1 **Manuscript**

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3 **Enzymatic hydrolysis of polyester thin films: real-time analysis of film mass**
4 **changes and dissipation dynamics**

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Abstract

Cleavage of ester bonds by extracellular microbial hydrolases is considered a key step during the breakdown of biodegradable polyester materials in natural and engineered systems. Here, we present a novel analytical approach for simultaneous detection of changes in the masses and rigidities of polyester thin films during enzymatic hydrolysis using a Quartz Crystal Microbalance with Dissipation monitoring (QCM-D). In experiments with poly(butylene succinate) (PBS) and the lipase of *Rhizopus oryzae* (RoL), we detected complete hydrolysis of PBS thin films at pH 5 and 40 °C that proceeded through soft and water-rich film intermediates. Increasing the temperature from 20 to 40 °C resulted in a larger increase of the enzymatic hydrolysis rates of PBS than that of non-polymeric dibutyl adipate. This finding was ascribed to elevated accessibility of ester bonds to the catalytic site of RoL due to increasing polyester chain mobility. When the pH of the solution was changed from 5 to 7, initial hydrolysis rates were little affected, while a softer film intermediate that lead to incomplete film hydrolysis was formed. Hydrolysis dynamics of PBS, poly(butylene adipate), poly(lactic acid), and poly(ethylene terephthalate) in assays with RoL showed distinct differences that we attribute to differences in the polyester structure.

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Introduction

The accumulation of synthetic, non-biodegradable plastic materials in the environment is a major environmental concern.¹⁻³ While plastic accumulation in aquatic systems is well documented and has received considerable research interest,^{3,4} comparatively little is known about plastic accumulation in terrestrial systems.² Among these, agricultural soils may receive direct inputs of plastics because these are heavily used in many agricultural practices to increase crop yields.^{5,6} The importance of plastics in agriculture applications is also reflected by the coined term ‘plasticulture’. One major application of polymers in agriculture are mulch films at a global use of approximately 1 million tons/year.⁶⁻⁸ These films are commonly made of non-degradable polymers (e.g. low-density polyethylene) that, if not completely removed from agricultural fields after use, pose the risk of accumulating in soils.^{7,9,10} Such accumulations are both environmentally and economically undesirable (i.e., the accumulation is expected to decrease soil productivity).^{2,11} One possibility to avoid accumulation of plastics in agricultural soils is to use plastics that biodegrade in soils.^{9,11-13} The released oligomers and monomers could be assimilated by soil microbes and used as carbon and energy sources, thereby converting the polymeric carbon into microbial biomass and carbon dioxide.

Polyesters composed of repeated units of aliphatic diols and aliphatic diacids or of aliphatic diols and aliphatic and aromatic diacids are commercially important biodegradable polymers.¹³⁻¹⁶ Ester bond cleavage (**Scheme 1**) is considered the rate-determining step in polyester biodegradation in soils and engineered systems.¹⁷

Scheme 1. Reaction equation for the hydrolytic cleavage of ester bonds in polyesters.

This reaction step can be catalyzed by extracellular microbial esterases including triacylglycerol lipases that have evolved to hydrolyze triacylglycerols into the corresponding diacylglycerols and fatty acids.^{18,19} Lipases are commonly considered to require a hydrophobic surface to reach full hydrolytic activity, a process known as ‘interfacial activation’.¹⁸ However, lipases have also been shown to hydrolyze dissolved esters in solution.²⁰

While the hydrolysis of synthetic polyesters by extracellular lipases from soil microorganisms has been reported as early as the 1970’s,¹⁹ this process has been systematically studied only recently. Deckwer and coworkers studied the enzymatic hydrolysis of polyesters using an automated pH-stat titration setup in which the amount of acid released during hydrolysis (**Scheme 1**) is quantified by titration.²¹ The rate of enzymatic polyester hydrolysis was shown to decrease with increasing difference between the melting point, T_m , of the tested polyester and the experimental temperature:²¹⁻²³ while aliphatic polyester films with low T_m between 57 and 72 °C completely hydrolyzed within 20 hours in the presence of lipases at experimental temperatures between 35 and 50 °C, polyesters with higher T_m (e.g., poly(butylene succinate) (PBS) with T_m = 114 °C) did not hydrolyze at detectable rates even at high experimental temperatures of 50 °C.²¹ The strong T_m -dependence was explained by the general decrease in polyester chain mobility with increasing T_m : only polyester chains with a sufficient degree of mobility adapt conformations required to enter the active site of the lipase.

