

# 1 Biochemical characterization and NMR study of a 2 PET-hydrolyzing cutinase from *Fusarium solani pisi*

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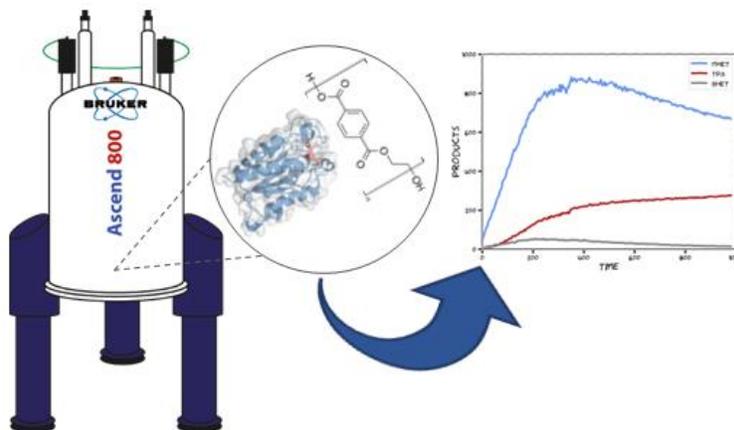
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11 **TOC Graphic (For Table of Contents use only)**



12

## 13 **ABSTRACT**

14 In recent years, the drawbacks of plastics have become evident, with plastic pollution becoming  
15 a major environmental issue. There is an urgent need to find solutions to efficiently manage  
16 plastic waste by using novel recycling methods. Biocatalytic recycling of plastics by using  
17 enzyme-catalyzed hydrolysis is one such solution that has gained interest, in particular for  
18 recycling polyethylene terephthalate (PET). To provide insights into PET hydrolysis by  
19 cutinases, we have here characterized the kinetics of a PET-hydrolyzing cutinase from *Fusarium*  
20 *solani pisi* (FsC) at different pH values, mapped the interaction between FsC and the PET analog  
21 BHET by using NMR spectroscopy, and monitored product release directly and in real time by  
22 using time-resolved NMR experiments. We found that primarily aliphatic side chains around the  
23 active site participate in the interaction with BHET, and that pH conditions and mutation around  
24 the active site (L182A) can be used to tune the relative amounts of degradation products.  
25 Moreover, we propose that the low catalytic performance of FsC on PET is caused by poor  
26 substrate binding combined with slow MHET hydrolysis. Overall, our results provide insights  
27 into obstacles that preclude efficient PET hydrolysis by FsC and suggest future approaches for  
28 overcoming these obstacles and generating efficient PET-hydrolyzing enzymes.

## 29 **INTRODUCTION**

30 Enzymatic depolymerization of polyethylene terephthalate (PET) by cutinases (EC 3.1.1.74)<sup>1,2</sup>  
31 and cutinase-like PETases (EC 3.1.1.101)<sup>3,4</sup> have recently received enormous attention because  
32 of their ability to hydrolyze the scissile ester bonds in PET, yielding well-defined products  
33 (BHET: bis(2-hydroxyethyl) terephthalate; MHET: mono(2-hydroxyethyl) terephthalic acid;  
34 TPA: terephthalic acid; EG: ethylene glycol) that can be reused to make new plastics.<sup>1,5</sup> These  
35 enzymes have thus provided a novel alternative to thermomechanical recycling of plastics, a

36 process in which only clear, homogenous plastic can be recycled with quality loss in each cycle  
37 (i.e., downcycling)<sup>6</sup>.

38 Important enzymes for PET hydrolysis include a PETase from *Ideonella sakaiensis* (IsP)<sup>2-4</sup>,  
39 and cutinases from *Thermobifida fusca* (TfC)<sup>2,7,8</sup>, *Humicola insolens* (HiC)<sup>2,9,10</sup>, *Fusarium solani*  
40 *pisi* (FsC)<sup>9,11</sup>, and leaf-branch compost cutinase (LCC)<sup>1</sup>. Cutinases are serine esterases that  
41 possess a Ser-His-Asp catalytic triad<sup>12</sup>. They have a characteristic  $\alpha/\beta$ -hydrolase fold and  
42 naturally hydrolyze ester bonds in cutin, an insoluble polyester in plant cuticle composed of  
43 hydroxy and epoxy fatty acids<sup>13</sup>.

