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Article

Enzyme Benchmarking with Polyethylene Furanoate Soluble Scaffolds for Directed Evolution of PEFases

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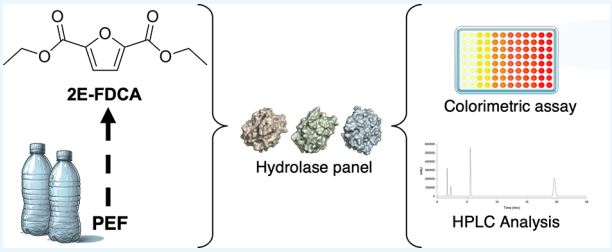
ABSTRACT: Plastic waste is a major threat in our industrialized world and is driving research into bioplastics. The success of biobased polyethylene furanoate (PEF) as a viable alternative to polyethylene terephthalate (PET) of fossil origin will depend on designing effective enzymes to break it down, aiding its recycling. Here, a panel of fungal and bacterial cutinases were functionally expressed in a tandem yeast expression system based on *Saccharomyces cerevisiae* and *Pichia pastoris*. The activity of the enzyme panel was tested with soluble PEF model scaffolds, observing a correlation with the degradation of real PEF powder. A high-throughput colorimetric screening assay based on the PEF scaffold diethyl furan-2,5-dicarboxylate was developed, establishing the basis for future directed evolution campaigns of PEFases.



INTRODUCTION

The development of sustainable materials is essential to mitigate current environmental challenges, particularly in relation to global plastics use. Among the emergent bioplastics that could replace those derived from fossil sources, polyethylene furanoate (PEF) is a clear frontrunner, as it has comparable physicochemical characteristics to the widely used polyethylene terephthalate (PET),[1] whose annual production surpassed the 75 million metric tons in 2015, becoming the second most produced plastic after polyethylene (PE).[2] Despite the fact that PEF surpasses PET in several environmental and technical aspects, PEF-based materials are not commercially available as of today, circumscribing its production to pilot plant scale at most. Unlike petroleum-based PET, PEF is produced through the copolymerization of 2,5-furandicarboxylic acid (FDCA) and monoethylene glycol, both of which are derived from renewable biomass.[2] PEF is roughly 30 times less permeable to CO_2 than PET and is a very resilient material that boasts high-performance melting and glass transition temperatures, features that contribute to its enhanced mechanical properties in various applications such as packaging and textile production.[3,4] However, the successful implementation of PEF as an alternative to PET depends not only on its renewable sourcing, but also on the establishment of a closed-loop circular economy. Despite being a biobased polymer, PEF biodegradation is not straightforward. Studies into its degradation have focused on industrial composting, conditions under which PEF is broken down after approximately 1 year. Nonetheless, there have been few environmental tests on PEF, and hence, little is known about its natural depolymerization. Accordingly, at present, the end-of-life management of PEF relies on chemical and mechanical recycling.[5,6]

Enzyme biotechnology may play a pivotal role in the comprehensive management of PEF.[7] Indeed, the use of depolymerizing enzymes could considerably relax the bottleneck in the degradation of this biopolymer, while allowing more precise control over the recovery of valuable by-products.[8] The few available studies into PEF degradation have predominantly focused on the use of cutinases (EC 3.1.1.74), a group of enzymes that catalyze the breakdown of cutin, a waxy biopolymer of plant cuticles. In particular, cutinases from *Humicola insolens* (HiC) and *Thermobifida cellulosilytica* (Thc_cut1) can degrade PEF both in amorphous materials and thin films with varying crystallinities.[9,10] PETase, an aromatic polyesterase from *Ideonella sakaiensis* (recently renamed as *Piscinibacter sakaiensis*) that is involved in PET depolymerization, was also shown to degrade PEF, as well as its related variant FAST PEtase.[11,12] Along these lines, the leaf and branch compost cutinase (LCC) has proved to degrade PEF with high efficacy.[12] While these enzymes show some promise in the degradation of PEF and its related bioplastics,

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fine-tuning their properties by protein engineering could convert them into more efficient industrial "*PEFases*".

Directed evolution currently represents the most efficient and versatile way to engineer proteins, permitting the rapid optimization of enzymes by mimicking natural evolution in a test tube and generating superior biocatalysts that dramatically outperform their natural counterparts. While significant endeavors have focused on the engineering of enzymes to meet the demanding operational conditions for PET degradation and recycling,[13−16] the field of PEF-degrading enzymes remains relatively unexplored. Here, we analyzed a variety of potential PEF depolymerases with a view to setup a directed evolution platform for PEF degradation. The panel of enzymes, comprising fungal and bacterial hydrolases, was expressed in the tandem yeast expression system (*Saccharomyces cerevisiae*/*Pichia pastoris*), benchmarking their functional expression and hydrolytic activity with different *p*-nitrophenol derivatives esters. The enzyme panel was tested with soluble PEF scaffolds, representative of structural motives of the polymer, as well as with real PEF powder, while a colorimetric high-throughput screening (HTS) assay for directed evolution was validated. Taken together, these results will enable us to perform laboratory evolution in a search for improved PEF depolymerases.

