ACS Sustainable Chemistry & Engineering

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Research Article

Efficient Depolymerization of Poly(ethylene 2,5-furanoate) Using Polyester Hydrolases

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Cite This: *ACS Sustainable Chem. Eng.* 2024, 12, 9658−9668

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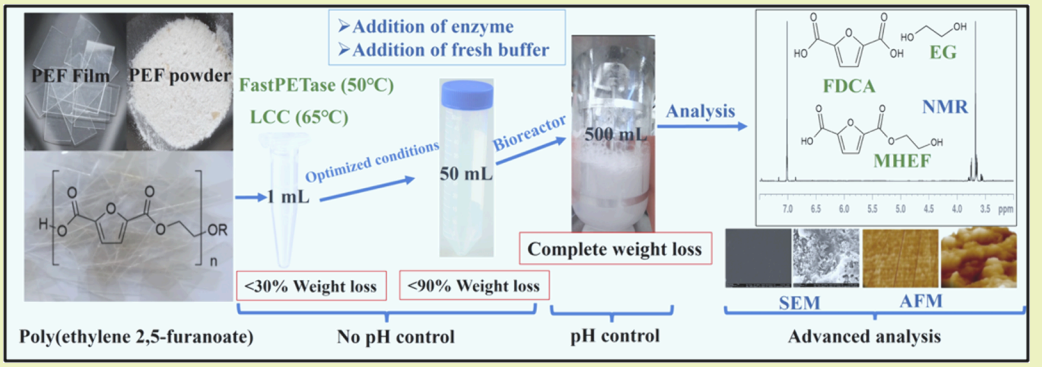


ABSTRACT: Poly(ethylene 2,5-furanoate) (PEF) is considered to be the next-generation green polyester and is hailed as a rising star among novel plastics. It is biobased, is nontoxic, and has comparable or improved properties compared to polyethylene terephthalate (PET). Biobased PEF offers lower life-cycle greenhouse gas emissions than PET. However, with its industrial production starting soon, relatively little is known about its actual recyclability. This work reports on the near complete depolymerization of PEF using two efficient PET hydrolases, FastPETase and leaf compost-cutinase (LCC), at loadings 4.5−17 times lower than previously reported. FastPETase and LCC exhibited maximum depolymerization of PEF, measured by weight loss and 2,5-furandicarboxylic acid (FDCA) production, using potassium phosphate−NaOH buffer at 50 and 65 °C, respectively. The 98% depolymerization of 13 g L^{-1} PEF film was achieved by three additions of the LCC in 72 h, while 78% weight loss was obtained using FastPETase in controlled conditions. Nonetheless, 92% weight loss was obtained with FastPETase when using only 6 g L^{-1} PEF. The main reaction products were identified as FDCA, ethylene glycol, and mono(2-hydroxyethyl)-furanoate. LCC performed better than FastPETase, in terms of both FDCA release and weight loss. The effect of crystallinity was evident on the enzymes' performance, as only 4% to 7% weight loss of crystalline PEF (32%) was recorded. Microscopy studies of the treated PEF films provided information on the surface erosion processes and revealed higher resistance of the crystalline phase, explaining the low level of depolymerization. The study presents important insights into the enzymatic hydrolysis of biobased PEF material and paves the path toward more viable applications within biopolymer waste recycling.

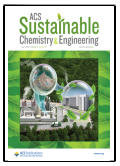
KEYWORDS: *PEF, Enzymatic depolymerization, Bioplastics, Plastic recycling, Circular economy, Polyester*

INTRODUCTION

Over the past years, the need for new eco-friendly polymers has gained momentum to replace fossil-based ones.[1,2] Furan-based polymers are viewed as a promising alternative, with 2,5-furandicarboxylic acid (FDCA) based materials like polyethylene furanoate (PEF) leading the way.[1,3,4] PEF is a thermoplastic polyester comparable to polyethylene terephthalate (PET), but with the advantage of being derived from renewable sources, making it a more environmentally friendly alternative.[5] PEF is typically produced by polymerizing ethylene glycol (EG) and FDCA through transesterification, followed by a polycondensation reaction.[6] FDCA is a biobased monomer derived from renewable resources such as sugars, while EG is commonly a petrochemical or biobased-derived monomer, used in the production of various polymers.[7]

PEF has several advantages including high mechanical strength, excellent gas barrier properties, and good thermal stability. PEF films have superior gas and water barrier properties compared to PET, making them suitable for

Received: January 31, 2024
Revised: June 1, 2024
Accepted: June 3, 2024
Published: June 20, 2024



https://doi.org/10.1021/acssuschemeng.4c00915
ACS Sustainable Chem. Eng. 2024, 12, 9658−9668

packaging applications, particularly in the food and beverage industry.[5] PEF also exhibits good thermal stability, allowing it to withstand high temperatures, comparable to PET, making it fit for existing recycling facilities. Moreover, one of the main advantages of PEF is its improved environmental profile: it can be derived 100% from renewable resources, reducing dependence on fossil fuels,[1,4] while its improved barrier properties allow the use of less material. However, industrial production and widespread adoption of PEF are still in the early stages, as it is not available on the market yet. Avantium, a European-based company, announced a FDCA Flagship Plant, which will enable the commercial launch of plant-based, recyclable, and high-performance PEF to customers worldwide in 2024. Stora Ensö is also building up its first production plant in Belgium, which should be operative within a year, as well.

Despite its interesting properties and substitution potential, the biotechnological depolymerization of PEF and subsequent recycling have not been extensively studied yet. There are only a few reports published in the last years that investigated the use of cutinases from *Thermobifida cellulosilytica* (Thc_cut1) and of *Humicola insolens* (HiC)[2,8,9] for its enzymatic depolymerization. Notably, a techno-economic analysis revealed that increasing recycling rates of bioplastics such as PEF (which have 4−5 times higher costs than the fossil-based alternatives) would significantly decrease the minimum selling price and make it comparable to that of recycled PET.[6] Therefore, it is important to better understand the potential of biotechnological tools for PEF recycling. In the current study, two very efficient PET hydrolases, namely, FastPETase,[10] a variant of wild-type *Ideonella* PETase, and the leaf-branch compost cutinase LCC,[11,12] were used to effectively depolymerize PEF film and powder in optimized reaction settings. To characterize the depolymerization, the hydrolyzed products were identified by using advanced analytical tools, and the surface properties of the enzyme-treated material were examined.

MATERIALS AND METHODS

Chemicals, Bacterial Strains, and Plasmids. All of the chemicals used in the study were of analytical grade. p-Nitrophenol butyrate (pNPB) and 2,5 FDCA were purchased from Sigma-Aldrich, USA. All the plasmids and gene constructs were synthesized by Gene Universal, USA. The sequences for FastPETase[10] and LCC[11,12] were retrieved from the PDB database, codon-optimized, and cloned into a pET26b (+) vector from Gene Universal, USA.