44 With increasing implementation of enzymes in plastic recycling processes, a “polyester  
45 biorefinery” may be envisioned in which hydrolysates from PET feed stocks can be used for  
46 different recycling and upcycling applications<sup>14</sup>. In this context, it would be desirable to not only  
47 increase the catalytic efficiency<sup>15</sup> and thermostability<sup>16</sup> of PET hydrolases, but also understand  
48 how reaction conditions influence product distribution. Moreover, overcoming factors limiting  
49 the catalytic efficiency of the enzymes is a requirement for their efficient use.

50 In order to shine light on these issues, we have used a combination of NMR spectroscopy and  
51 UV-based assays to characterize FsC (UniProtKB: P00590). Using continuous time-resolved  
52 NMR experiments we followed the hydrolysis of PET by FsC under different pD values, and  
53 used the backbone amide resonances to probe the interaction of an inactive S120A-FsC mutant  
54 with BHET. Moreover, we applied a suspension-based assay<sup>17</sup> to derive inverse Michaelis-  
55 Menten kinetic parameters for FsC. Overall, our results provide useful biochemical insights into  
56 PET hydrolysis by FsC.

## 57 MATERIALS AND METHODS

### 58 *Particle size measurement*

59 The particle size distribution of the PET powder was measured with a Mastersizer 3000 Hydro  
60 MV (Malvern) instrument. Approximately 0.5 g of PET powder was dissolved in 10 mL 96%  
61 ethanol. Solutions were added to the cell dropwise until an obscuration of 4% was obtained.  
62 Refractive indices of 1.636 and 1.360 were used for PET and ethanol, respectively, and a particle  
63 absorption index of 0.010 was used. The data from five measurements was analyzed using the  
64 Mastersizer software, which provides average particle size parameters (volume mean diameter  
65 and surface mean diameter), as well as the specific surface area of the particles.

### 67 *Protein production and purification*

68 Recombinant *E. coli* ER2566 cells (New England Biolabs T7 Express) harboring the  
69 pFCEx1D plasmid (containing wild-type FsC, S120A-FsC or L182A-FsC) were incubated in 5  
70 mL precultures containing LB supplemented with 100 µg/mL ampicillin at 30 °C and 225 rpm  
71 for 16 hours. Main cultures were made by inoculating 500 mL of either 2xLB (20 g/L tryptone,  
72 10 g/L yeast extract, 5 g/L NaCl) or <sup>15</sup>N-enriched minimal M9 medium (6 g/L Na<sub>2</sub>HPO<sub>4</sub>, 3 g/L  
73 KH<sub>2</sub>PO<sub>4</sub>, 0.5 g/L NaCl supplemented with 98% (<sup>15</sup>NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 4 g/L D-(+)-glucose, 5 mL Gibco  
74 MEM Vitamin Solution (100x), 300 mg/mL MgSO<sub>4</sub>, 2 mg/L ZnSO<sub>4</sub>, 10 mg/L FeSO<sub>4</sub>, 2 mg/L  
75 CuSO<sub>4</sub>, and 20 mg/L CaCl<sub>2</sub>) with 1% preculture, followed by incubation at 25 °C and 225 rpm.  
76 At OD<sub>600</sub> = 1.7 – 1.9, the cells were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside  
77 followed by further incubation at 25 °C and 225 rpm overnight.

78 Cells were harvested by centrifugation for 5 min at 5000 g and 4 °C, and periplasmic fractions  
79 were prepared by the osmotic shock method as follows. The pellet was gently resuspended on ice

80 in 50 mL TES buffer (100 mM Tris HCl, 500 mM sucrose, 0.5 mM ethylenediaminetetraacetic  
81 acid (EDTA), pH 7.5) with a cOmplete ULTRA protease inhibitor tablet (Roche). After 10 min  
82 centrifugation at 6500 g, the pellet was resuspended on ice in 50 mL ultrapure water. The  
83 suspension was then centrifuged for 15 min at 15000 g followed by 30 min at 21000 g. The TES  
84 and water fractions were dialyzed at 4 °C in 2 L reverse-osmosis water overnight. Equilibration  
85 buffer (25 mM Na-acetate pH 5.0) was added to both fractions followed by centrifugation at  
86 7000 g and 4 °C for 5 min. The supernatant was filtered using a filter (0.2 µm pore size) prior to  
87 further protein purification.