RESULTS AND DISCUSSION

Enzyme Panel and Initial Biochemical Characterization. The primary objective of this study was to validate a colorimetric HTS assay for enzymatic PEF degradation based on PEF soluble scaffolds. Accordingly, we opted to benchmark a diverse array of bacterial and fungal cutinases to showcase the broad applicability of this method to any enzyme with PEF degrading capabilities. Based on previous studies on plastic degradation, seven well-known hydrolytic enzymes were selected to form a panel of bacterial and fungal hydrolases,[9−11,12,17−25] Table 1 and Supplementary Table S1.

Table 1. Panel of Enzyme Candidates for PEF Degradation

enzyme	accesion number	origin	reported plastic degradation	refs
Fusarium solani cutinase (FsC)	P00590	fungal	PET, PCL, PBS	17−20
Cryptococcus sp. cutinase (CC)	Q874E9	fungal	PLA, PCL, PBS, PHB	21
Thielavia terrestris cutinase (TtC)	G2RAE6	fungal	PET, PCL, PBS	22
Humicola insolens cutinase (HiC)	A0A075B5G4	fungal	PET, PEF, PU-PE	9, 23, 24
PETase from *Ideonella sakaiensis* (PETase)	A0A0K8P6T7	bacterial	PET, PEF	11, 12
Thermobifida cellulosilytica cutinase 1 (ThC_cut1)	E9LVH8	bacterial	PET, PEF	10, 25
Leaf and Branch Compost cutinase (LCC)	G9BY57	bacterial	PCL, PET, PEF	12

Among the fungal hydrolases, we selected the cutinases from *Fusarium solani* (FsC), *Cryptococcus* sp. (CC), *Thielavia terrestris* (TtC), and *Humicola insolens* (HiC), which display a wide spectrum of plastic degradation activity. As bacterial hydrolases, we included a PETase from *Ideonella sakaiensis* (PETase), Leaf and Branch Compost cutinase (LCC), and cutinase 1 from *Thermobifida cellulosilytica* (ThC_cut1). Of this repertoire of enzymes, HiC, PETase, LCC, and ThC_cut1 have already shown PEF degrading activity.[9−11,12]

As described in previous studies with fungal ligninases[26] (and references therein), we introduced this set of enzymes into a tandem-yeast secretion system, based on *Saccharomyces cerevisiae* (a suited host for mutant library construction and laboratory evolution), and on *Komagataella phaffii* − *Pichia pastoris* − (appropriate host for overproduction in a fed-batch bioreactor). Among the main advantages of yeast production systems vs bacterial counterparts are the ease of secretion of foreign proteins to the extracellular medium together with sophisticated eukaryotic machinery to perform complex posttranslational modifications, including N and C-terminal processing and glycosylation. The latter is of special interest to provide recombinant enzymes with improved stability, albeit it may complicate biochemical characterization in those cases where hyperglycosylation and/or different glycoforms arise. After cloning the enzymes in our tandem-yeast secretion platform, the selected hydrolases were produced by *P. pastoris* and purified to homogeneity in order to benchmark the enzyme panel. Initially, an array of commercial *p*-nitrophenol derivatives was used to evaluate the general hydrolytic activity and functional expression levels of each enzyme. These compounds included *p*-nitrophenyl esters with alkyl radicals of increasing length (*i.e.*, *p*-nitrophenyl butyrate (*p*-NPB), *p*-nitrophenyl octanoate (*p*-NPO), *p*-nitrophenyl dodecanoate (*p*-NPD), and *p*-nitrophenyl palmitate (*p*-NPP)), as well as those with bulkier branched (*p*-nitrophenyl trimethylacetate (3MA)) and aromatic (*p*-nitrophenyl benzoate (*p*-NPBZ)) functional groups. All the enzymes were expressed up to the g/L scale in the fed-batch bioreactor being active to a greater or lesser extent with the different esters (Figure 1).