Synthesis of PEF and Processing into Thin Films. Since PEF is not available on the market yet, it was produced in the lab. PEF synthesis was performed by using a previously reported modified protocol,[8] based on the work by Papageorgiou et al.[13,14] All PEF materials were obtained as powders. Only for the high molecular weight PEF (40 kDa) that was obtained as a brown glassy powder was the material purified by dissolution in concentrated TFA and then precipitated in a large volume of ice-cold H_2O to yield a white powder. The high molecular weight PEF powder was then processed into thin films as previously reported.[2,8] The characteristics of different PEF powders and films used in this study are provided in Table 1.

Enzymatic Depolymerization of PEF Films and Powders. FastPETase and LCC were expressed in the *Escherichia coli* strain BL21 (DE3) and purified thereafter (Supporting Information, S1.1).[10−12] The protein concentration and enzyme activity were determined (Supporting Information, S1.2). Unless stated differently, 13.0 ± 0.5 mg of PEF amorphous film (size 0.5 × 1 cm, thickness 0.2 mm) or powder (40 kDa) were incubated with 250 nM and 1000 nM of purified enzyme (FastPETase and LCC) in 1.0 mL of buffer (50 mM KH_2PO_4/NaOH, pH 8.0) in separate reactions. The Eppendorf tubes were incubated in a thermo-shaker (constant vertical shaking) at an optimum temperature of 50 and 65 °C for FastPETase and LCC, respectively.[10,12] The blank (buffer) and control (film/powder and buffer without enzymes) were also incubated. The reaction was monitored for 96 h. After completion of the reaction, the polymer weight loss was calculated (Supporting Information, S1.3) and the released FDCA was quantified using HPLC-DAD analysis.

Table 1. Different PEF Materials Used in the Study and Their Characteristics

Type	Thickness (mm)	Crystallinity (%)
Powder (40 kDa)	nd[a]	16
Film (amorphous)	0.2 ± 0.02	2
Film (crystalline)	0.2 ± 0.02	32

[a]nd: not determined.

Evaluation of Different Reaction Variables for PEF Hydrolysis. Different enzyme concentrations for FastPETase and LCC (100, 250, 500, 1000, 2000, and 5000 nM) were used to optimize the effect of enzyme loading on PEF depolymerization. A total of 13.0 mg of PEF (powder or film) were incubated in 1 mL KH_2PO_4-NaOH buffer (50 mM, pH 8.0) for 96 h in different enzyme concentrations. Different 50 mM buffer systems, such as KH_2PO_4-K_2HPO_4 (pH 6.5−7.5), KH_2PO_4-NaOH (pH 8.0−9.0), glycine-NaOH (pH 9.0−10.0), and $NaHCO_3$−-Na_2CO_3 (pH 10.0−11.0), were tested for the depolymerization of PEF. T_m of the enzymes was determined using nanodifferential scanning fluorimetry (Supporting Information, S1.4). The temperature of incubation (30, 40, 50, 55, 65, and 70 °C), time of reaction (6, 12, 18, 24, 48, 72, and 96 h), and substrate loading (6.0, 12.0, and 18.0 mg) were also tested for the hydrolysis of PEF (powder, amorphous film, and 32% crystalline film) to identify optimized conditions. The crystallinity of the treated and untreated samples was measured using differential scanning calorimetry (Supporting Information, S1.5, Figure S5).

Effect of Enzyme-Addition Strategy on Depolymerization of PEF. After optimization of key parameters, the reaction volume was increased 50 times, and enzymatic hydrolysis of the PEF amorphous film was performed, using three different strategies for enzyme addition. In the first set, 750 nM FastPETase and 3000 nM LCC were added only at the beginning of the reaction. In the second set of experiments, new enzymes (250 and 1000 nM of FastPETase and LCC) were added to the reaction at 0, 24, and 48 h. In the third set, both the buffer and enzymes were replaced in the reaction mixture at 0, 24, and 48 h. The reaction was performed in a final volume of 50 mL. The reaction products were quantified after completion of the hydrolytic reaction (72 h), and weight loss was calculated gravimetrically. The hydrolytic ability of both enzymes was also tested at a 10% substrate loading in 0.5 mM phosphate−NaOH buffer to evaluate the industrial potential of the enzymes.

PEF Depolymerization in a Bioreactor. The depolymerization of PEF films was performed in a bioreactor (Biostat A, Sartorius) under controlled conditions. The 13 g L^{-1} PEF film was added to 500 mL of 50 mM phosphate−NaOH buffer (pH 9.0) and 250 nM (FastPETse) and 1000 nM (LCC) in separate reactions. The reaction was performed at 50 and 65 °C for FastPETase and LCC, respectively. The initial pH of the reaction was adjusted to 9.0 using 1 M NaOH. The enzyme addition (250 nM FastPETase, 1000 nM LCC) was made after 24 and 48 h. The samples were collected at different time intervals, and monomers were quantified using HPLC analysis. The reaction was stopped after 96 h, residual PEF films were recovered from the reaction, and the weight was measured.

Advanced Analytical Methods. *High Performance Liquid Chromatography (HPLC).* HPLC analysis was performed for all the PEF hydrolysis reactions to quantify the aromatic monomer (FDCA) released. The 500 μL samples were taken and centrifuged at 20,000 rpm for 10 min, and the supernatant was diluted 1:1 with methanol. FDCA standard was prepared in 50% methanol (v/v) to ensure equivalence with the samples. The samples were analyzed using a

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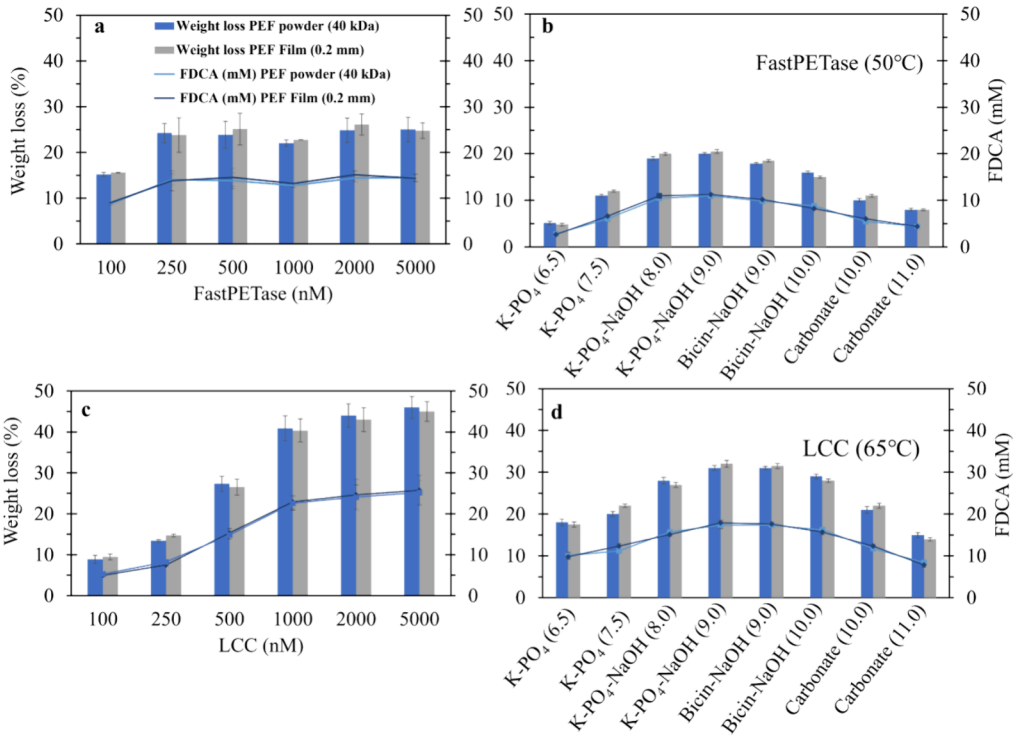

Figure 1. Depolymerization (weight loss and FDCA release) profile of 13 g L^{-1} of PEF powder (40 kDa) and amorphous PEF film (0.2 mm) using (a) FastPETase at 50 °C and (c) LCC (65 °C) using different concentrations (100, 250, 500, 1000, 2000, and 5000 nM). (b) FastPETase (50 °C) and (d) LCC (65 °C) in different buffers and pH.