88 The proteins were purified by loading the periplasmic extracts in a 20 mM Na-acetate buffer  
89 pH 5.0 onto a 5 mL HiTrap CM FF cation exchanger (Cytiva) connected to an ÄKTApure FPLC  
90 system (Cytiva). All steps were performed at a flow rate of 5 mL/min. Proteins were eluted by  
91 using a linear salt gradient (0 – 500 mM NaCl). FsC and S120A-FsC eluted at 40 – 120 mM  
92 NaCl. Eluted fractions were analyzed using sodium dodecyl sulphate-polyacrylamide gel  
93 electrophoresis (SDS-PAGE; Figure S1) gels run under denaturing conditions using SurePAGE  
94 Bis-Tris gels (GenScript) and MES-SDS running buffer (GenScript) followed by staining using  
95 the eStain L1 Protein Staining System (GenScript). PAGE-MASTER Protein Standard Plus  
96 (GenScript) was used for the identification of target proteins.

97 The fractions containing wild-type FsC, S120A-FsC or L182A-FsC were pooled and  
98 concentrated using centrifugal concentrators (10 kDa cut-off, Sartorius). The protein  
99 concentration was calculated by measuring  $A_{280}$  using Nanodrop and the theoretical extinction  
100 coefficient ( $\epsilon = 14690 \text{ M}^{-1} \text{ cm}^{-1}$ ), which was estimated using the ProtParam server  
101 (<https://web.expasy.org/protparam/>)<sup>18</sup>. The yields were calculated to be approximately 40 mg  
102 protein per L cell culture.

### 103 *Interactions with BHET*

104 Interactions between S120A-FsC and BHET were probed by measuring chemical shift  
105 perturbations (CSP) as follows. A  $^{15}\text{N}$ -HSQC spectrum of  $^{15}\text{N}$ -labeled S120A-FsC (175  $\mu\text{M}$ ) in a  
106 buffer consisting of 25 mM phosphate pH 5 and 10 mM NaCl with 10% D<sub>2</sub>O, was recorded at  
107 313K as a reference. BHET was dissolved in another sample of  $^{15}\text{N}$ -S120A-FsC (175  $\mu\text{M}$ ), and  
108 the two samples were combined in different proportions to obtain the following BHET  
109 concentrations: 0.3 mM, 1.1 mM, 3.6 mM, 5.5 mM, and 7.6 mM while keeping the protein  
110 concentration constant.  $^{15}\text{N}$ -HSQC spectra were recorded for each BHET concentration. CSP in  
111 amide pairs were expressed as the combined chemical shift change,  $\Delta\delta_{comb} =$   
112  $\sqrt{(\Delta\delta H)^2 + (\Delta\delta N/R_{scale})^2}$ .

113 where  $\Delta\delta H$  and  $\Delta\delta N$  are the CSP of the amide proton and nitrogen, respectively, and  $R_{scale}$   
114 was set 6.5<sup>19</sup>. The dissociation constant,  $K_D$ , was calculated by fitting CSP to a two-site fast  
115 exchange model,  $\Delta\delta_{comb} = \Delta\delta_{max} \frac{([P]+[L]+K_D) - \sqrt{([P]+[L]+K_D)^2 - 4[P][L]}}{2[P]}$ .

116 where  $\Delta\delta_{max}$  is the CSP at full saturation, and  $[P]$  and  $[L]$  are respectively the concentration of  
117 S120A-FsC and BHET.

118 These NMR spectra were recorded in a Bruker Ascend 600 MHz spectrometer equipped with  
119 an Avance III HD console and a 5-mm cryogenic CP-TCI z-gradient probe at the NV-NMR  
120 laboratory at NTNU.