PEF Soluble Scaffolds and Their Direct Correlation with PEF Enzymatic Degradation: Screening Assay for Directed Evolution of PEFase. A variety of PET hydrolase screening assays have been reported to date.[27] These methods rely on laborious techniques such as embedding of fluorogenic probes in the polymer,[28,29] microfluidics, or are just dependent on cascade reactions that impede the continuous monitoring of the reaction.[30−32] In other cases, they are based on the release of terephthalic acid[33,34] and consequently not applicable to PEF. Screening methods employing the solid polymer have been also developed,[15,35] which in the case of PEF would translate into additional steps of chemical synthesis, characterization and more challenging scalability due to lower homogeneity and limited enzymatic activity. Aiming for a faster and more sensitive method, we selected two soluble scaffolds that are components of the PEF matrix as potential surrogate substrates: dimethyl furan-2,5-dicarboxylate (2M-FDCA) and diethyl furan-2,5-dicarboxylate (2E-FDCA). Soluble PEF model scaffolds offer significant advantages for enzyme activity assays. Their high solubility in water allows for the creation of homogeneous reaction mixtures, while their well-defined structure ensures consistent and reliable activity measurements. This ease of use and scalability make them ideal for high-throughput screening applications.

We first monitored the stepwise hydrolysis of these molecules by HPLC with most of the enzymes of the panel showing activity on these compounds. Indeed, enzymatic activity on 2M-FDCA and 2E-FDCA released methyl furan-2,5-dicarboxylate (1E-FDCA) and ethyl furan-2,5-dicarbox-

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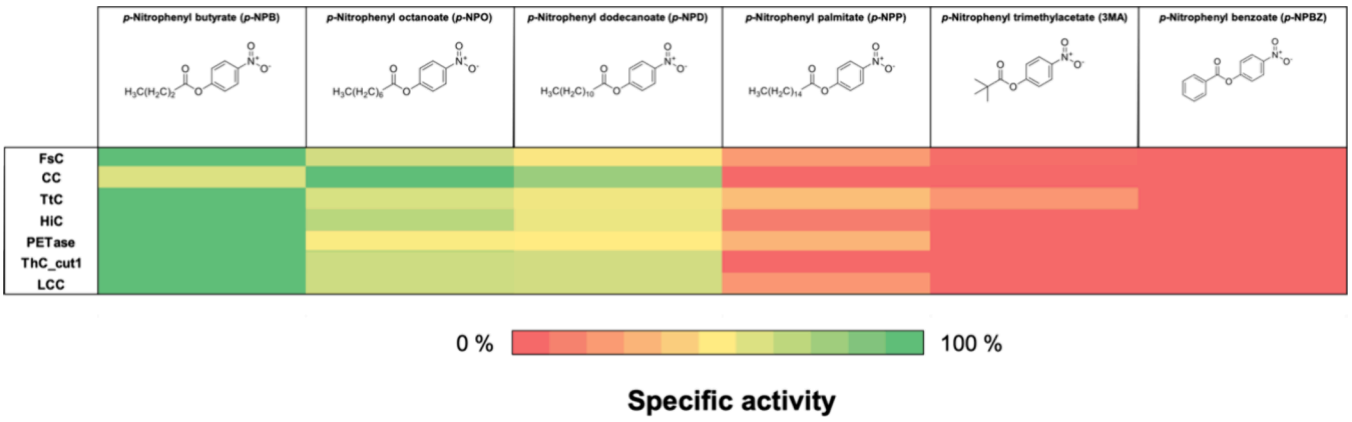
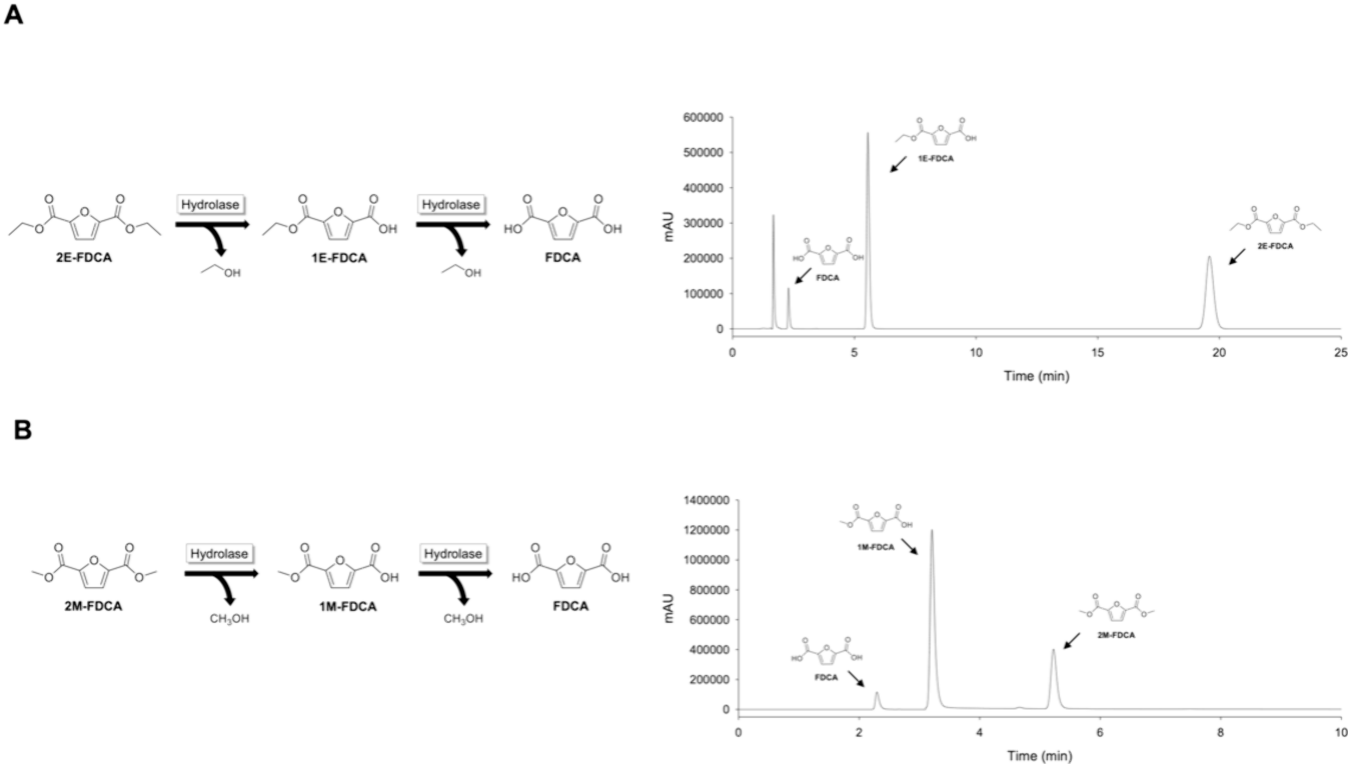