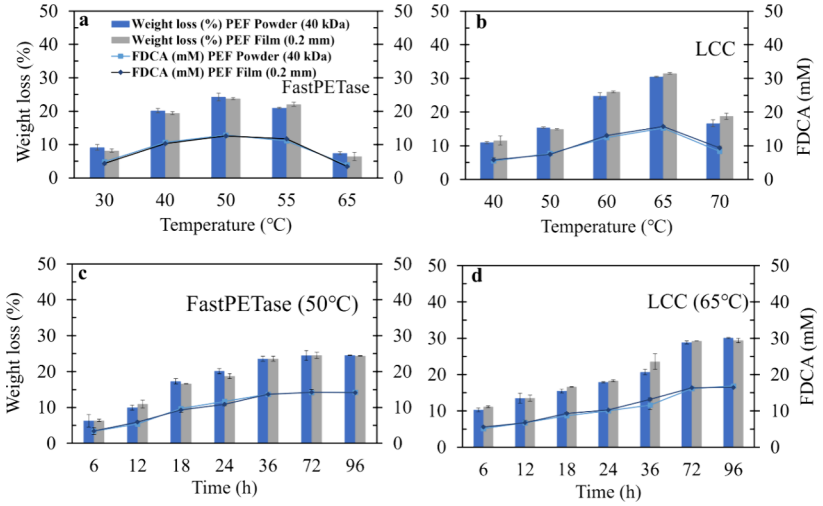

Figure 2. Depolymerization (weight loss and FDCA release) profile of PEF powder (40 kDa) and amorphous PEF film (0.2 mm) of (a) FastPETase (250 nM) and (b) LCC (1000 nM) at different temperatures of incubation. (c) FastPETase (250 nM) and (d) LCC (1000 nM) at different time intervals (6, 12, 18, 24, 36, 72, and 96 h). The reaction was performed in 50 mM KH_2PO_4–NaOH buffer (pH 9.0) and using 13 mg of PEF powder or film.

Dionex Ultimate 3000 system fitted with a diode array detector. A C18 column (Phenomenex Luna 5 μm, 250 mm × 4.6 mm) at 30 °C was used for the separation of the products of the reaction with 0.1% formic acid in water (A) and methanol (B) as the mobile phase. The solvent gradient was as follows: 10% B (0–5 min), increased linearly to 50% (5–17 min), and 100% B (17–20 min). The flow rate was 1 mL min^{-1} and the injection volume was 2 μL, with detection at 241 nm and a total run time of 20 min.

Liquid Chromatography–Mass Spectrometry (LC-MS). LC-MS was performed on a Hitachi LaChrom Elite HPLC system using a C6-phenyl (150 × 4.6 mm Ascentis Xpress 2.7 μm, Sigma-Aldrich) column coupled to a high-resolution mass spectrometer (compact qTOF, Bruker) with an electrospray source (Capillary: 4500 V; end plate offset 500 V; dry gas 4.0 L min^{-1}, 200 °C) in positive mode using a 3:97 flow splitter. The flow was kept constant at 1.2 mL min^{-1}. Gradient elution started with 90% solvent A (H_2O HiPerSolv, VWR) and 10% solvent B (MeCN HiPerSolv, VWR), both supplemented with 0.1% formic acid (Sigma-Aldrich, LC-MS grade). Over 15 min, the fraction of solvent B was increased linearly to 99%; hereafter, it was kept constant for another 5 min.

Nuclear Magnetic Resonance (NMR). Samples (24 h) were prepared by mixing 500 μL of supernatant with 25 μL (200 mM) of 3-(trimethylsilyl)propionic-2,2,3,3 acid sodium salt D4 (TSP-D4) in D_2O. ^{1}H NMR spectra were recorded on a BRUKER AVIII-600 MHz

https://doi.org/10.1021/acssuschemeng.4c00915
ACS Sustainable Chem. Eng. 2024, 12, 9658–9668

NMR spectrometer equipped with a cryogenic probe. Spectra were recorded at 298.1 K with a standard 1D pulse sequence with an acquisition time of 2.73 s (64k complex data points, spectral width of 20 ppm). The relaxation delay was set to 15.5 s. During the last 5 s of the relaxation delay, a weak continuous-wave pulse of $\gamma B1/2\pi = 70$ Hz was applied for water suppression. Resonance assignment in product mixtures was done by comparing chemical shifts with spectra of pure standards.

Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM). A Zeiss EVO 60 scanning electron microscope was used to study the surface morphology of the samples. To avoid charging the polymers with an electron beam, a thin layer of gold was sputtered across the surface before imaging. The AFM measurements were carried out using the Ntegra Aura system (NT-MDT) in tapping mode and utilizing commercial silicon cantilevers with tip curvature radius < 10 nm. The obtained data were processed by Image Analysis P9 software supplied with the microscope.

RESULTS AND DISCUSSION

Optimization of Key Hydrolysis Parameters. *Buffer, pH, and Enzyme Loading.* FastPETase and LCC were produced in *E. coli* BL21 (DE3) and purified. These enzymes are among the best polyester hydrolases reported so far and are known to depolymerize aliphatic and aromatic polyesters, including PET. The purified enzymes were used for PEF depolymerization experiments. The powder (40 kDa) was selected, based on the previous results of Pellis et al. (2016) where better depolymerization was observed using this material when compared to the 6 and 10 kDa polymers.[8,15]

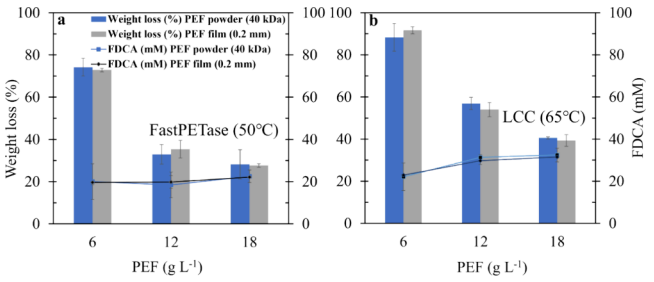

Figure 3. Depolymerization (weight loss and FDCA release) profile of PEF powder (40 kDa) and amorphous PEF film (0.2 mm) of (a) FastPETase (20 nM g^{-1} PEF) and (b) LCC (80 nM g^{-1} PEF) at different substrate concentrations for 72 h. The reaction was performed in 50 mM KH_2PO_4–NaOH buffer (pH 9.0).