121

### 122 *Suspension-based assay*

123 The kinetics of the FsC reaction on PET were measured by using a suspension-based assay  
124 originally described by Bååth et al<sup>17</sup>, at three different pH values (5.0, 6.5 and 9.0). Reactions  
125 were set up in triplicate in Eppendorf tubes with a total volume of 600  $\mu\text{L}$ , containing 10  $\text{g L}^{-1}$

126 crystalline PET powder (GoodFellow product code ES306031;  $37.7 \pm 2.6\%$  crystallinity<sup>20</sup>),  
127 enzyme concentrations varying between 0 – 1  $\mu\text{M}$ , and either a 25 mM sodium acetate buffer pH  
128 5.0, a 25 mM sodium phosphate buffer pH 6.5 containing 50 mM NaCl, or a 50 mM glycine  
129 buffer pH 9.0.

130 The reactions were incubated in an Eppendorf ThermoMixer C at 40 °C and 450 rpm for 7  
131 hours. At 0, 1, 3, 5 and 7 hours 100  $\mu\text{L}$  of were transferred from each reaction to a 96-well  
132 MultiScreen<sub>HTS</sub> HV Filter Plate (0.45  $\mu\text{m}$  pore size; Millipore), and the reactions were stopped by  
133 vacuum filtering using a Vac-Man 96 Vaccum Manifold (Promega) onto a 96-well Clear Flat  
134 Bottom UV-Transparent Microplate (Corning). PET hydrolysis products were quantified by  
135 measuring  $A_{240}$  in a Spectramax Plus 284 microplate reader (Molecular Devices) and  
136 concentrations were calculated by using a standard curve made with 15, 30, 60, 90, 120 and 150  
137  $\mu\text{M}$  TPA (Figure S2).

### 138 139 *Time-resolved <sup>1</sup>H-NMR experiments*

140 Time-resolved <sup>1</sup>H-NMR experiments were carried out in a Bruker Avance III HD 800 MHz  
141 spectrometer equipped with a 5-mm cryogenic CP-TCI z-gradient probe at the NV-NMR  
142 laboratory at NTNU.

143 The buffers used were the same as for the suspension-based assay, but they were lyophilized  
144 and redissolved in 99.9% D<sub>2</sub>O (pD 5.0) prior to use, giving pD values at 5.0, 6.5, and 9.0.  
145 Reactions (600  $\mu\text{L}$ ) were prepared in 5 mm NMR tubes and contained an amorphous PET film  
146 (GoodFellow product code ES301445;  $2.0 \pm 1.6\%$  crystallinity<sup>20</sup>) cut into a size of 30x4x0.25  
147 mm, buffer, FsC (10  $\mu\text{M}$ ) and TSP (trimethylsilylpropanoic acid; 400  $\mu\text{M}$ ).

148 After adding wild-type FsC or FsC-L182A, samples were immediately inserted into the  
149 spectrometer, where they were incubated for 17.5 hours at 40 °C. A solvent-suppressed <sup>1</sup>H

150 spectrum was acquired every 5 min by using a modified version of the 1D NOESY pulse  
151 sequence with presaturation and spoil gradients (noesygprr1d). Briefly, a 2D matrix was made  
152 with the direct dimension (TD2 = 32k) corresponding to the 1D  $^1\text{H}$  experiment spectrum, and the  
153 indirect dimension (TD1 = 196) corresponding to number of individual experiments. The  
154 experiment time was determined by the acquisition time (AQ = 1.7 s), number of scans (NS =  
155 32), the NOESY mixing time (D8 = 10 ms), the relaxation delay (D1 = 4 s), and an  
156 interexperiment delay (D14 = 130 s).

157 Signals corresponding to the aromatic protons of BHET, MHET and TPA were integrated  
158 using the serial integration (intser) routine in Bruker TopSpin version 4.1.3.

159

## 160 **RESULTS AND DISCUSSION**

### 161 *Particle size distribution of PET powder*

162 Enzymatic activity on PET is affected by the physical properties of the substrate, like percent  
163 crystallinity, particle size, and accessible surface area<sup>20</sup>. To provide more information about  
164 commonly used PET substrates we used laser diffraction to measure the particle size distribution  
165 (volume-weighted mean diameter,  $D[4,3] = 103 \pm 1 \mu\text{m}$ ; surface area-weighted mean diameter,  
166  $D[3,2] = 65.3 \pm 0.7 \mu\text{m}$ ) and specific surface area ( $92 \pm 1 \text{ mm}^2 \text{ mg}^{-1}$ ) of crystalline PET powder  
167 (Figure S3).