Figure 1. Heat map of the candidate variants' specific activity toward different *p*-nitrophenyl esters.

Scheme 1. Left: HPLC Analysis of the 2E-FDCA (A) and 2M-FDCA (B) Enzymatic Reactions;[a] Right: Product Distribution of 2E-FDCA (A) and 2M-FDCA (B) Hydrolysis for Each Enzyme



[a]Reactions were performed at 30 °C and incubated overnight with shaking at 220 RPM. Each reaction mixture contained 0.2 μM of pure enzyme, 2 mM of either 2E-FDCA or 2M-FDCA and 10% DMSO in 100 mM potassium phosphate buffer pH 8.0 in a total volume of 1 mL.

ylate (1M-FDCA), respectively, which were converted to FDCA upon further hydrolysis, Scheme 1.

Since both molecules were susceptible to hydrolysis, we opted to proceed with the 2E-FDCA due to its closer resemblance to the PEF structural motif. In order to validate the use of this soluble scaffold as a surrogate substrate for PEF-degrading activity, we measured the activity of the enzyme panel with both 2E-FDCA and real PEF powder, Figure 2. FsC, TtC, and CC hardly showed hydrolytic action. By contrast, LCC, PETase, ThC_cut1, and HiC did hydrolyze both 2E-FDCA and PEF, with a general good correlation.

In order to adapt the enzymatic degradation of 2E-FDCA to a HTS assay, one potential approach could involve coupling the release of ethanol to an alcohol dehydrogenase, enabling the measurement of NAD^+ consumption at 340 nm.[36] Aiming for a rapid and less expensive method, we opted for the use of pH indicators, as it has been effectively and successfully exemplified by Beech et al.[37] for the PET diester moiety BHET. In our case, the HTS assay would be based on the release of acidic species from 2E-FDCA after enzymatic attack. Since α/β hydrolases usually exhibit stronger activity under alkaline conditions,[38] the hydrolytic reaction was set at pH 8.0 using phenol red as pH indicator and EPPS as a buffer of choice due to their close pK_a values. Phenol red undergoes a gradual transition from red (λ_{max} = 550−560 nm) to yellow (λ_{max} = 430−450 nm) as the media acidifies, with linear responses obtained in the range from pH 8.2 to 6.8. Thus, the acidification resulting from the hydrolysis of 2E-FDCA can be