While these studies were successful in relating enzymatic hydrolyzability to the physicochemical properties of polyesters, the used pH-stat titration approach provides limited information on enzyme-polymer interactions and potential changes in polyester structure during hydrolysis. For instance, it remained unclear whether the enzymes primarily caused lateral or vertical erosion in the polyester bulk material. These methodological limitations could be overcome by using highly sensitive *in situ* surface techniques provided that they can be used to directly monitor enzymatic hydrolysis of thin polyester films. Among these techniques is Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) which continuously monitors the resonance frequencies of piezoelectric quartz crystal sensors that are mounted in flow-through cells. The frequencies increase and decrease in response to decreasing and increasing masses that are adsorbed to the sensor surfaces, respectively. QCM-D is sensitive to changes in the adsorbed mass of as little as 1 ng/cm² and may thus detect the adsorption of hydrolytic enzymes to polyester thin films as well as the subsequent mass loss of the thin film during hydrolysis due to the release of monomers and oligomers. In addition, QCM-D monitors changes in the viscoelastic properties of adlayers by continuously detecting the energy that is dissipated during the sensor's oscillation.^{24,25} In general, energy dissipation values increase with decreasing rigidity and increasing softness of the adlayer. While QCM-D has successfully been used to study the enzymatic degradation of the polymers cellulose²⁶, polyhydroxyalkanoates (PHA)²⁷, poly(L-lactide) (PLLA)²⁸, and chitin²⁹, we are not aware of its previous use to assess the enzymatic hydrolysis of polyesters composed of diols and dicarboxylic acids.

The overall goal of a larger research effort in our group is to advance a more complete understanding of the fate of biodegradable polyesters in soils. Because enzymatic hydrolysis is considered a key process in polyester degradation, the specific

goal of the work presented here was to systematically study this process for selected polyester-lipase combinations. More specifically, this work focused on the hydrolysis of thin films of poly(butylene succinate) (PBS) by the lipase of *Rhizopus oryzae* (RoL), a soil fungus, under varying solution conditions (i.e., temperature, pH and enzyme concentrations). PBS was chosen because it is a commercially important biodegradable material and because it belongs to the larger class of biodegradable aliphatic polyesters composed of diols and dicarboxylic acids. We used QCM-D as the primary analysis technique to obtain molecular level insights into the mass changed and dissipation dynamics of PBS films during hydrolysis. We complemented selected QCM-D hydrolysis experiments with Optical Waveguide Lightmode Spectroscopy (OWLS) measurements. This method is based on detecting the hydrolysis of polyester films on OWLS sensors by monitoring the changes in the refractive indices across the sensor-solution interfaces.²⁴ To assess whether the QCM-D approach is broadly applicable and, thus has the potential to systematically assess the effects of polyester structure on enzymatic hydrolyzability in future work, we investigated the hydrolysis of three additional polyesters (i.e., poly(butylene adipate) (PBA), poly(lactic acid) (PLA) and poly(ethylene terephthalate) (PET) by RoL.

Materials and Methods

Chemicals. All chemicals used were of high purity (> 95%) and are listed in the Supporting Information, SI.

Lipase. Lipase from *Rhizopus oryzae* (RoL) was obtained from Sigma Aldrich (product number 62305) and used as received. Lipase stock solutions (concentrations of 10 mg/mL), prepared by dissolving the lipase in a pH 7 buffered solution (see below), were stored in small volume aliquots at -20 °C until use. Experimental lipase solutions (100 µg/mL, if not indicated differently) were obtained by diluting RoL stocks into sterile solutions (see below).

The effects of solution pH and temperature on RoL activity were assessed using dibutyl adipate (DBA) and glyceryl trioleate as model substrates and a pH-stat titration assay adapted from Marten et al.²¹ In short, 500 µl of DBA or glyceryl trioleate and 9.5 mL of an emulsifying solution (0.32 g/mL glycerol, 3 mg/mL gum arabicum, 10 mM KCl) were added to a thermo-jacketed beaker and emulsified using an Ultra-Turrax T18 dispersing instrument (IKA; operated for 1 min at 13500 rpm). Following equilibration of the solution temperature and pH, 20 µL of a RoL stock solution were added to the emulsion. DBA hydrolysis was monitored by continuous titration of the generated acids (**Scheme 1**) with a 20 mM KOH solution using an automated titration system (Titrando 907, Metrohm). The hydrolysis rate was calculated from the number of hydroxide ions titrated to maintain a constant pH. RoL inactivation over time was assessed by comparing the DBA hydrolysis rates between 0 and 5 minutes and between 5 and 10 minutes after enzyme addition, respectively. DBA titration assays run at pH < 7 considered the protonation equilibrium of the liberated monomethyl adipate ($pK_a = 4.69$ at 25 °C).

Polyesters. The polyesters included in this study were PBS (obtained as film), PBA, PET, and PLA (containing 11-13 % D-lactide) (obtained as granules). These materials were kindly provided by BASF SE. Key physicochemical properties of the polymers are provided in **Table 1**.

Solutions. All solutions for hydrolysis experiments were prepared in Milli-Q water (resistivity = 18.2 M Ω cm; Barnstead NANOpure Diamond) and contained 10 mM KCl as background electrolyte and 3 mM of pH buffering species (i.e., acetate (pH 4 and 5), phosphate (pH 6), or tris-(hydroxymethyl)aminomethane (pH 7 and 8)). Temperature-corrected solution pH values were adjusted to within ± 0.03 units of the nominal values using concentrated KOH or HCl. All solutions were filter sterilized (0.2 μ m cellulose acetate, Target) and degassed prior to use in hydrolysis experiments.