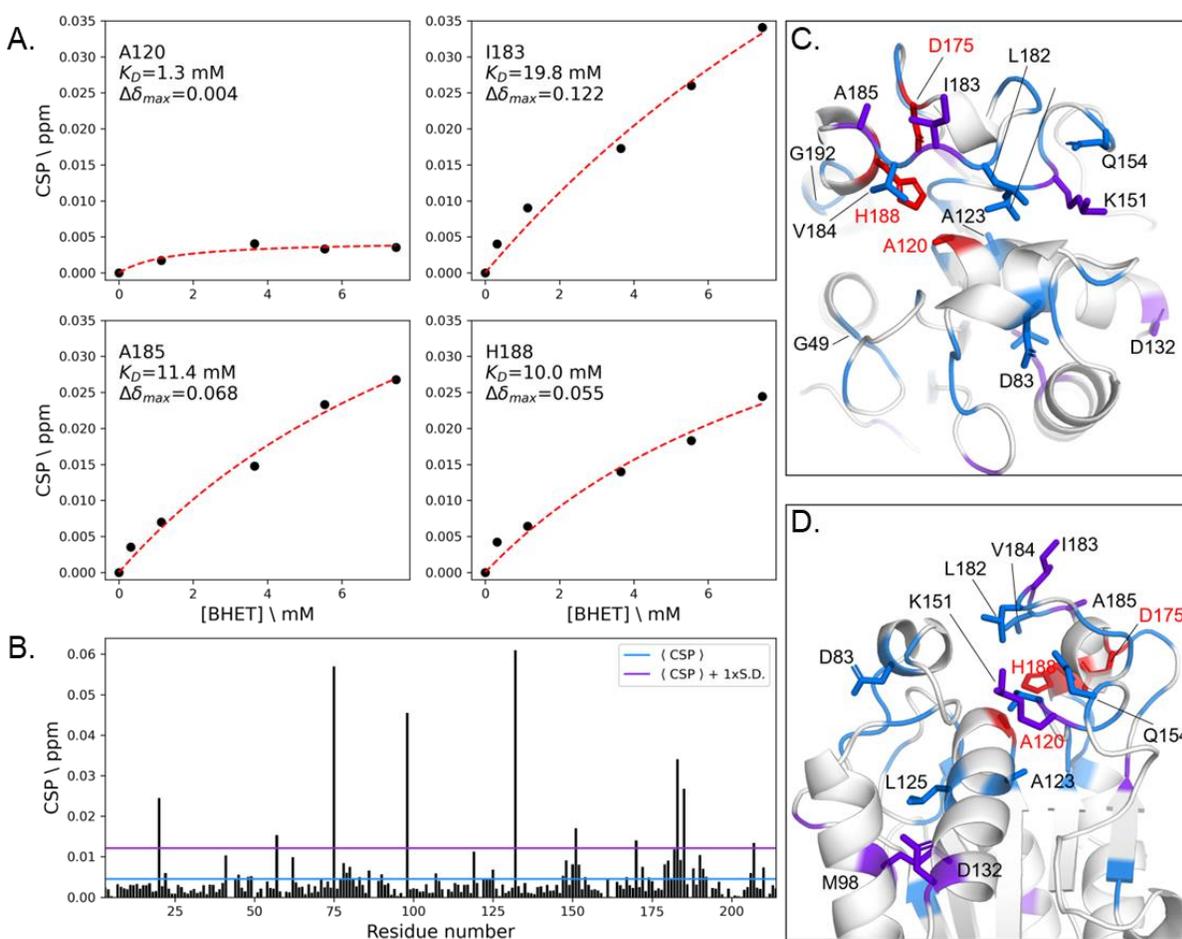
168

### 169 *Interactions between S120A-FsC and BHET*

170 To identify the substrate-binding residues on FsC we titrated BHET, as an analog of PET, on  
171 the inactive S120A-FsC mutant and followed chemical shift perturbations (CSP) on the amide  
172 proton-nitrogen pairs by using  $^{15}\text{N}$ -HSQC spectra. Upon substrate binding, changes in the

173 chemical environment around protein nuclei cause corresponding changes in  $^{15}\text{N}$ -HSQC signals.  
174 The previously published<sup>21</sup> chemical shift assignment of FsC (Biological Magnetic Resonance  
175 Data Bank (BMRB) accession 4101) was used for analysis of  $^{15}\text{N}$ -HSQC data.