Preliminary tests were made, using the purified enzyme, and applying the best conditions previously reported for PET depolymerization.[10,12] The initial results showed 10 and 15% depolymerization of the material. Therefore, we tried to further optimize the reaction conditions for achieving the optimum hydrolysis of PEF.

Enzyme loading for efficient conversion is an important parameter of enzymatic processes, including PEF hydrolysis. PEF weight loss and FDCA production reached a maximum (25% weight loss and 13.8 mM FDCA) at 250 nM FastPETase concentration at 50 °C in potassium phosphate–NaOH buffer (pH 8.0) after 96 h (Figure 1a). Interestingly, the PEF hydrolysis did not improve when the concentration of FastPETase was increased to 500 and 1000 nM and even to 5000 nM. However, LCC showed increased depolymerization of both powder and film (Figure 1c), with the increase in enzyme loading, reaching a maximum weight loss of 35% at a concentration of 1000 nM, and did not increase further. Notably, previous studies reported a concentration for Tf_cut1 and HiC of 5 μM for PEF depolymerization.[2,9] FastPETase and LCC clearly show higher depolymerization when used at lower enzyme concentrations. FastPETase showed optimum weight loss at lower enzyme concentrations (250 nM) than LCC (1000 nm); however, LCC performed better in terms of FDCA production and total depolymerization. This could be due to low product tolerance, thermostability, and loss of activity at a low pH for FastPETase. A recent study showed the low thermostability of FastPETase during PET hydrolysis at different reactor scales compared to other variants.[16] Interestingly, both powder and film showed nearly the same depolymerization by using both enzymes. However, this is likely due to the crystallinity of the PEF powder (16%) used here (Table 1). Additionally, a recent study by Brizendine et al. (2022) also showed that particle size reduction of PET affects only the initial rate of hydrolysis but not the overall conversion.[17] However, it would be interesting to see the effect of varying particle sizes and crystallinities of PEF on the hydrolytic abilities of these new highly efficient enzyme variants.

The effect of using different buffers for PEF hydrolysis was also investigated (Figure 1b,d). Both FastPETase (50 °C) and LCC (65 °C) treated PEF showed weight loss using buffer solutions with a pH ranging from 6.5 to 11.0. The weight loss was low (5%) in sodium phosphate buffer (at pH 6.5) for both

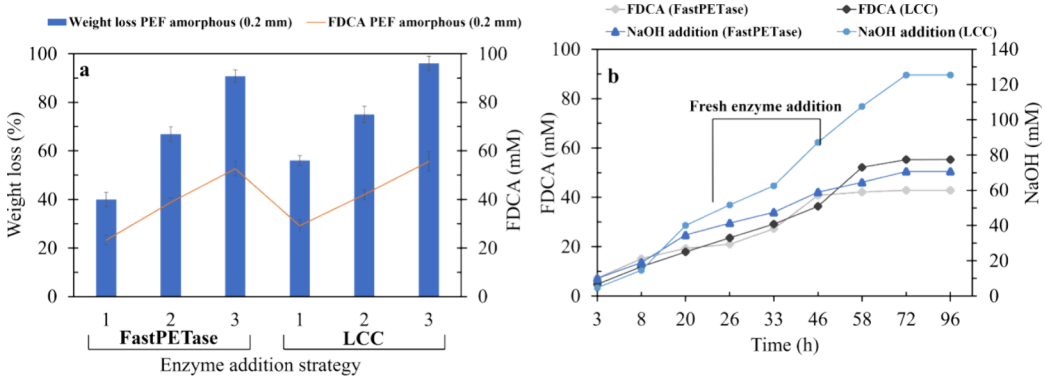

Figure 4. Depolymerization and FDCA measurements of 13 g L^{-1} of PEF film (a) using three different approaches for FastPETase and LCC: (1) adding enzyme (750 nM for FastPETase and 3000 nM for LCC) only at the beginning and (2) at 0, 24, and 48 h (250 and 1000 nM) and (3) adding fresh buffer plus enzyme (250 and 1000 nM) at 0, 24, and 48 h. The reactions were performed for 72 h at 50 and 65 °C respectively. (b) The release of FDCA and NaOH consumption during depolymerization in a bioreactor, using approach (2).

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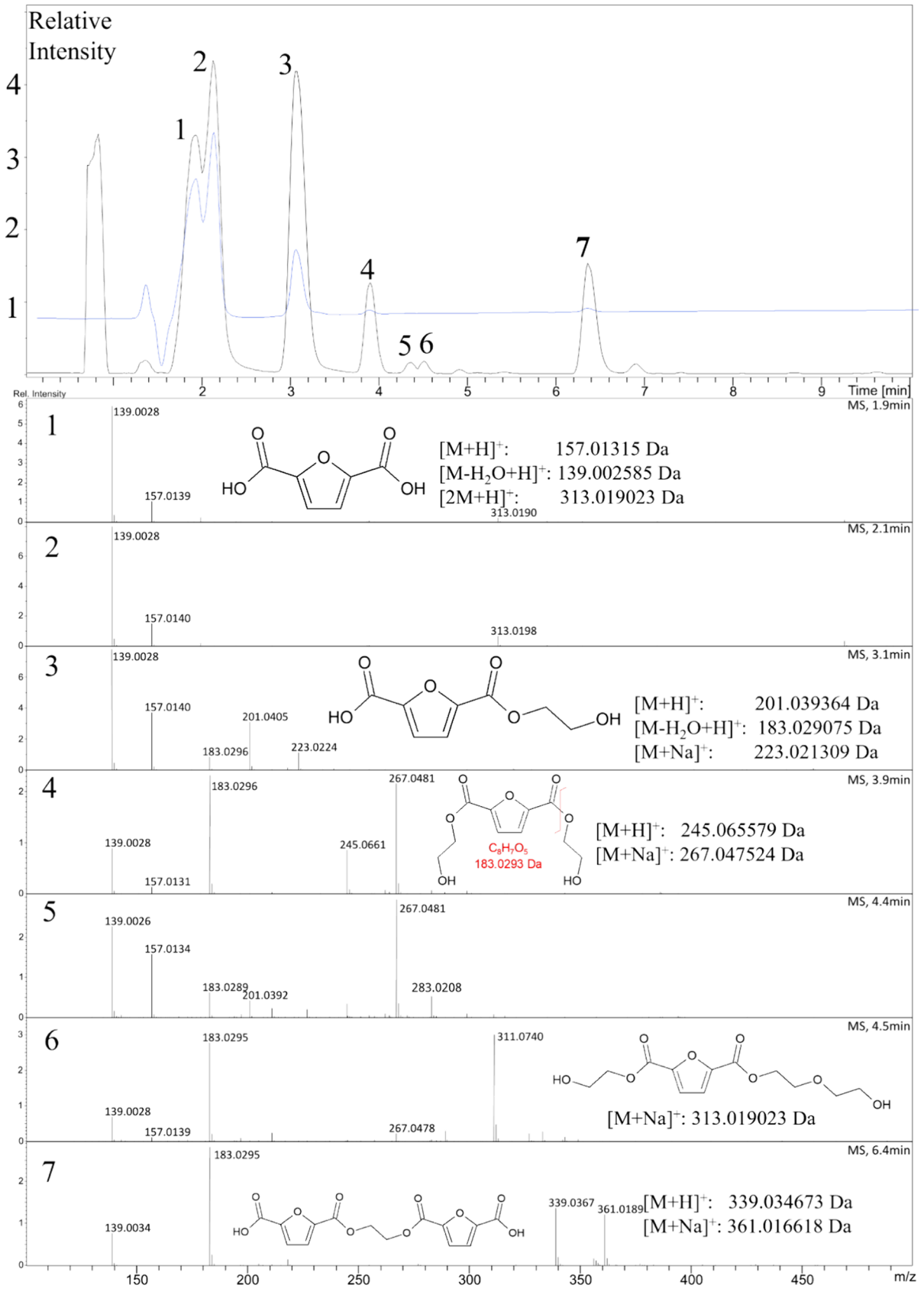

