val174	1.6	nm	nm	nm
GroGu		$_{ATG}$Met174	1	1	1	1

- Mapping mutations

Mutants obtained during the evolutionary campaigns were sequenced and structurally analyzed. In relation to 31F12's mutation, it appears to be around the Mg^{2+} binding site, an important structural determinant in peroxygenases. In the case of 4AC, the variant is mutated next to the heme channel. This represents a pivotal area for the catalysis of the enzyme because of the high interaction with the substrate as pathway to the active site. (**Figure 16**).

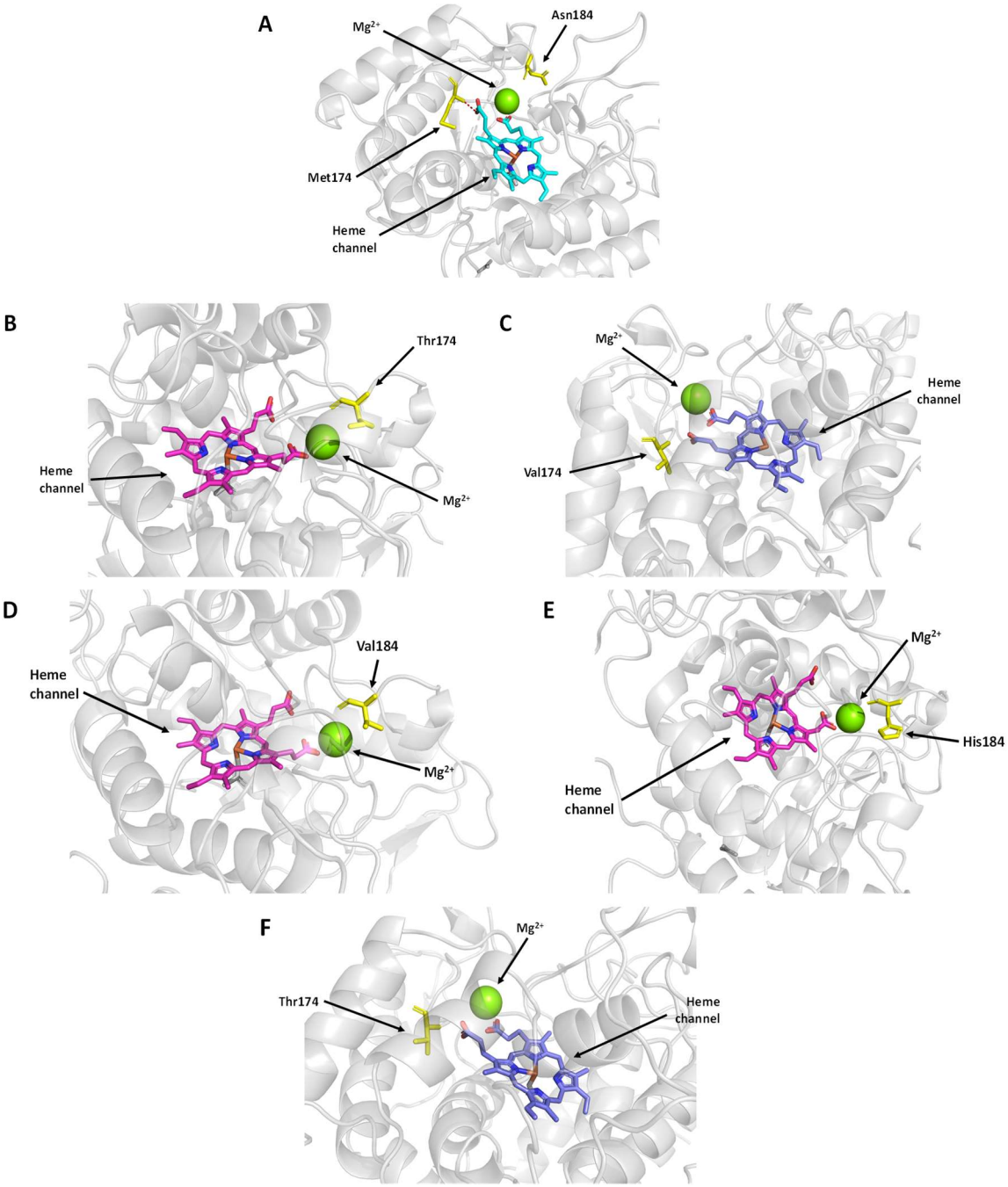


Figure 16. *Structural representations of GroGu and the winners obtained from random and Site Saturation mutagenesis, retrieved from Swiss Model Tool (https://swissmodel.expasy.org/) modeled based on PaDa-I crystallized (PDB: 6EKZ), sharing a 66.0% identity in 329 residues overlap; Score: 1143.0; Gap frequency: 0.6% (data recovered from SIM - Alignment Tool for protein sequences. https://web.expasy.org/sim/.* ***A.*** *GroGu model. On yellow: Met174, Asn184.* ***B.*** *Mutant 31F12 model. On yellow: Ile184.* ***C.*** *Mutant 4AC model. On yellow: Val174.* ***D.*** *Mutant 2A8 model. On yellow: Val184.* ***E.*** *Mutant 1C9 model. On yellow: His184.* ***F.*** *Mutant 1E8 model. On yellow: Thr174.*

5. Conclusions and perspectives