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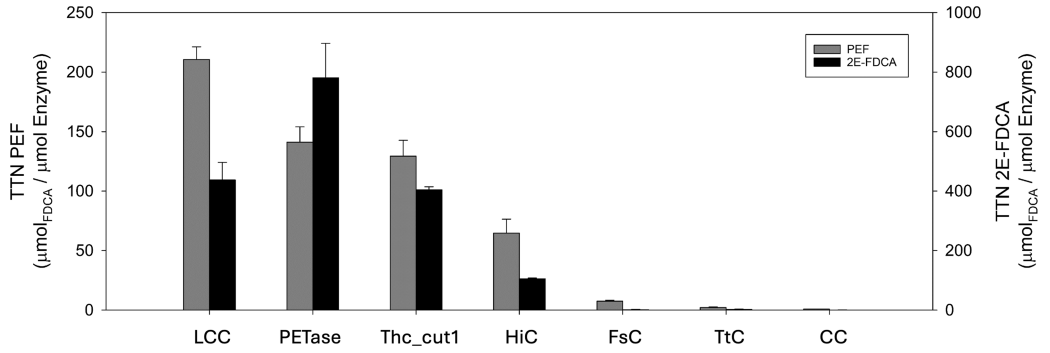

Figure 2. Comparison between PEF and 2E-FDCA enzymatic hydrolysis. Reactions were carried out at 30 °C. Each reaction mixture contained a known amount of enzyme, 5 mg/mL PEF or 2 mM 2E-FDCA (dissolved in 100% DMSO v/v, leading to a final concentration of 10% in the mixture) in 100 mM potassium phosphate buffer, pH 8.0. The Total Turnover Numbers (TTNs, $\mu mol_{product} \cdot \mu mol_{enzyme}^{-1}$) were estimated from FDCA concentration after 240 min in the case of PEF and 150 min in the case of 2E-FDCA. Each point and the standard deviation were derived from three independent measurements.

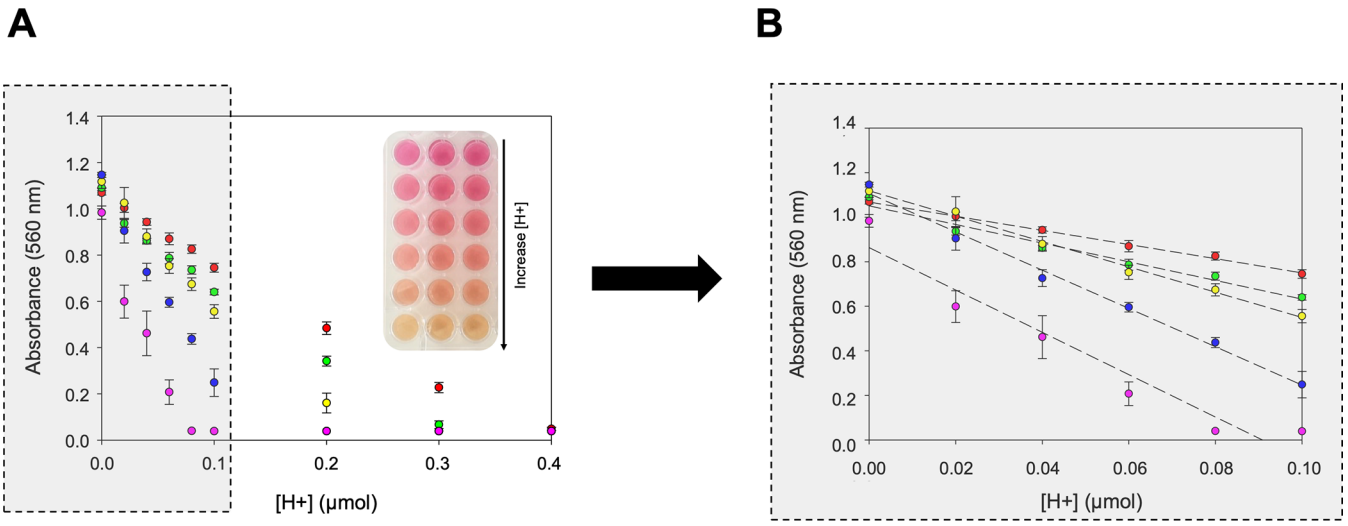

Figure 3. Response of the HTS colorimetric assay to increasing concentrations of FDCA. To mimic the enzyme reaction, the remaining concentration up to 2 mM FDCA was completed with 2E-FDCA. (A) Effect of ionic strength on the sensitivity of the colorimetric assay for FDCA. (B) Inset shows lower FDCA concentrations. The different conditions tested were: EPPS 5 mM (red circles), EPPS 4 mM (green circles), EPPS 3 mM (yellow circles), EPPS 2 mM (blue circles), and EPPS 1 mM (pink circles). Each point and the standard deviation was derived from three independent measurements.

easily measured as it corresponds to a decrease in absorbance at 560 nm.[39] The effect of different EPPS concentrations (from 1 to 5 mM) was studied with increasing concentrations of FDCA (i.e., mimicking the proton release upon 2E-FDCA enzymatic hydrolysis), Figure 3.