Figure 5. LC-MS analysis of LCC-catalyzed PEF depolymerization products after 24 h. Blue line: UV-chromatogram at 243 nm; black: base peak chromatogram (MS, positive mode). The mass spectra at the seven indicated peaks with possible structures to fit the observed masses and possible structures are shown. FDCA is eluting in two separate peaks; the reason for this is unknown.

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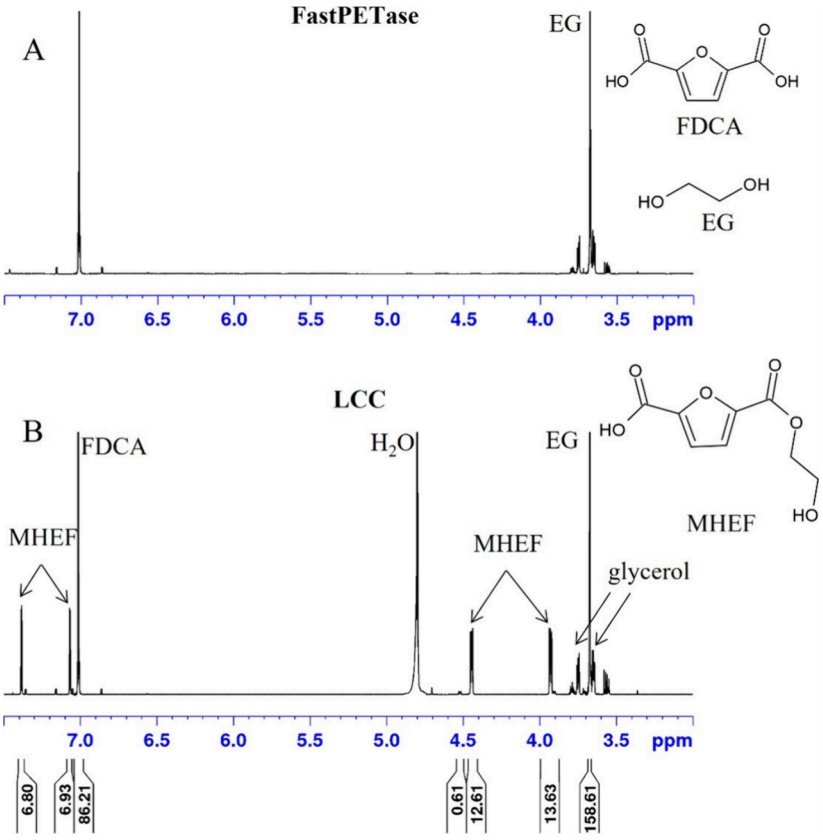

Figure 6. ^{1}H NMR spectra of supernatant after treatment of PEF with (A) FastPETase and (B) LCC. While the only hydrolysis product detected after FastPETase degradation is FDCA, LCC produces ∼14% MHEF and ∼86% FDCA under the conditions used. Other minor reaction products exist and could be detected by LC-MS (see Figure 5 and Figure S3).

enzymes, whereas LCC had slow hydrolysis of PEF in 50 mM carbonate−bicarbonate buffer, pH 11.0 (16%). Depolymerization was most efficient in 50 mM glycine−NaOH and KH_2PO_4−NaOH buffer (pH 9.0) for both enzymes (22 and 34%, respectively). The treatment with FastPETase and LCC resulted in maximum depolymerization activity at pH 8.0 (21%) and 9.0 (30%), showing a rapid decrease as soon as the pH dropped. This is due to the release of acidic hydrolysis products, which decrease the pH of the reaction mixture. FastPETase and LCC led to 24% and 30% weight loss of the polymer film and powder within 96 h. PEF hydrolysis was also tested at 0.5 M phosphate−NaOH buffer (Figure S3) where FastPETase catalyzed reaction reached 8% weight loss, whereas LCC exhibited 52% weight loss. The hydrolytic ability of FastPETase was influenced negatively with an increase in buffer molarity, whereas LCC hydrolytic ability was not, which is in agreement with previous studies.[15] Most PET hydrolases reported so far exhibited optimum activity between pH 7.0 and 9.0.[18−20] To date, the highest PEF hydrolysis efficiency was observed at pH 8.0, using 1 M potassium phosphate buffer when Thc_cut1 and HiC were used.[2,9] The use of higher buffer strength is also not preferred at the industrial scale, to ease downstream processing, as well as operating expenses or expenditure (OPEX), and therefore, the enzyme systems should be evaluated at lower buffer concentration.[16]

Temperature and Time Course of Reaction. The effect of incubation temperature showed a pattern where maximum weight loss and FDCA release were observed at the optimum temperature reported for these enzymes during PET hydrolysis.[10,12] FastPETase and LCC performed maximum PEF depolymerization at 50 and 65 °C, respectively (Figure 2a and Figure 2b). The depolymerization efficiency decreased as the temperature was increased or decreased. These findings are consistent with the melting temperature (T_m) of both enzymes, measured by nano-DSF (Figure S1). The half-life of FastPETase at 50 °C and LCC at 70 °C was reported to be 22.5 and 51 h, respectively.[10,21] As expected, FastPETase had lower thermostability and lost activity very rapidly above 50 °C, which was in good agreement with previous observations.[16] On the other hand, LCC was more stable and showed a melting point of 79 °C. The T_m of LCC is closer to the glass transition temperature (T_g) of PEF (80 °C) which could be an advantage, as temperature near glass transition is supposed to increase the chain mobility. However, PET/PEF hydrolysis is a surface erosion process, and polymer surface amorphization, the water plasticization effect, and T_g of the surface layer play an important role in polymer hydrolysis. A recent study by Tarazona et al. (2022) showed that PET nano films having comparable properties to the PET surface layer had lower T_g (40 °C) compared to bulk PET T_g (65−81 °C).[22] Another study reported that T_g of amorphous PET disks decreased from 75 to 60 °C upon presoaking in water at 65 °C, while the crystallinity was unaffected.[23] There was no effect on the reaction rate on presoaked disks at 68 °C, i.e., above the T_g catalyzed by LCC ICCG.[23] It clearly demonstrates that there are factors other than crystallinity, like water plasticization and T_g of the surface layer, which also affect polymer hydrolysis. In future studies of PEF hydrolysis and other related polymers, these factors should also be considered to achieve efficient