176 Addition of BHET to  $^{15}\text{N}$ -labeled S120A-FsC led to gradual changes in the  $^1\text{H}$ - $^{15}\text{N}$  resonances  
177 consistent with fast exchange between the free and bound states<sup>22</sup>. Analysis of CSP allows  
178 estimation of dissociation constants, but interpretation of CSP with a small  $\Delta\delta_{max}$  (A120 in  
179 Figure 1A) can lead unreliable estimates. Analyzing CSP with higher  $\Delta\delta_{max}$  values on residues  
180 near the active site (Figure 1A) led to an estimate of around  $K_D = 10 - 20$  mM. This is a very  
181 weak interaction and, as discussed below, it may be one of the reasons for the low catalytic  
182 activity of FsC. However, suitable estimation of  $K_D$  values requires full saturation of the protein,  
183 which was unreachable due to the poor solubility of BHET<sup>22</sup>.



184

185 **Figure 1. Interactions between S120A-FsC and BHET.** Panel A show chemical shift  
 186 perturbations (CSP; black dots) at increasing BHET concentrations for four representative  
 187 residues near the active site. The dissociation constant ( $K_D$ ) and maximum CSP ( $\Delta\delta_{max}$ ) are  
 188 derived from the fit of the data to a two-site fast exchange model (red line). Panel B shows the  
 189 CSP per residue; residues with CSP larger than the average CSP,  $\langle CSP \rangle$ , are colored blue in  
 190 Panels C and D, whereas residues with CSP larger than the average CSP plus one standard  
 191 deviation are colored purple in Panels F and G. Residues in the active site are colored red in  
 192 Panels C and D.

193

194 Titration with 7.6 mM BHET led to CSP (Figure 1B) mainly on residues located around the  
195 active site of FsC (S120A, D175 and H188), where several aliphatic residues (A123, L125,  
196 L182, I183, V184, A185), as well as some polar residues (D83, T150, K151, Q154) were  
197 affected by the interaction with BHET (Figure 1C–D). This suggests that the binding is  
198 predominantly mediated by hydrophobic interactions. CSP on more distant residues (M98,  
199 D132) are likely the result of structural rearrangements upon binding with BHET, rather than  
200 direct interactions.

201 There are similarities between these findings and those of a recent study in which Charlier et  
202 al<sup>23</sup> used NMR to probe binding of LCC to MHET. Regions around LCC's V212–A213  
203 (equivalent to L182–V184 in FsC) and H191 (equivalent to K151 in FsC) were also found to be  
204 important for binding MHET, but based on our results (Figure 1C–D) BHET binding seems to  
205 require a more extended binding pocket in the regions around G49 and G192.

206

### 207 *Effect of pH on FsC-catalyzed PET hydrolysis*

208 The electrostatic potential inside and around the active site of cutinases has been hypothesized  
209 to be closely linked to catalytic efficiency<sup>24</sup>. To test this hypothesis, we assayed the enzymatic  
210 activity of FsC on PET powder at different pH values and enzyme concentrations, and analyzed  
211 the data by fitting an inverse Michaelis-Menten model<sup>2</sup> that has previously been used to  
212 characterize cutinase hydrolysis of PET. In contrast to the conventional Michaelis-Menten model  
213 in which  $V_{max}/E_0$  describes the catalytic rate when all enzymes are substrate-bound, the inverse  
214 model uses  $^{inv}V_{max}/S_0$  to define the rate when all sites on the insoluble PET substrate are  
215 saturated with enzymes (for a detailed description of these models in the context of PET  
216 degradation see Bååth et al.<sup>2,25</sup>). The model described the data well (Figure 2, Table 1), and