As expected, we found the assay to be more sensitive at lower buffer concentrations, with the steepest slope at 1 mM EPPS, albeit at the cost of saturating the signal at low [H$^+$] concentrations. Higher buffer concentrations also produced good linear correlations, roughly up to 2 mM FDCA. Based on the 2E-FDCA enzymatic degradation rate, 2 mM EPPS was considered a reasonable compromise between sensitivity and accuracy for this assay.

To validate the HTS assay, we followed the enzymatic hydrolysis of 2E-FDCA over time and with increasing concentrations of the enzyme, Figure 4. A strong correlation was evident between the enzyme concentration and the colorimetric signal. Moreover, the assay was very sensitive, with a limit of detection ($\Delta_{Abs} \approx 0.02$) and a limit of quantification ($\Delta_{Abs} \approx 0.01$), which should guarantee accurate screening of mutant libraries. Finally, to confirm the fidelity of our assay, initial turnover rates were measured and compared to those obtained by HPLC. Pleasingly, results were very similar regardless of the method (colorimetric or HPLC) used, as shown in Figure 5.

CONCLUSIONS

Employing sustainable materials is fundamental to addressing the challenges presented worldwide from the use of plastics. Among the biobased plastics currently available that can be hydrolyzed, PEF is an attractive candidate to replace petroleum-based PETs due to the similarity in their physicochemical traits, making it a compelling opportunity in various industrial sectors. However, despite its origin from biomass, PEF is highly recalcitrant, and there is an urgent need for a robust enzymatic method to guarantee its complete degradation and recycling. Here, we studied several potential PEF degrading enzymes while setting up a platform that leverages a tandem yeast-expression system and a screening assay to perform directed evolution. With these tools, the engineering of a robust, active, and stable PEFase could be

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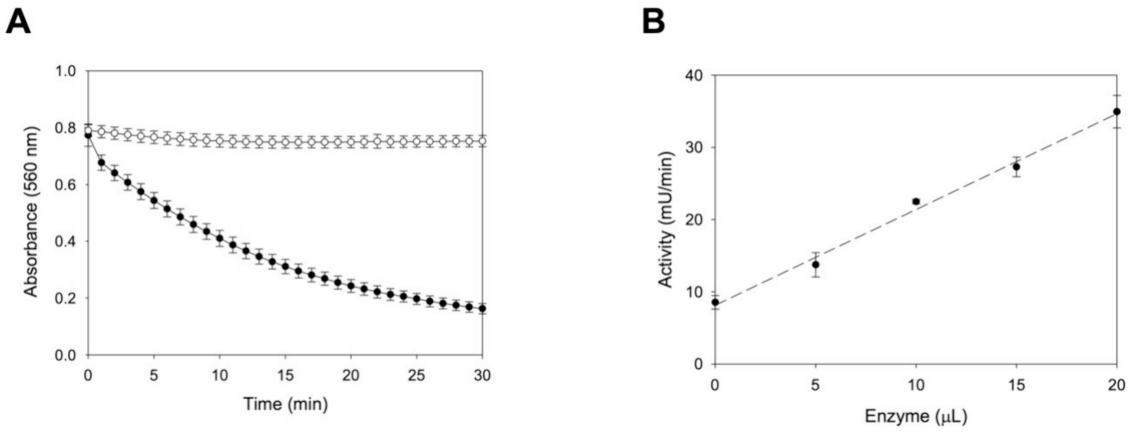

Figure 4. Validation of the HTS assay. Time course (A) and linearity (B) tests of the 2E-FDCA hydrolysis. Assay validation (linearity and sensitivity tests) was performed in 96-well plates with 180 μL of working solution per well containing 20 μL of 2E-FDCA, 20 mM (dissolved in 100% DMSO v/v), 20 μL of phenol red, 0.05 mM (dissolved in 2 mM EPPS buffer pH 8.0), and 140 μL of 2 mM EPPS buffer, pH 8.0. Reactions were started by adding up to 20 μL of enzyme, and they were incubated at 30 °C and 220 rpm for 5 or 30 min (for the linearity and time course reaction experiments, respectively). Color development was monitored in a plate reader at 560 nm, and each point and standard deviation was derived from three independent measurements.