Formation and characterization of polyester thin films. Polyester thin films were prepared by spin coating. While this procedure results in films that are thinner than materials used in agricultural applications, enzymatic hydrolysis is a surface process. The results obtained with thin films are therefore expected to also apply to the hydrolysis of larger polyester specimen. For spin coating, the polyesters PBS, PBA, and PLA were dissolved in chloroform and PET in hexafluoroisopropanol under sonication (concentrations: 0.5% (w/w)). Subsequently, 40 μ L of the respective polyester solution were transferred onto the surface of a commercial, gold-coated QCM-D sensor (QSX 301, QSense) that was vacuum fixed on a spin coater (WS-650MZ-23NPP, Laurell Technologies). The sensor was then spun at 4000 rpm for 1 minute (acceleration: 1500 rpm/s). A similar approach was used to form thin polyester films on OWLS waveguides, as detailed in the SI.

The reproducibility of the spin coating was assessed by determining the thicknesses of selected thin films using spectroscopic ellipsometry on a M-2000F

system (J.A. Woollam C. Inc.), as detailed in the SI. For each sensor, the coated mass was calculated from the decrease in the resonance frequency measured in air at 20 °C before and after spin coating using the Sauerbrey equation, as detailed below.

The surface polarities of the spin-coated films were determined by measuring the contact angles of 2 µL sessile water droplets on the films using a DSA 100 system (Krüss GmbH) and employing the Laplace model of the instrument software (DSA 3).

In situ surface techniques. A QCM-D E4 system (Q-Sense) equipped with four flow cells was used to investigate lipase adsorption onto polyester thin films and subsequent lipase-catalyzed film hydrolysis. QCM-D continuously monitors changes in the resonance frequencies, Δf_n (Hz) and the corresponding energy dissipation values, ΔD_n , of the fundamental tone ($n=1$) and several overtones ($n= 3-13$) of an oscillating piezo-quartz crystal resonator embedded in the QCM-D sensor. Changes in the areal mass density of the adlayer on the sensor surface, Δm (ng/cm²), were determined using the Sauerbrey equation:

$$\Delta m = C * -\frac{\Delta f_n}{n} \quad \text{Eq. 1}$$

where C (17.7 ng*cm⁻²*Hz⁻¹) is the sensor-specific mass sensitivity constant and Δf_n (Hz) is the frequency shift of the n^{th} overtone. The 5th overtone was used for calculations and plotting. We note that the Sauerbrey equation is applicable only if adlayers are relatively rigid (i.e., $-\Delta D_n/(-\Delta f_n /n) \ll 4*10^{-7} \text{ Hz}^{-1}$)²⁵ while it underestimates the mass of soft and elastic adlayers.³⁰

Prior to the QCM-D hydrolysis experiment, spin-coated sensors were immersed in pH 7 solutions for approximately 14 h at 25 °C. This pre-equilibration step increased the reproducibility of the subsequent hydrolysis. After mounting the sensors into the flow cells, the experiments were started by running enzyme-free, pH-buffered solutions

over the mounted sensors at constant volumetric flow rates (20 $\mu\text{l}/\text{min}$) and temperatures. Upon attainment of stable Δf_n and ΔD_n readings, RoL-containing solutions of the same pH were delivered over the sensor surface at the same flow rate. When predefined termination criteria were reached (e.g., re-attainment of stable Δf_n and ΔD_n readings), the experiment was stopped and the sensors were removed from the flow cells, briefly rinsed with Milli-Q water and dried under a N_2 stream. Finally, the resonance frequencies of the dried sensors were determined in air at 20 $^\circ\text{C}$. The differences in the resonance frequencies before and after hydrolyses were converted into changes in the adlayer dry mass over the hydrolysis experiments. QCM-D sensors were cleaned as detailed in the SI. OWLS hydrolysis experiments were conducted in a similar manner, as detailed in the SI.

Results and discussion

Polyester thin film properties. Spin coating of each of the four polyesters investigated resulted in thin films with reproducible masses, thicknesses and surface polarities, as determined by QCM-D, ellipsometry, and contact angle measurements, respectively (**Table 1**). The film thicknesses and water contact angles were polyester-dependent and varied between 61 and 90 nm and between 65 and 76°, respectively. Small contact angles of 65° on the PBS films were consistent with the oxygen content (C/O ratio of 2) of the films. The larger contact angles on PBA and PET films were consistent with higher C/O ratios (2.5) of those films. The PLA surface was more apolar than expected based on its low C/O ratio of 1.5, possibly caused by a preferential orientation of the backbone methyl groups towards the surface.

Table 1. List of investigated polyester thin films and their characteristics. The masses, thicknesses and surface polarities of the films were determined by Quartz Crystal Microbalance with Dissipation monitoring (QCM-D), ellipsometry, and contact angle measurements, respectively. Averages ($\pm$ standard deviations) of four replicates are shown (note: separate sensors were used for the different techniques). T_m and T_g refer to the melting points and glass transition temperatures, respectively. Asterisk (*) indicates a purely amorphous polymer and therefore the absence of a T_m . M_n and M_w are the number average molecular weight and the weight average molecular weight, respectively.