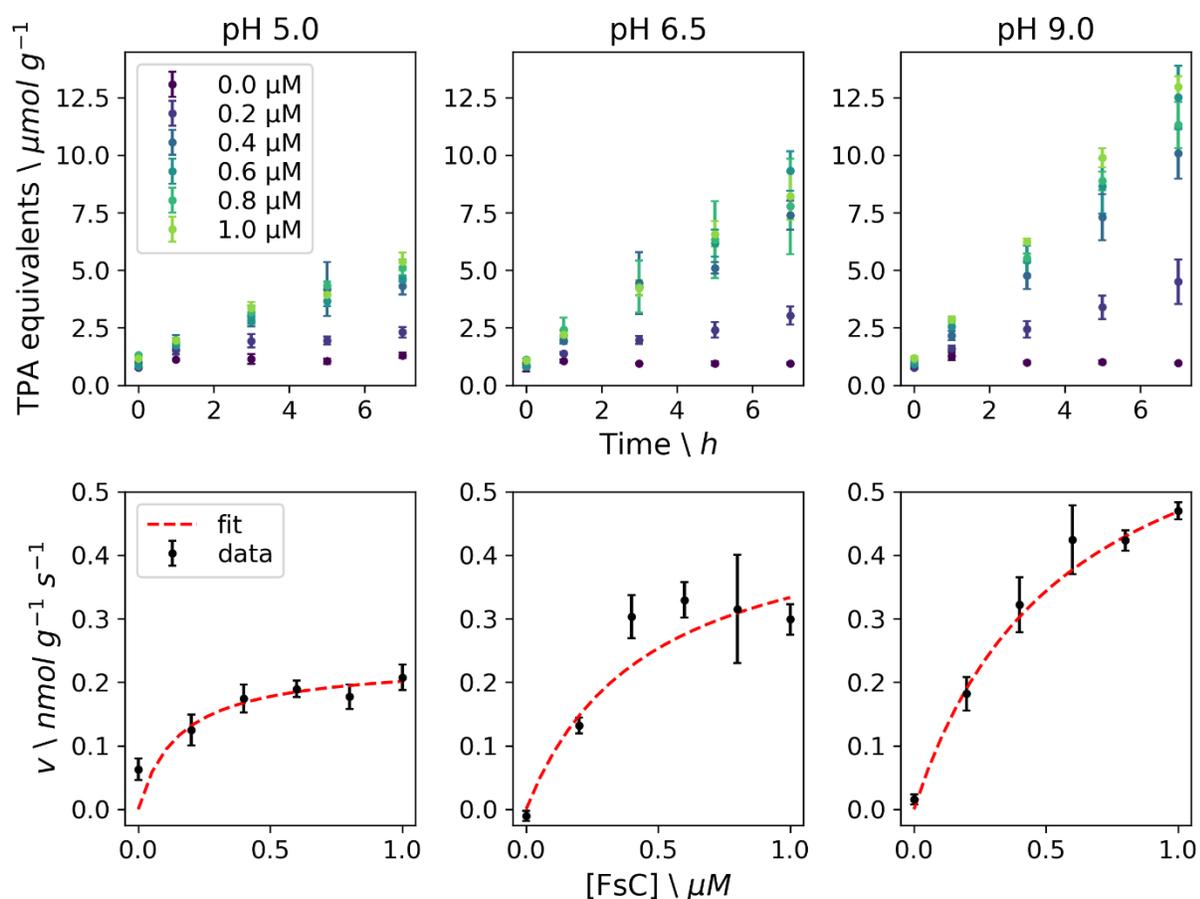
217 maximum activity in terms of  $^{inv}V_{max}/S_0$  was found at pH 9.0. At this alkali pH, the concentration  
218 of solubilized products was approximately 3-fold higher than at pH 5, and 1.5-fold higher than at  
219 pH 6.5. This observation is consistent with previous reports on the “electrostatic catapult”  
220 mechanism of esterases and lipases<sup>26</sup>, where electrostatic repulsion (favored by high pH) of  
221 negatively charged hydrolysis products (MHET and TPA in the case of PET) from negative  
222 charges in the active site cleft favors catalytic performance. Reduction in pH was accompanied  
223 by a decrease in enzymatic activity together with an increase in binding affinity (i.e., a reduction  
224 of  $^{inv}K_M$  values) (Table 1). This observation finds explanation in the neutralization of negative  
225 charges on the substrate, hydrolysis products and active site, which reduce electrostatic repulsion  
226 effects<sup>24</sup>. This may lead to too tight binding of the enzyme to substrate and/or products,  
227 precluding efficient catalysis. The validity of this interpretation hinges on the assumption that  
228  $^{inv}K_M$  can be used as a proxy to describe enzyme-substrate affinity.