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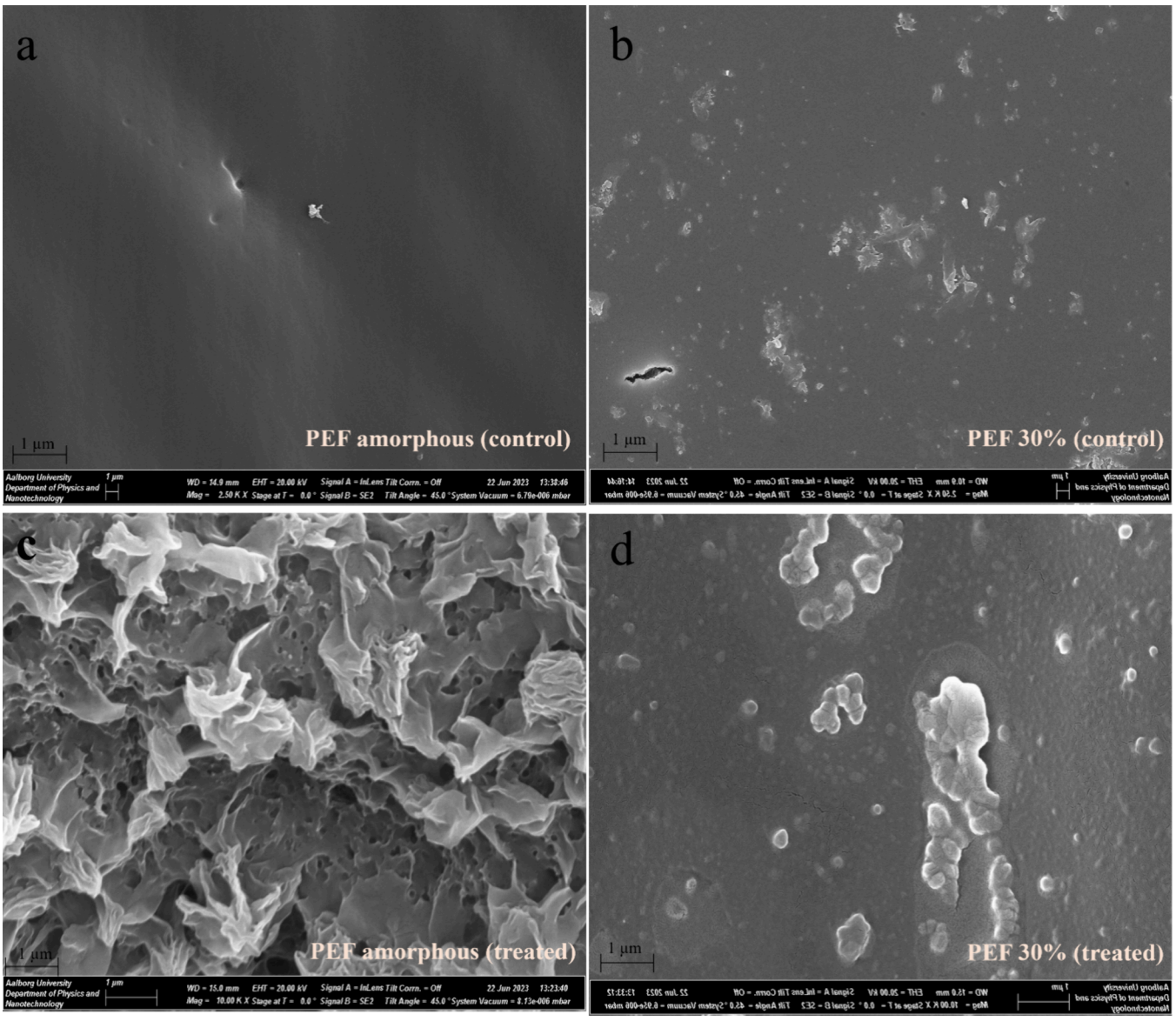

Figure 7. SEM images of (a and b) untreated and (c and d) LCC-treated amorphous and crystalline PEF films, respectively.

hydrolysis in a reasonable time. Other thermostable cutinases, such as Thc_cut1 and HiC, also exhibited higher depolymerization at 65 °C than 50 °C.[1] After the effect of temperature was investigated, the time of incubation of the enzymes with PEF material was considered. FastPETase achieved a 24% weight loss in 36 h. The depolymerization process slowed down after 36 h and no significant changes in weight loss nor FDCA release were observed after longer incubation times (Figure 2c). A reason for this could be the poor thermostability and short half-life of FastPETase, besides the changes in pH and/or product accumulation.[16] LCC showed a different trend during the time frame tested (Figure 2d). The polymer weight loss and produced monomer (for both powder and film) increased with prolonged incubation time and reached 34% in 72 h. Comparable results were obtained by Pellis et al., who reported that Thc_cut1 exhibited maximum hydrolysis on 40 kDa PEF powder in 72 h.[8] This confirms the hypothesis that either thermostability or tolerance to product accumulation in FastPETase is lower, which affects the overall depolymerization process with this enzyme.

Substrate Concentration. The depolymerization of 6 g L^{-1}, 12 g L^{-1}, and 18 g L^{-1} of PEF powder and film was performed using an equal enzyme-to-substrate loading for FastPETase (20 nM g^{-1} PEF) and LCC (80 nM g^{-1}). Interestingly, FastPETase and LCC were able to depolymerize 74% and 90% of PEF releasing 19 and 23 mM of FDCA (Figure 3a,b), respectively, when using 6 g L^{-1} PEF. As the PEF concentrations were increased to 12 g L^{-1} and 18 g L^{-1}, the weight losses were 35% and 30%, respectively, using FastPETase. With LCC, the weight losses were 56% at 12 g L^{-1} and 40% at 18 g L^{-1}. The released FDCA did not increase with increasing substrate concentration, possibly indicating product inhibition, thermal denaturation of the enzymes, and/or low activity due to a change in the pH of the reaction. In previous studies, MHET was the major inhibiting factor for the efficient hydrolysis of the Tf_cut2 in the PET degradation process.[24] A similar mechanism might be expected for PEF but should be tested by incubating the enzymes in the presence of specific hydrolysis products. After optimization of all the above-mentioned parameters, a final reaction with 13 g L^{-1} PEF powder,