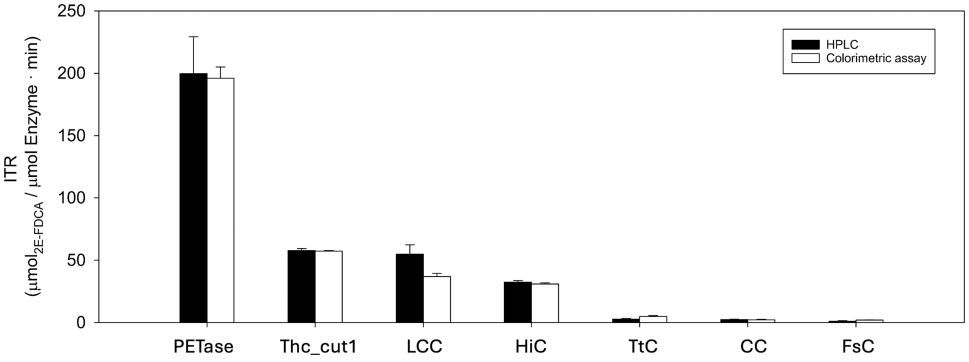

Figure 5. Comparison between HPLC analytical method and Phenol Red-based colorimetric assay to measure 2E-FDCA enzymatic hydrolysis. Initial Turnover Rates (ITR) were measured as $\mu mol_{product} \cdot \mu mol_{enzyme}^{-1} \cdot min^{-1}$. Each point and the standard deviation was derived from three independent measurements.

carried out with a view to ultimately situate PEF as an essential bioplastic on an industrial scale.

MATERIALS AND METHODS

Materials and Strains. The *P. pastoris* strain X-33 and the antibiotic zeocin were purchased from Invitrogen (U.S.A.). *Escherichia coli* strain XL1-Blue competent cells were obtained from Agilent Technologies (U.S.A.). The protease-deficient *S. cerevisiae* strain BJ5465 was from LGCPromochem (Barcelona, Spain). iProof High-Fidelity DNA Polymerase was purchased from Bio-Rad (U.S.A.). Cutinase genes were synthesized by Integrated DNA Technologies (U.S.A.). The NucleoSpin plasmid kit and NucleoSPin Gel and PCR Clean-up kit were purchased from Macherey Nagel (Germany). *p*-NPB, *p*-NPO, *p*-NPD, *p*-NPP, 3MA, and *p*-NPBZ were purchased from Merck Life Science (U.S.A.). 2E-FDCA was purchased from VWR chemicals (U.S.A.). 2M-FDCA was purchased from TCI chemicals (U.S.A.). All chemicals and medium components were of the highest purity available.

Expression of the Enzyme Panel in a Tandem Yeast System. Cutinase genes were designed to include the α-mating factor signal peptide from *S. cerevisiae* at the N-terminus and a histidine-tag at the C-terminal. Genes were cloned and functionally expressed in a tandem yeast (*S. cerevisiae*/*P. pastoris*) expression system as reported elsewhere.[40] *P. pastoris* clones containing hydrolytic enzymes were fermented followed previous protocols.[40] Enzyme supernatants were concentrated by sequential steps of tangential flow filtration (Minimate EVO System, Cytiva) and ultrafiltration (Amicon Stirred Cell 50 or 200 mL, Millipore). Resulting concentrated fraction was centrifuged and filtered (0.22 μM) prior to purification. Recombinant enzymes were purified in a one-step method on an ÄKTA Pure instrument (GE Healthcare) by Ion Metal Affinity Chromatography (IMAC) using a prepacked 5 mL HisTrap FF column (GE Healthcare). Column was equilibrated, and samples were washed in Buffer A (20 mM Tris-HCl pH 8.0, 250 mM NaCl, 20 mM imidazole). Bound proteins were eluted within a linear gradient from 0% to 40% of Buffer B (20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 M imidazole). The grade of purity of the preparations was confirmed by SDS-PAGE using precast gels (Bio-Rad, Mini-PROTEAN TGX Gels, 12%).

Enzyme Activity Test with *p*-Nitrophenyl Esters. Specific activity of purified enzymes was measured with *p*-nitrophenyl butyrate (*p*-NPB), *p*-nitrophenyl octanoate (*p*-NPO), *p*-nitrophenyl dodecanoate (*p*-NPD), *p*-nitrophenyl palmitate (*p*-NPP), *p*-nitrophenyl benzoate (*p*-NPBZ), and *p*-nitrophenyl trimethylacetate (3MA). Reaction mixtures

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contained 1 mM pNPB or 0.25 mM *p*-NPO/*p*-NPD/*p*-NPP/*p*-NPBZ/3MA, and purified enzyme in 100 mM KH_2PO_4 buffer, pH 8.0. Reactions were performed at room temperature in a 96-well plate (Standard, Flat Base, Sarstedt, Germany) in a final volume of 200 μL. Reactions were carried out in triplicate and followed spectrophotometrically in a plate reader (SpectraMax ABS plus, Molecular Devices, Sunnyvale, CA) at 400 nm (ε_{400} for *p*-nitrophenol = 21.000 M^{-1} cm^{-1}). We defined one unit of activity as the amount of enzyme able to release 1 μmol of *p*-nitrophenol per minute under the corresponding conditions.