Polyester	Name	Structure	T_m, T_g (°C)	M_n, M_w (g/mol)	Coating weight ($\mu\text{g}/\text{cm}^2$)	Thickness (nm)	Contact angle (°)
PBS	Poly(butylene succinate)		111.4, -35	23100, 106000	6.82 (± 0.08)	61.1 (± 1.2)	65 (± 4)
PBA	Poly(butylene adipate)		58.7, -59	19800, 52100	6.5 (± 0.3)	60.6 (± 1.4)	75 (± 3)
PLA	Poly(lactic acid)		*, 57.1	67400, 229000	8.29 (± 0.21)	72.5 (± 1.5)	75.9 (± 0.9)
PET	Poly(ethylene terephthalate)		254.7, 75	16800, 50700	10.74 (± 0.20)	90 (± 4)	74.3 (± 0.9)

Hydrolysis of PBS films by RoL. **Figure 1a** shows the results of a representative

QCM-D experiment on PBS hydrolysis by RoL at pH 5 and 40 °C. The hydrolysis experiment entailed four consecutive phases. At the onset of the experiment (phase I; $t < 0$ min), RoL-free solution was run over the coated sensor to equilibrate the PBS film to the experimental solution and temperature conditions. This procedure resulted in stable mass and dissipation readings.

Figure 1. Mass changes and dissipation dynamics of a thin poly(butylene succinate) (PBS) film during hydrolysis by the lipase of *Rhizopus oryzae* (RoL) at 40 °C and pH 5.0, as measured by Quartz Crystal Microbalance with Dissipation monitoring (QCM-D). **a.** Changes in the mass, Δm (green, left ordinate), and dissipation values, ΔD (red, right ordinate), of the adlayer over time. The time was set to zero when the lipase-containing solution was delivered to the flow cell. Roman letters indicate four distinct phases during hydrolysis, as detailed in the text. The insert shows a blow-up of the changes in the adlayer mass over the first 35 minutes after introducing the enzyme. **b.** Comparison of wet and dry mass loss during hydrolysis. At several time points during the RoL hydrolysis (as indicated by the arrows), the QCM-D experiments were stopped and the sensors were dismantled from the flow cells and dried. The insert relates the mass loss during the QCM-D experiment (ordinate; wet mass loss) to the dry mass loss of the adlayers determined on the dry sensor (abscissa, dry mass removed). **c.** Schematic models for the four phases of PBS hydrolysis by RoL. The dry mass of the sensor adlayer before (I) and after (IV) hydrolysis including standard deviations of five independent measurements indicated close to complete hydrolysis of the PBS films.

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265 Subsequent delivery of a RoL-containing solution of the same pH resulted in an
266 increase in the adsorbed mass and the energy dissipation, consistent with RoL
267 adsorption to the PBS surface (phase II; started at $t=0$ min). The dissipation increase
268 implies that PBS film with adsorbed RoL was slightly less rigid than the original PBS
269 film. Following enzyme adsorption, the adlayer mass remained constant up to
270 approximately $t=80$ minutes, while ΔD values increased slightly but continuously over
271 time. These findings are consistent with the onset of lipase-catalyzed ester hydrolysis
272 that softened the PBS surface but formed hydrolysis products that did not yet detach
273 from the film into the solution. Phase III started approximately 80 minutes after
274 switching to the RoL-containing solution: the adlayer mass started to decrease,
275 indicating substantial hydrolytic cleavage of the PBS ester bonds and the release of
276 soluble hydrolysis products from the film into the solution. During this phase, the rate
277 at which mass was lost from the sensor surface (i.e., $-\Delta(\Delta m)/\Delta t$) and the adlayer
278 dissipation value, ΔD , increased and reached maximum values at the same time (i.e.,
279 approximately 150 min after enzyme addition). The effective mass loss rate was
280 therefore highest when the film was in its most dissipative (i.e., soft) state. During phase
281 III, the PBS film was still sufficiently rigid for the Sauerbrey equation to be applicable,
282 as detailed in the SI (i.e., Eq. 1 underestimated the adlayer mass by at most 10% as
283 compared to the Voigt-Kelvin viscoelastic modeling³¹).³² After the mass loss rates and
284 ΔD reached maxima, both values decreased continuously. Finally, stable Δm and Δ
285 D values were attained in phase IV, suggesting that the surface was depleted in
286 hydrolysable ester bonds. Comparisons of the dry masses of the sensors before and after
287 PBS coating step and after hydrolysis (i.e., phase IV) revealed that the mass loss during
288 hydrolysis corresponded to $93.0 \pm 2.0\%$ (average $\pm$ standard deviation; $n=5$) of the spin-

coated PBS mass. The fractional loss of PBS was estimated to be even higher (i.e., 97%) considering that the sensor after hydrolysis had mass contributions from adsorbed RoL, which was independently assessed by adsorbing RoL to a bare QCM-D sensor (**Figure S1**). We therefore conclude that RoL completely hydrolyzed the spin-coated PBS films within three hours at pH 5 and 40 °C.

A continuous delivery of RoL-containing solutions to the flow cell was required for continuous PBS hydrolysis: control experiments showed only minor decreases in adlayer masses when the delivery of the RoL solution was stopped during hydrolysis or when the RoL solution was replaced by RoL-free solution following initial RoL adsorption to the PBS film (**Figure S2**). Additional control experiments showed decreasing PBS hydrolysis by RoL in the presence of increasing solution concentrations of urea, a chaotropic agent that is commonly used to inactivate enzymes by destabilizing their structures (**Figure S3**). These findings suggest that sustained hydrolysis required a continuous delivery of active lipase to the flow cells and removal of hydrolysis products and/or formed acids from the flow cells. Therefore, all experiments were conducted under constant delivery of a RoL solution.