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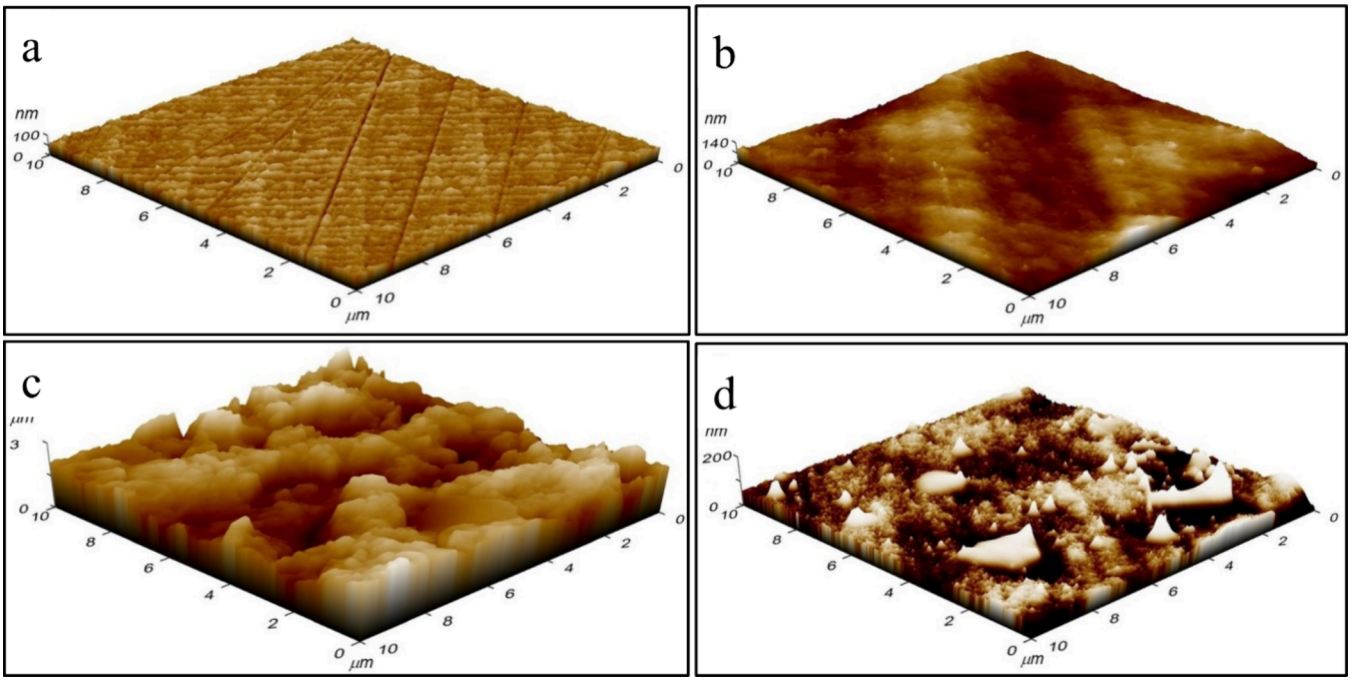

Figure 8. AFM topography images of amorphous (a) virgin and (c) treated as well as crystalline (b) virgin and (d) treated PEF films.

amorphous film, as well as crystalline film (32%) (Figure S2a) was performed. Approximately 36% weight loss of powder and the amorphous film was observed for FastPETase and 53% of polymer weight loss was observed for the LCC catalyzed reaction. The release of FDCA correlated well with the weight loss of PEF. However, crystalline PEF had only 4% and 8% weight loss, using FastPETase and LCC, respectively. The effect of the crystallinity of aromatic polyesters like PET on enzymatic hydrolysis is well-known.[22−26] Also, in the case of PEF, Weinberger et al. showed that an increase in crystallinity from 10 to 20% significantly reduced weight loss and monomer release, which is coherent with the present findings.[2]

PEF Depolymerization in 50 mL Scale with Repeated Enzyme and Buffer Addition. All the previous experiments were performed on a 1 mL scale (50 mM phosphate−NaOH, pH 9.0) with incubation at 50 and 65 °C for FastPETase and LCC, respectively, without a constant pH control, which may have a significant effect on the overall depolymerization of these materials. Therefore, a first scale-up (50 mL) was performed under the same reaction conditions, adopting a different strategy to further improve PEF depolymerization. In the first set of experiments, 750 nM and 3000 nM of FastPETase and LCC were added at the beginning of the reaction and approximately 37% and 55% of PEF film was depolymerized (Figure 4a). In the second set of experiments, 250 and 1000 nM FastPETase and LCC were added every 24 h, and the reaction was stopped after 72 h. This strategy led to 65% and 75% PEF depolymerization, respectively, after three enzyme additions (Figure 4b).

Both FastPETase and LCC showed similar depolymerization, and the pH of the reaction solution decreased drastically (from pH 9.0 to 6.0). Therefore, we performed another experiment, where fresh buffer (50 mM potassium phosphate−NaOH) and the enzyme were added every 24 h. This strategy allowed approximately 92% and 98% depolymerization of 13 g L^{-1} PEF film to be achieved, using FastPETase and LCC (Figure 4b). To assess the high substrate loading, 10% (w/v) PEF film (amorphpus) was incubated in 0.5 M phosphate−NaOH buffer following approach 2 for FastPETase (20 nM g^{-1}) and LCC (80 nM g^{-1}) fresh enzyme was added after 24 h and 48 h. After 72 h, FastPETase showed 8% weight loss and 40 mM FDCA, whereas LCC showed 52.2% weight loss and 216 mM of FDCA (Figure S3). However, a reaction with continuous pH control would most likely have positive effects without requiring replacement of the buffer. So, the next step would be to assess the depolymerization under pH-controlled conditions and study the effect of released FDCA on enzyme activity during hydrolysis. Further, mass balance analysis of PEF suggests that most of the product is FDCA (86% for LCC and 81% for FastPTase) and ethylene glycol with smaller contributions of the oligomer MHEF (Figure S2b,c). As expected, the formation of FDCA and other products decreased as the crystallinity of the material increased. To the best of our knowledge, only three studies were reported exclusively targeting PEF hydrolysis with polyester hydrolases, and all were performed on a small scale without any pH control.[2,8,9] Our findings suggested that PEF depolymerization would improve significantly using a pH-controlled reaction system, which was further investigated.

Depolymerization in a Bioreactor. PEF depolymerization using both FastPETase and LCC showed interesting results. FastPETase-catalyzed hydrolysis exhibited a 78.2% weight loss (Figure S2d) and the reaction progressed very fast in the initial hours and slowed down after 24 h (Figure 4b). The addition of fresh enzyme at 24 h resulted in a further increase in FDCA release and NaOH consumption until 35 h. Another enzyme addition was made at 48 h, but the consumption of NaOH and FDCA release did not increase substantially. After 56 h, NaOH consumption almost stopped and a maximal FDCA concentration of 43 mM was reached and 40 mM EG and 0.8 mM MHEF (determined by quantitative NMR).

The LCC catalyzed reaction showed a clear trend, where NaOH consumption and FDCA release increased gradually and the addition of fresh enzyme at 24 and 48 h led to a further

https://doi.org/10.1021/acssuschemeng.4c00915
ACS Sustainable Chem. Eng. 2024, 12, 9658−9668

increase in depolymerization (Figure 4b). The FDCA concentration did not change after 72 h. The PEF films were almost completely depolymerized (98%) (Figure S2d) with a final FDCA titer of 55 mM (Figure 4b) and were 58 mM EG and 5 mM MHEF. The product inhibition (evident after 48 h) rather than the lower thermostability of FastPETase seems to be the main reasons for less depolymerization of PEF, despite the pH control. This would explain the higher weight loss (92%) obtained with FastPETase, when adding fresh buffer every 24 h. On the other hand, LCC confirmed its higher product tolerance (and thermostability) during the reaction, which was evident from the weight loss and release of FDCA. The previous studies on PEF reached comparable depolymerization results (though in mL scale), using 1000 nM g^{-1} PEF (5 mM concentration) of commercial enzymes,[2,8,9] whereas the enzyme loading in the present study was 58 nM g^{-1} and 230 nM g^{-1} PEF for FastPETase and LCC, respectively. The current work represents the first report of efficient PEF depolymerization in a bioreactor using multiple enzyme additions (0, 24, and 48 h) of in-house produced LCC/FastPETase, with an overall enzyme loading 4.5−17 times lower than previous studies.