In the present study, a HTS assay towards the hydroxylation of the epoxy resin Araldite/Aradur was developed making use of the surrogate substrate 1Phe (**ta 17**). By means of directed evolution and the aforementioned screening platform, several enhanced variants were discovered and the most interesting were preliminary characterized. The future combination of the most synergic mutations should render improved mutants that will be studied and used in lab-scale degradation tests against epoxy resins.

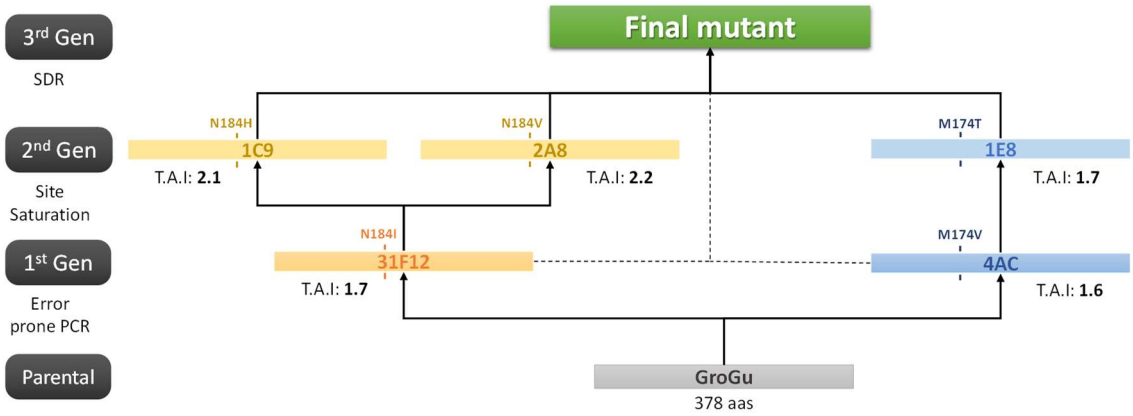


Figure 17. *Directed evolution campaign of GroGu towards improved 1Phe hydroxylation activity. Mutations are named and displayed as a perpendicular line. TAI represents the improvement in activity detected in* S. cerevisiae *cultures for each mutant in comparison with parental type GroGu. Arrows indicate the laboratory evolution workflow. For 2nd generation 31F12 & 4AC are the parental types. For 3rd generation Site-Directed Recombination, dashed arrows indicate all the parental types.*

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Sustainability contribution

In regards to the project's contribution to sustainability, its primary goal is to develop a feasible method for degrading resin polymers in an efficient and environmentally beneficial manner. As a result, the outcomes of this initiative presented a new way to try to undo some of the damage that had previously been difficult to tackle.

Supplementary Materials:

Link S1: Amino acid selection for recombinant strains and further information about media purposes at: *Introduction to Yeast Media (sigmaaldrich.com)* .