We further note that PBS hydrolysis rates at pH 5 and 40 °C increased with increasing RoL concentrations from 50 to 200 µg/mL (**Figure S4**). Plots of the adlayer mass decreases versus the cumulative amount of RoL passed through the flow cell resulted in overlapping curves for experiments run with RoL concentrations of 50 and 100 µg/mL, demonstrating that the rate of PBS hydrolysis was controlled by the amount of enzyme delivered to the PBS films. The same normalization revealed slower relative PBS hydrolysis at 200 µg RoL/mL suggesting that hydrolysis rates started to be limited by the accessibility of ester bonds rather than RoL concentration.

We complemented the QCM-D experiments on PBS hydrolysis by RoL at pH 5 and 40 °C with OWLS experiments. The two instruments have different measurement principles: With OWLS, one is able to monitor changes in the refractive index at the sensor-solution interface and, hence, to sense only changes in the interfacial masses of PBS and RoL (i.e., the ‘dry’ adlayer mass). Conversely, QCM-D measurements are based on the detection of changes in the oscillation frequency of the sensor. Thereby, the entire mass of the adlayer that couples to the oscillation, including adlayer-associated water (i.e., the ‘wet’ adlayer mass) is measured.^{24,25,33-35} The OWLS data showed the same four phases in the adlayer mass dynamics that we identified before employing QCM-D (**Figure S5**, SI). A direct comparison of the rates and final extents of hydrolyses measured by both techniques is, however, impossible given that the spin-coated masses on the QCM-D and OWLS sensors differed and that the QCM-D and OWLS flow cells have different geometries and thus different hydrodynamic flow conditions. The different measurement principles are an explanation for the earlier detection of PBS mass loss employing OWLS as compared to the QCM-D experiments, in which initial PBS mass loss was likely compensated by water incorporation into the adlayer.

The contribution of water to the QCM-D-sensed adlayer mass was separately determined in QCM-D experiments in which PBS hydrolysis was terminated at different time points. After removal of the sensors from the flow cells, the dry mass of the remaining adlayer was determined by QCM-D in air. The dry mass loss of the adlayer was calculated from the mass difference between the dry sensor before and after hydrolysis. **Figure 1b** shows that the loss of dry mass during intermediate phases of hydrolysis was much larger than the loss of wet mass, implying that the PBS thin films incorporated significant amounts of water during hydrolysis. For example, 120 minutes

after RoL addition only $1.6 \mu\text{g}/\text{cm}^2$ of the wet mass but $3.4 \mu\text{g}/\text{cm}^2$ of dry mass was removed from the sensor. The mass of water incorporated into the film thus compensated for more than half of the dry mass lost during hydrolysis. The high water contents of the films during hydrolysis were consistent with the high ΔD values in intermediate phases of the hydrolysis. The good agreement between wet and dry mass losses when phase IV was reached, indicated similar extents of water coupling to the film in phases I and IV.

The hydrolysis dynamics of the PBS thin films based on the QCM-D data are shown schematically in **Figure 1c**. Phase I corresponded to a rigid and compact PBS thin film. The adsorption of RoL to the PBS surfaces and the subsequent onset of hydrolysis resulted in slightly more dissipative films (phase II). Continuous hydrolysis resulted in sponge-like, soft and water-rich PBS adlayers (phase III) that contained significant amounts of water. It is possible that hydrolysis during the onset of this phase was autocatalytic in that the PBS surface area accessible to RoL increased over time. We note that the formation of such PBS intermediates would not have been apparent from OWLS data, thereby highlighting the advantages of QCM-D. Similar soft intermediates have previously been reported for the hydrolysis of cellulose and chitin films by cellulases and chitinases, respectively, measured by QCM-D.^{26,29} Finally, the entire PBS film has been hydrolyzed when phase IV was reached.

Effect of solution pH on PBS hydrolysis by RoL. **Figure 2a** shows the results of representative QCM-D experiments on the hydrolysis of PBS by RoL at 40 °C and different pH-values of the enzyme solutions. The corresponding changes in the dissipation values are given in **Figures 2b** (pH 5 and 7) and **S6** (all pH values). The pH dependency of PBS hydrolysis rates and extents by RoL are summarized in **Figure 2c**. For each experiment, the hydrolysis rate was estimated by the inverse of the time

required to reach maximal film dissipation, $1/t(\Delta D_{\max})$, as detailed in the SI (**Figure S7**).