Advanced Analysis of Depolymerization Products. HPLC and LC-MS. To further confirm the HPLC results of product formation, we analyzed the hydrolysate with additional methods. LC-MS of enzymatically treated PEF samples showed FDCA as the most abundant product, followed by mono(2-hydroxyethyl)-furanoate (MHEF), in good agreement with HPLC analysis. In particular, FastPETase treated samples showed only FDCA and MHEF signals, and no larger oligomers were detected (Figure S3). Interestingly, different products, such as FDCA, MHEF, and large oligomers, were detected in the samples treated with LCC (Figure 5). Longer oligomers were also expected but not detected, most likely due to poor solubility in the reaction buffer. Similarly, Pellis et al. observed FDCA as a major product and several oligomers in the PEF samples treated with cutinase 1 from *Thermobifida cellulosilytica*.[8]

NMR Analysis. To further elucidate the hydrolysate composition, NMR analysis was performed on FastPETase and LCC treated amorphous PEF film (0.2 mm), showing interesting results (Figure 6). As expected, FastPETase treatment showed conversion of PEF to the monomers FDCA and ethylene glycol, but there was no signal for oligomers like MHEF or larger oligomers (Figure 6a). This, together with the lower weight loss, might indicate that FastPETase has a different hydrolysis mechanism, with potentially higher specificity for smaller oligomers and/or an "exo-lytic" activity. This might partially explain the lower enzyme loading requirement observed in the case of FastPETase. In fact, PETase has been recently considered a surface-modifying hydrolase,[15] suggesting that it might be preferably cutting the loose ends of polymer chains that stick out of the polymer surface.

NMR analysis also confirmed that LCC treated hydrolysate presented FDCA, MHEF, and EG signals, possibly suggesting a higher endolytic activity and/or a lower specificity for shorter oligomers, compared to the PETase (Figure 6b). The combination of the two enzymes might therefore lead to a synergistic effect with faster and complete depolymerization of PEF to its monomers. This might however not be required: from a recycling and energetic point of view, it might be more reasonable to repolymerize MHEF or BHEF (bis(2-hydroxyethyl)-furan-2,5-dicarboxylate) directly, rather than starting from the monomers. Therefore, hydrolysis leading to efficient and selective depolymerization of MHEF or BHEF would be preferable and would facilitate both the downstream processing and recycling.

SEM and AFM. The surface morphology and topography of untreated amorphous and crystalline PEF samples, as well as the enzyme-treated ones, were analyzed by using SEM and AFM. It can be observed in Figure 7a,b that the surfaces of untreated PEF films (amorphous and crystalline) are relatively smooth, showing some individual features related to the synthesis of the polymer films. However, the enzyme-treated polymer materials show evident erosion of the surfaces (Figure 7c,d). As expected, this erosion was higher for amorphous films compared to the crystalline ones. Analyzing the images obtained on a larger scale allows us to conclude that the change of morphology is more uniform for the amorphous samples compared to the films with a certain degree of crystallinity, suggesting that the enzymes are highly active on amorphous parts. At the same time, the depolymerizing activity is reduced with an increase in crystallinity. In other words, the depolymerization undergoes selectively leaving the crystalline parts to be less affected. Weinberger et al. reported a high surface roughness for low crystalline films. The roughness of the polymer decreased as the crystallinity increased.[2]

This selective erosion, as well as the change of topography and surface roughness, was studied by AFM. Pick-to-pick values (minimum-to-maximum height) for the untreated PEF were 93.2 and 119.3 nm, with root mean squares of 7.8 and 14.9 nm, for the amorphous and crystalline samples, respectively (Table S1). Like the SEM observations, AFM revealed significant changes in the surface morphology and topography after the enzyme treatment (Figure 8a−d). The roughness increased dramatically, and pick-to-pick values varied from area to area within the same sample. For this reason, it would be not correct to provide mean values. We would rather discuss an interval of the measured values. The height variations for the enzyme-treated amorphous PEF were exceptionally large, going from 2600 to 3500 nm, while for the crystalline samples they were more moderate, between 140 and 420 nm. When the topography of the treated crystalline films is analyzed, one can see significant inhomogeneity (selectivity) in the depolymerization (Figure 7d). The protrusions were high with lateral dimensions on the micrometer (sometimes on the submicrometer) scale. Comparing the topography and phase images showed a clear difference in the contract between the protrusions and the rest of the surface (Figure S4). This observation suggests that the protrusions correspond to crystalline fractions that are less affected by the enzymes, which explains the low depolymerization ratios of the crystalline samples.

In conclusion, the enzymatic hydrolysis of PEF amorphous films (0.2 mm) was successfully performed, and the possible mechanism was discussed. Different reaction variables, viz., buffer, pH, enzyme loading, time, and substrate concentration, were optimized to increase the depolymerization process. Up to 98% weight loss was achieved within 72 h after the optimization when LCC was used. LCC also shows its potential for PEF hydrolysis at increased substrate loading. The presence of a crystalline phase was found to negatively influence the depolymerization process, significantly decreasing the weight loss ratio. Overall, LCC was more active than FastPETase, as shown by the weight loss and FDCA

https://doi.org/10.1021/acssuschemeng.4c00915
ACS Sustainable Chem. Eng. 2024, 12, 9658−9668

production, possibly owing to its higher thermostability, activity at higher temperatures, and product tolerance. FDCA was the major product of PEF hydrolysis, followed by MHEF and other oligomers, as validated using HPLC, LC-MS, and NMR. Microscopy studies of the treated films showed drastic surface erosion and revealed higher resistance of the crystalline phase to the enzymes, thus explaining the observed low depolymerization ratios. The investigations provided important insights into the hydrolysis mechanism of PEF by FastPETase and LCC, at a very low enzyme loading, improving the potential for the industrial enzymatic recycling of this new and promising Bioplastic.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.4c00915.

> Expression and production of enzymes; activity and protein assay; polymer weight loss; Nano DSF and DSC; AFM measurements; depolymerization measurements; mass balance; weight loss of PEF films in the bioreactor; LC-MS data; AFM figures; and DSC analysis of films and powders (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge the European Union for granting the H2020 UPLIFT project (Grant agreement no. 953073). The authors are also thankful to Lars Wagner Städe for his assistance with the HPLC analysis and Klaus Ringsborg Westphal for LC-MS. The NMR laboratory at Aalborg University is supported by the Obel Family, SparNord, and Carlsberg foundations.

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