Document S2: Nucleospin Gel and PCR Clean-Up protocol: *Instruction-NucleoSpin-Gel-and-PCR-Clean-up.pdf (mn-net.com)* .

Link S3: For further information about salmon testes DNA and its use on hybridation go to: *Deoxyribonucleic acid, single stranded for hybridization 68938-01-2 (sigmaaldrich.com)* .

Link S4: Yeast Transformation Kit (Sigma-Aldrich), product description, components and use at: *Yeast Transformation Kit (sigmaaldrich.com)* .

Document S5: Zymoprep™ Yeast Plasmid Miniprep I protocol: *Zymoprep™ Yeast Plasmid Miniprep I (zymoresearch.com)* .

Document S6: Nucleospin Plasmid protocol: *Instruction-NucleoSpin-Plasmid.pdf (mn-net.com)* .

Document S7: *E. coli* transformation protocol*: Manual: XL1-Blue Competent Cells (chem-agilent.com)*

Annex

Table A1. *Pipetting scheme for plasmid digestion.*

Reagent	Volume (µL)	Total amount
Digestion buffer – NEBuffer CutSmart (10x)	5	1x
Plasmid pJRoC30 (X ng/µL)		≈ 10 ng
***Bam*H1 HF (20 U/µL)**	2	40 U
***XhoI* (20 U/µL)**	2	40 U
***dd*H_2O**	Bring to 50	-

Table A2. *GeneMorph II Random Mutagenesis pipetting scheme.*

Component	V (µL)	Final concentration
Buffer Mutazyme II (10x)	5	1x
DMSO (50%)	3	3%
40 mM dNTPs mix	1	0.8 mM
Template DNA	X (300 ng)	-
Primer RMLN	1.85	370 nM
Mutazyme II polymerase	1.85	370 nM
***dd*H_2O**	Bring to 50	-

Table A3. *Site Saturation Mutagenesis PCR formula.*

Component	V (µL)	Final concentration
Buffer (5x)	10	1x
DMSO (50%)	3	3%
40 mM dNTPs mix	1	0.8 mM
Template DNA (10 ng/µL)	1	0.2 ng/µL;10 ng
10 µM Primer RMLN	1.25	250 nM
10 µM OligoSatRev	1.25	250 nM
Polymerase (2U/µL)	0.5	0.02U/µL
***dd*H_2O**	Bring to 50	-

Component	V (µL)	Final concentration
Buffer (5x)	10	1x
DMSO (50%)	3	3%
40 mM dNTPs mix	1	0.8 mM
Template DNA (10 ng/µL)	1	0.2 ng/µL;10 ng
10 µM OligoSatFw	1.25	250 nM
10 µM Primer RMLC	1.25	250 nM
Polymerase (2U/µL)	0.5	0.02U/µL
***dd*H_2O**	Bring to 50	-

Table A4. *Protocol to achieve* S. cerevisiae *competent cells (from Sigma-Aldrich).*

Steps	V (mL)	Conditions
Inoculate yeast from YPD plates into YPD	20	Into 100 mL flask (sterile)
Grow		*ON* 30 ^{o}C
Dilute culture into YPD (initial OD_{600} should be >2)	100	OD_{600} ~ 0.3 500 mL flask (sterile)
Grow		3-6 h 30 ^{o}C
Harvest by centrifugation		RT 5 min
Discard supernatant		
Resuspend cells in water (sterile)	50	
Harvest by centrifugation		RT 5 min
Remove supernatant		
Resuspend cells in Transformation Buffer	1	
Add glycerol to 15%		
Store		-80 ^{o}C Indefinitely

Table A5. S. cerevisiae *transformation protocol (from Sigma-Aldrich).*

Steps	V (µL)	Conditions
Aliquot salmon testes DNA (10 mg/mL)	10	
Add plasmid and insert	X (100 ng)	
Add competent cells	80	
Vortex		~ 10 sec
Incubate		30 min 30 ºC
Add DMSO to 10%		
Heat shock		15 min
Spin		6-9 sec
Remove supernatant		
Resuspend cells in water	200	
Plate on SC plates	50	
Incubate face down		30 ºC