The hydrolysis of PBS thin films by RoL at pH 6 exhibited comparable mass and dissipation dynamics as well as rates and final extents as previously discussed for pH 5. At pH 7, initial PBS hydrolysis rates were comparable to those at pH 5 and 6. However, hydrolysis plateaued when approximately $3.8 \mu\text{g}/\text{cm}^2$ of the initial mass was released from the sensor. The corresponding loss in adlayer dry mass determined after dismounting and drying of the sensors was much larger (i.e., approximately $6 \mu\text{g}/\text{cm}^2$ or 77% of the coated dry mass; **Figure S8**). This finding implies that the final PBS adlayer at pH 7 contained significant amounts of water, consistent with the high final ΔD values (**Figure 2b**). We note that the Sauerbrey equation may have underestimated the wet mass of the remaining adlayer due to its high energy dissipation. No hydrolysis of the PBS films by RoL was detected by QCM-D at $\text{pH} \geq 7.5$ (**Figure 2a and 2c**). The slight increase in adlayer mass at such pH values reflects the adsorption of RoL to the PBS films. At pH 4, PBS hydrolysis was slightly slower and less extensive (i.e., removal of 82% of the dry adlayer mass) than at pH 5 and 6.

To complement the PBS data, we also determined the pH dependence of RoL-mediated hydrolysis of dibutyl adipate (DBA) and glyceryl trioleate (as a model for triglycerides typically hydrolyzed by RoL) using a pH-stat titration assay. RoL hydrolyzed DBA from pH 5 to 9 with temperature-dependent activity maxima at pH 7 (40 °C) and pH 8 (20 °C) (**Figure 2d**). Between pH 5 and 8, overall RoL activities at 40 °C were higher than those at 20 °C. This is consistent with an increase in reaction rates with increasing temperature according to the Arrhenius rate law. From pH 7 to 9, DBA hydrolysis rates at 40 °C decreased strongly, whereas those at 20 °C changed more gradually. We ascribe the loss in RoL activity at $\text{pH} > 7$ and 40 °C to thermal

inactivation of RoL at high pH. This explanation is supported by the finding that DBA hydrolysis between five and ten minutes after RoL addition is much slower than that over the first five minutes after RoL addition (**Figure 2e and Figure S9**). The pH and T dependencies of glyceryl trioleate hydrolysis by RoL were similar to those of DBA, as detailed in the SI (**Figure S10**).

Measurable DBA hydrolysis by RoL at pH 8 rules out that incomplete PBS hydrolysis at pH 7 and the absence of PBS hydrolysis at $\text{pH} \geq 7.5$ were due to a general inactivity of RoL at these pH values. Instead, incomplete PBS hydrolysis at pH 7 may have resulted from electrostatic repulsion between the negatively charged carboxylate groups that were formed during the film hydrolysis and the more negatively charged RoL surface at pH 7 than pH 5 (graphical representations of enzyme isopotential surfaces are shown in **Figure S11**). This explanation is supported by control experiments in which a decrease in the pH of the RoL solution from 7 to 5 after attainment of the dissipation maximum at pH 7 resulted in decreasing dissipation and continuous release of wet mass from the sensor (**Figure S12**). It is conceivable that deprotonation of liberated carboxylic acid moieties in the formed dissipative film at pH 7 resulted in a large negative charge that impaired RoL adsorption due to electrostatic repulsion. Absence of PBS hydrolysis at $\text{pH} \geq 7.5$ may have resulted from a decreased thermal stability of RoL adsorbed to PBS as compared to RoL in the DBA emulsion. At pH 4 and 40 °C, RoL hydrolyzed a large extent of the PBS film within 5 hours, although DBA hydrolysis results showed a strong decrease in RoL activity with decreasing pH (we note that DBA hydrolysis at pH 4 was experimentally inaccessible by the pH-stat titration assay due to extensive protonation of the liberated carboxylic acids). These differences between the PBS and DBA data highlights that esterase activities measured in solutions or emulsions with low molecular weight substrates are

413 not very good predictors for esterase activity on polyester films with dynamic
 414 hydrolysis features.

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Figure 2. Effects of solution pH and temperature on the hydrolysis of poly(butylene succinate) (PBS) thin films by the lipase of *Rhizopus oryzae* (RoL). **a.** Representative curves showing the pH-dependent changes in adlayer masses during the hydrolysis of PBS by RoL at 40 °C, as investigated by Quartz Crystal Microbalance with Dissipation monitoring (QCM-D). **b.** Mass (solid) and dissipation (dotted) dynamics during the hydrolysis of PBS at 40 °C and pH 5 (green) or pH 7 (red) as determined by QCM-D. **c.** Estimated PBS hydrolysis rates and fractions of PBS removed as a function of solution pH as determined by QCM-D. Error bars represent standard errors of at least triplicate measurements. Asterisks indicate pH values at which no PBS hydrolysis was detected over 10 h (only duplicate measurements, error bars for dry mass fractions show ranges of duplicates). **d.** pH dependent activity of RoL, as determined in pH-stat titration experiments on the hydrolysis of dibutyl adipate (DBA). The activity unit of 1 kat corresponds to 1 mol of butanol liberated per second. **e.** DBA hydrolysis activity ratios of RoL with respect to solution pH as investigated by pH- stat titration experiments. **f.** Temperature dependent changes in the adlayer wet masses during the hydrolysis of PBS thin films by RoL at pH 5, as measured by QCM-D. **g.** Estimated PBS hydrolysis rates based on data from the QCM-D experiments. Error bars represent standard errors of at least triplicate measurements (or ranges of duplicate experiments, where indicated by ‡). Asterisks (*) indicate conditions at which no PBS hydrolysis was detected over 10 h by QCM-D (only duplicate measurements). **h.** Temperature-dependency of the hydrolytic activity of RoL on DBA, determined at pH 5 and 7 using the pH-stat titration assay. 1 kat corresponds to 1 mol of butanol liberated per second. Mass and dissipation curves for pH 5 and 40 °C were re-plotted from Figure 1a for comparison.