PEF Synthesis. PEF synthesis was carried out as previously described by Pellis et al.[10] Following the protocol that involves the dissolution of the synthesized polymer in TFA and its precipitation in water, a white powdery polymer with a high molecular weight was obtained.

PEF Depolymerization Reactions and HPLC Analysis. Ten mg of PEF powder (crystallinity 2%) were incubated in 100 mM KH_2PO_4 buffer, pH 8.0, with 2 μM of hydrolase in a final volume of 2 mL. Reactions were carried out for 6 h in an orbital shaker (New Brunswick Innova 44, Eppendorf) at 30 °C and 150 RPM. Proteins were subsequently removed using ice-cold methanol precipitation.[41] Then, samples were centrifuged at 12700 RPM at 0 °C for 15 min. The resulting supernatant was acidified by adding 10 μL of 6 M HCl and then transferred to HPLC vials. Released products were analyzed by reverse high-performance liquid chromatography (HPLC; 3000 Series, Agilent Technologies, Palo Alto, CA) coupled with a UV DAD detector and equipped with a poroshell C-18, Agilent). Twenty μL of samples were injected and run using a CH_3OH/H_2O linear gradient at a flow rate of 0.35 mL/min keeping the column at 40 °C and detection at 260 nm. The retention time of the target compound (FDCA) was determined by injecting a standard solution of the known compound and comparing the elution times of the target analyte to the standard. A series of standard solutions containing known concentrations of the target compounds were prepared in the range from 0 to 5 mM for each analyte. Blank reactions were carried out in a buffer. All reactions were performed in triplicate.

2E-FDCA Hydrolysis Reactions and HPLC Analysis. Reaction mixture contained culture supernatant of known enzyme concentration, 2 mM of either 2M-FDCA or 2E-FDCA (in DMSO, 10% final concentration), 100 mM KH_2PO_4 buffer, pH 8.0, and *dd*H_2O up to 1 mL. Reactions were carried out at 30 °C for 5 h. Depolymerization rates were followed over time, analyzing samples every 30 min by reverse high-performance liquid chromatography (HPLC; Shimadzu LC-2050C-3D), equipped with an InfinityLab Poroshell 120 EC-18 column (150 mm × 4.6 mm, 4 μm) and a photodiode array (PDA). Ten μL of samples were injected and run in isocratic mode with 1% (v/v) TFA in CH_3OH/H_2O (4:6) at a flow rate of 1 mL/min, keeping the column temperature at 30 °C and detection at 240 nm. The injections were performed by an autosampler. The retention times of the target compounds were determined by injecting standard solutions of the known compounds and comparing the elution times of the target analytes with those of the standards. We employed a standard curve method to quantify the target analyte concentration in our samples. A series of standard solutions containing known concentrations of the target compounds were prepared with a range of 0.1 to 5 mM for each analyte. Blank reactions were carried out in buffer. For each concentration level, triplicate injections were made to ensure precision.

HTS Colorimetric Assay. EPPS buffer, pH 8.0, was evaluated at several concentrations (1, 2, 3, 4, and 5 mM) versus increasing FDCA concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 1.5, and 2 mM). In order to mimic the enzymatic reaction, increasing FDCA concentrations were compensated with either 2E or 2M-FDCA decreasing concentrations (2, 1.9, 1.8, 1.7, 1.6, 1.5, 1, 0.5, and 0 mM). Enzyme validation (linearity and sensitivity tests) and depolymerization rate calculations were performed in 96-well plate (Standard, Flat Base, Sarstedt, Germany) with 180 μL of working solution per well containing 20 μL of 2E-FDCA, 20 mM (dissolved in 100% DMSO v/v), 20 μL of phenol red, 0.05 mM (dissolved in 2 mM EPPS buffer pH 8.0), and 140 μL of 2 mM EPPS buffer, pH 8.0. Reactions started by adding up to 20 μL of enzyme and were incubated at 30 °C and 220 RPM (Minitron-Infors, Biogen, Spain) for 5 or 30 min (for linearity and time course reaction experiments, respectively). Color development was monitored in a plate reader at 560 nm (SpectraMax ABS Plus, Molecular Devices, Sunnyvale, CA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c09053.

Table S1 containing a sequence identity matrix of hydrolases of this study (PDF)

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ACS Omega 2024, 9, 45633−45640

Notes

The authors declare no competing financial interest.

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