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A directed evolution platform to engineer fungal peroxygenases for the degradation of thermoset composite epoxy resins.

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1. BACKGROUND

Thermoset composites are known for their exceptional mechanical properties, along with their thermal and chemical resistance. Nevertheless, their inherent complexity limits their end-of-life management to incineration and landfill storage, exposing the need for environmentally-friendlier solutions [1].

Fig. 1 Current life cycle of epoxy resins.

BIZENTE project aims for the biodegradation of these complex plastics by the action of **oxidative enzymes as Unspecific peroxygenases (UPOs, EC 1.11.2.1)** which are known for their wide range of C-H oxyfunctionalization reactions [2] and are reported to degrade complex and recalcitrant structures as lignin [3].

The development of this novel technology emerges as a greener solution for the thermoset composites recycling from a **Circular Economy** perspective.

2. CASE OF STUDY

In this study, **Hexflow® RTM6** was selected as a representative epoxy resin as it is an aerospace qualified resin widely used as a thermoset composite. Due to the inherent complexity and high hydrophobicity of the resin, it cannot be used as a substrate to develop a **High-Throughput Screening** (HTS) assay so rather, we analyzed its structural arrangement to find a water-soluble scaffold that contains key resin motifs susceptible to enzymatic attack.

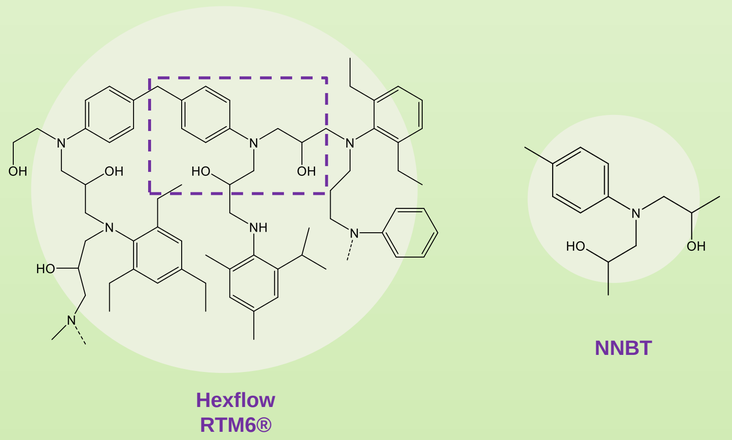

Fig. 2 Chemical structure of Hexflow RTM6® (left) and NNBT (right).

After careful examination, N,N-bis(2-hydroxypropyl)-p-toluidine (**NNBT**) was chosen as a potential substrate to design the HTS assay (**Figure 2**). UPO mutants from the short and long families were initially benchmarked to determine their potential N-dealkylation activity and, therefore, develop a **directed evolution platform** for their engineering.

3. METHODOLOGY

As witnessed by GC-MS, the cleavage of NNBT through the N-dealkylating activity of UPO releases a secondary aromatic amine and lactaldehyde. The latter was coupled to the reporter Purpald® for its colorimetric identification (**Figure 3A**).

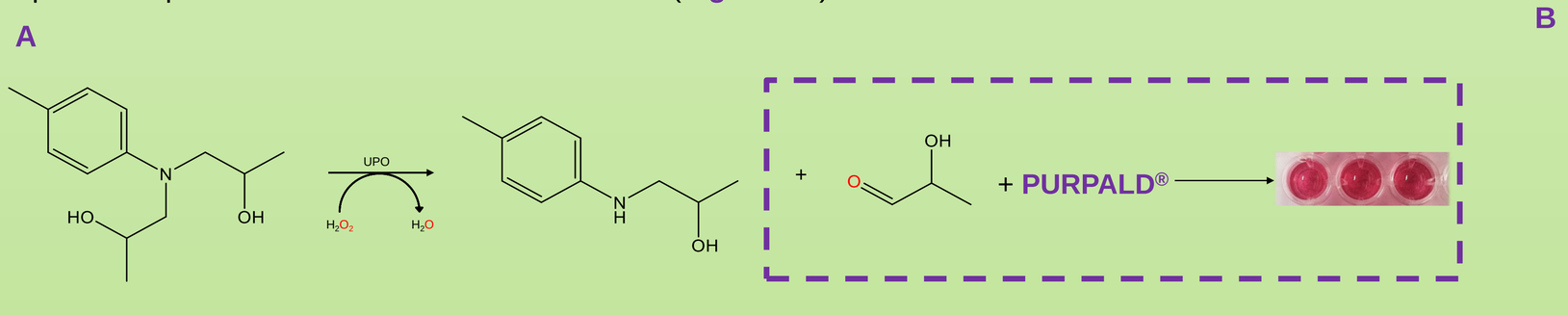

Fig. 3 Reaction scheme for the N-dealkylation of NNBT by UPO and the colorimetric assay to detect lactaldehyde (left). Validation of the HTS assay (right).

The screening method was then adapted to a 96-well microplate scale and validated determining its linearity and coefficient of variation (CV) (**Figure 3B**).

4. RESULTS

Finally, the HTS assay was then used to evaluate a small subset of mutant libraries (200 clones each) based on an evolved UPO from *Candolleomyces (Psathyrella) aberdarensis,* Grogu [4] and screened for NNBT N-dealkylation.

The mutagenic landscapes were constructed by error-prone PCR and in vivo shuffling and using polymerases with different mutational bias.

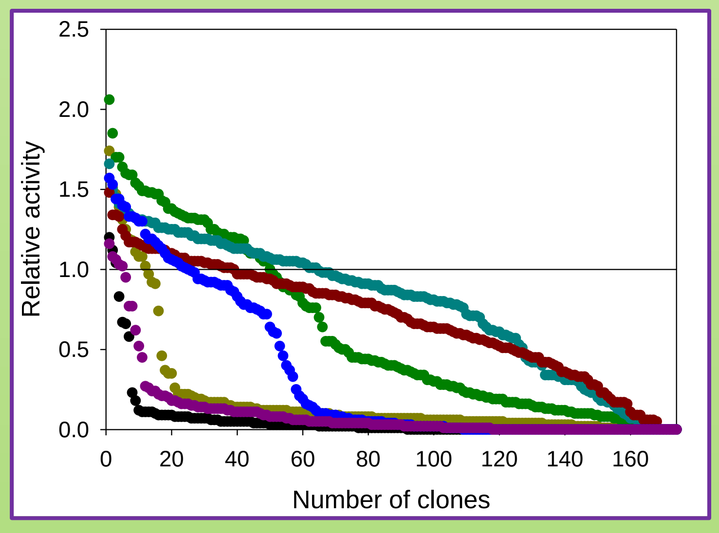

Fig. 4 Mutagenic landscapes of GroGu for the N-dealkylation of NNBT.

To achieve a broader assessment of the assay, we introduced different mutational loads which correlated with the aforementioned mutagenic landscapes.

The developed colorimetric HTS assay allowed us to select the best condition to proceed with directed evolution campaigns and to identify potential enhanced variants.

CONCLUSIONS

1. Using a model compound that included the main features of the epoxy resin Hexflow® RTM6, a colorimetric HTS assay was developed, and it was used to screen UPO mutant libraries constructed in and expressed by *S. cerevisiae.*

2. Given that activity improvements would be expected over the course of directed evolution, larger and more accurate model scaffolds could be synthesized and incorporated for study, thereby ensuring better focus on the whole polymer structure.

3. Thereby, the present work [1] paves the way for directed UPO evolution for epoxy resin biodegradation as well as to colorimetrically detect N-dealkylation activity on biological samples.

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