Effect of temperature on the hydrolysis of PBS by RoL. **Figures 2f** and **S13** show representative results of the effect of temperature on the PBS adlayer mass and dissipation dynamics during hydrolysis by RoL at pH 5, respectively. The temperature dependence of estimated hydrolysis rates are shown in **Figure 2g**. These data show that PBS hydrolysis at pH 5 was highly temperature dependent: while no hydrolysis by RoL was detectable at 20 °C, the rates of hydrolysis increased when the temperature was increased to 30 and 40 °C. A further temperature increase from 40 to 45 °C resulted in little further enhancement of the hydrolysis rates. Compared to PBS, the rates of hydrolysis of DBA by RoL at pH 5 were much less dependent on temperature: the rates continuously increased from $0.24 \cdot 10^{-7}$ kat/mg at 20 °C to $0.61 \cdot 10^{-7}$ kat/mg at 50 °C and then decreased slightly to $0.40 \cdot 10^{-7}$ kat/mg at 55 °C (**Figure 2h**). The more pronounced temperature dependence of PBS hydrolysis rates when compared to hydrolysis rates of DBA can be ascribed to temperature having not only a positive effect on enzyme catalysis (which affects the hydrolysis of both PBS and DBA) but also on the flexibility in the PBS polymeric backbone rendering it more susceptible to enter the

catalytic site of RoL.²¹ Consistent with this explanation, the absence of detectable PBS hydrolysis at 20 °C likely resulted from insufficient backbone flexibility at this temperature. The small enhancement in PBS hydrolysis at pH 5 upon increasing the temperature from 40 to 45 °C suggests that sufficient backbone mobility was reached already at 40 °C and/or that an additional increase in backbone flexibility was largely compensated by thermal RoL inactivation at these temperatures. Similar to the rates at pH 5, PBS hydrolysis rates at pH 7 also increased upon increasing the temperature from 20 to 40 °C (**Figures 2g** and **S14**). However, PBS hydrolysis rates at pH 7 decreased substantially when the assay was performed at 45 °C, likely due to decreased thermal stability of RoL at high pH, as detailed above.

Hydrolysis of PLA, PBA and PET. **Figures 3a** and **S15** show the results of representative mass and dissipation dynamics of PBA, PLA, and PET thin films over the course of QCM-D hydrolysis experiments with RoL at pH 5 and 40 °C. All polyester films showed initial mass increases upon RoL delivery, demonstrating that RoL adsorbed to all tested polyesters. RoL subsequently hydrolyzed PBA and PLA, but not PET, as detailed further below. Similar to PBS, RoL-containing solutions had to be continuously delivered to the PBA and PLA films to sustain enzymatic hydrolysis at well detectable rates (**Figure S16**).

The complete hydrolysis of PBA films by RoL required less time than the complete hydrolysis of PBS films of comparable thicknesses, as evidenced from both QCM-D and OWLS experiments (**Figures 3a** and **S4**). Faster hydrolysis of PBA as compared to that of PBS was expected based on findings of Marten et al.²¹ who detected hydrolysis of PBA but not of PBS using *Pseudomonas species* lipase and a pH-stat titration assay. Faster PBA hydrolysis can be rationalized based on its lower T_m (**Table 1**) as compared to PBS and, therefore, the presumably higher polyester backbone

mobility within crystalline domains at 40 °C in PBA than PBS. We note that the faster hydrolysis of PBA mainly resulted from a shorter time that was required to initiate mass loss of the PBA adlayer (shorter phase II), while the mass loss rates during phase III were comparable for PBS and PBA. The earlier mass loss observed for PBA, as compared to PBS, coincided with a steeper increase in the dissipation value per decrease in adlayer mass (i.e., larger $\Delta D/\Delta m$ values in **Figure 3b**), suggesting that the PBA film was more readily converted into a soft, sponge-like intermediate than the PBS film. This finding shows that the length of the dicarboxylic acid unit alters the hydrolysis dynamics of aliphatic polyesters. Future work is required to systematically assess the effect of the dicarboxylic acid unit on the hydrolysis dynamics.

Similar to PBS and PBA, PLA films showed stable mass readings in the absence of RoL at $t \leq 0$ min, suggesting the absence of non-enzymatic PLA hydrolysis under these experimental conditions. Consistently, literature studies suggest that chemical PLA hydrolysis require higher temperatures.^{36,37} The changes in PLA film mass and dissipation values during RoL-mediated hydrolysis at $t > 0$ min were distinctly different from those of PBS and PBA. Following initial RoL adsorption, the PLA wet adlayer mass started to decrease at an approximately constant rate over two hours. The mass loss rate decreased and the wet adlayer mass leveled off after about 5 h at a mass loss slightly above 4 $\mu\text{g}/\text{cm}^2$. Thereafter, the wet adlayer mass slowly increased. At the same time, the adlayer dissipation values showed a pronounced increase (**Figure 3b**). Finally, the adlayer wet mass leveled off at approximately -2.5 $\mu\text{g}/\text{cm}^2$. However, the wet mass of the final adlayer may have been underestimated due to its very high dissipation values (i.e., $\Delta D \approx 80 \cdot 10^{-6}$). Approximately 70% of the spin-coated dry PLA mass was hydrolytically removed (**Figure 3c**).

502 The increases in the mass and dissipation values in later phases of the PLA
503 hydrolysis strongly suggest that a significant amount of water was incorporated into the
504 film. The incorporation of water as the cause of the increases in wet adlayer mass is
505 supported by the absence of such mass increases in OWLS experiments run under the
506 same conditions (**Figure S5**). We speculate that the formation of a negatively charged,
507 polar and water-rich PLA adlayer impaired further hydrolysis due to the lack of
508 sufficiently apolar adsorption sites required for interfacial activation of RoL. We note
509 that the formation of a water-rich PLA film with impaired hydrolyzability may be RoL
510 specific. A previous study that combined atomic force microscopy and standard QCM
511 without dissipation monitoring to determine PLLA hydrolysis by proteinase K did not
512 report the formation of a water-rich film intermediate.²⁸

513 No mass loss was detected from the PET adlayer at 40 °C over a ten-hour
514 exposure to RoL solution. This finding suggests that the ester bonds in PET were not
515 accessible to RoL at this temperature, consistent with the high T_m (255 °C) of PET
516 (**Table 1**). Future studies are warranted to assess PET hydrolysis by esterases that have
517 previously shown to hydrolyze PET in batch experiments.³⁸⁻⁴⁰

Figure 3. Mass and dissipation dynamics of the polyesters poly(butylene succinate) (PBS), poly(butylene adipate) (PBA), poly(lactic acid) (PLA), and poly(ethylene terephthalate) (PET) by lipase from *Rhizopus oryzae* (RoL) at pH 5 and 40 °C. **a.** Representative changes in PBS, PBA, PLA and PET adlayer masses during exposure to RoL, as investigated by Quartz Crystal Microbalance with dissipation monitoring (QCM-D). **b.** Changes in adsorbed wet masses versus the corresponding changes in adlayer dissipation values. The same experiments as in panel (a.) are shown. **c.** Adlayer dry masses before (empty bars) and after (dashed bars) the hydrolysis experiments, as determined by QCM-D. Error bars represent the standard deviations of five replicates for PBS and the ranges of duplicate measurements for PBA, PLA, and PET. The PET experiment was stopped after seven hours of RoL exposure. The PBS data is re-plotted from Figure 1a for comparison.

Implications

On a fundamental process level, this work shows that the hydrolysis of polyester films catalyzed by a lipase isolated from a soil fungus forms films that are soft and water-rich. We postulate that the microenvironment created in these soft films greatly affects the accessibility of ester bonds to esterases and thereby controls the rates of ester hydrolysis. While these soft states were intermediates for some films that were completely hydrolyzed, the soft films slowed down hydrolysis for other films. Future work is required to systematically assess whether the formation of these soft films and the hydrolysis of ester bonds in these films are linked to specific polyester structures and/or to the properties of the hydrolytic enzymes. More importantly, however, our results suggest that initial hydrolysis rates are inadequate for predicting bulk film hydrolysis. Instead, we propose that film hydrolysis dynamics are investigated to obtain a better understanding of polyester hydrolysis by microbial extracellular enzymes in natural and engineered systems.

From a methodological perspective, this work presents a novel QCM-D based approach for studying the mass and dissipation dynamics of polyester thin films during enzymatic hydrolysis at high sensitivity. As compared to pH-stat titration assays and optical *in situ* techniques, the QCM-D approach has the advantage that not only information on the rate and extent of polyester film hydrolysis, based on detection of the sensor associated mass, but also on the changes in the viscoelastic properties of the

551 films during hydrolysis, based on detection of the energy that is dissipated during the
552 oscillation, can be obtained. As shown for selected polyester-lipase combinations in
553 this work, the employment of the approach enables deciphering the effects of polyester
554 composition, solution pH and temperature on the enzymatic polyester hydrolysis.

555 The experimental approach developed herein opens the possibility to
556 systematically assess enzymatic hydrolysis of different polyesters to correlate polyester
557 structure with hydrolyzability. Such structure-activity relationships will serve as
558 valuable references for complementary polyester degradation experiments that more
559 closely mimic natural conditions, including laboratory incubations of the polyesters in
560 soils and the burial of polyesters in agricultural fields. Furthermore, these relationships
561 can be used to identify potentially distinctive polyester hydrolysis mechanisms among
562 different esterase subclasses (e.g. lipases versus classical esterases⁴¹⁻⁴³).

565 **Associated content**

566 Supporting Information. Additional information and data on chemicals used,
567 QCM-D and OWLS experiments and pH-stat titration measurements. This material is
568 available free of charge via the Internet at <http://pubs.acs.org>.

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571 **Notes**

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