229  
230 **Table 1.** Inverse Michaelis-Menten parameters for FsC on PET powder at 40 °C and three pH  
231 values. The parameters were calculated based on fitting of the data in Figure 2. Catalytic rates  
232 are given with two units,  $^{inv}V_{max}/S_0$  in nmol products per gram PET powder per second, and  
233  $^{inv}V_{max}/S_0^*$  in nmol products per particle surface area in  $m^2$  per second. The error bars represent  
234 standard error from the fit (n=3).

pH	$^{inv}K_M$ ( $\mu\text{M}$ )	$^{inv}V_{max}/S_0$ ( $\text{nmol g}^{-1} \text{s}^{-1}$ )	$^{inv}V_{max}/S_0^*$ ( $\text{nmol m}^{-2} \text{s}^{-1}$ )
5.0	$0.15 \pm 0.09$	$0.23 \pm 0.03$	$2.5 \pm 0.3$
6.5	$0.46 \pm 0.13$	$0.49 \pm 0.07$	$5.3 \pm 0.8$
9.0	$0.57 \pm 0.18$	$0.74 \pm 0.09$	$8.0 \pm 0.9$

235

236



237

238 **Figure 2. Enzymatic activity for wild-type FsC on PET powder ( $10 \text{ g L}^{-1}$ ) at  $40 \text{ }^\circ\text{C}$  and three**

239 **pH values.** The top panels show the release of TPA equivalents per gram PET powder with

240 increasing enzyme concentration ( $0 - 1 \mu\text{M}$ ) at 0, 1, 3, 5 and 7 hours. The bottom panels show

241 the initial rate,  $v$  (based on a linear fit of the product concentration at 0, 1 and 3 hours), as a

242 function of enzyme concentration (black dots), and the corresponding fit of the inverse

243 Michaelis-Menten model (red dashed line). The error bars correspond to the standard deviation

244 ( $n = 3$ ).

245 Interestingly, the  ${}^{\text{inv}}K_M$  values here reported (Table 1) match the  ${}^{\text{inv}}K_M$  values found by Bååth et

246 al<sup>25</sup> for TfC and LCC in the presence of surfactant, resulting in maximum  ${}^{\text{inv}}V_{\text{max}}/S_0$  values of

247 about 9 (TfC) and 40 (LCC)  $\text{nmol g}^{-1} \text{s}^{-1}$ . However, these values are 10 – 100-fold higher than

248 the  $^{inv}V_{max}/S_0$  values for FsC (Table 1). The inferior performance of FsC on PET may be caused  
249 by its poor binding to BHET (Figure 1) and PET (similar to the surfactant-weakened binding  
250 affinities of TfC and LCC). A structure-based sequence alignment (Figure S4) of FsC (PDB  
251 1CEX) to TfC (PDB 5ZOA) and LCC (PDB 4EB0) reveals that FsC has a  $3_{10}$ -helix (L81–R88;  
252  $\eta_2$  in Figure S4) in its active site cleft, which is absent in TfC and LCC. This helix participates at  
253 least via D83 in the interaction of the enzyme with BHET (Figure 1). It may be that the presence  
254 of this helix is detrimental for the binding and catalytic activity of FsC on PET. Araújo et al<sup>27</sup>  
255 have previously shown that L81A (also part of the  $\eta_2$  helix) and L182A FsC mutants had higher  
256 hydrolytic activity on PET and polyamide 6,6 fibers than the wild-type cutinase, indicating that  
257 engineering a less crowded binding site could boost cutinase activity on PET.

258

#### 259 *Hydrolytic activity on PET films monitored by time-resolved NMR*

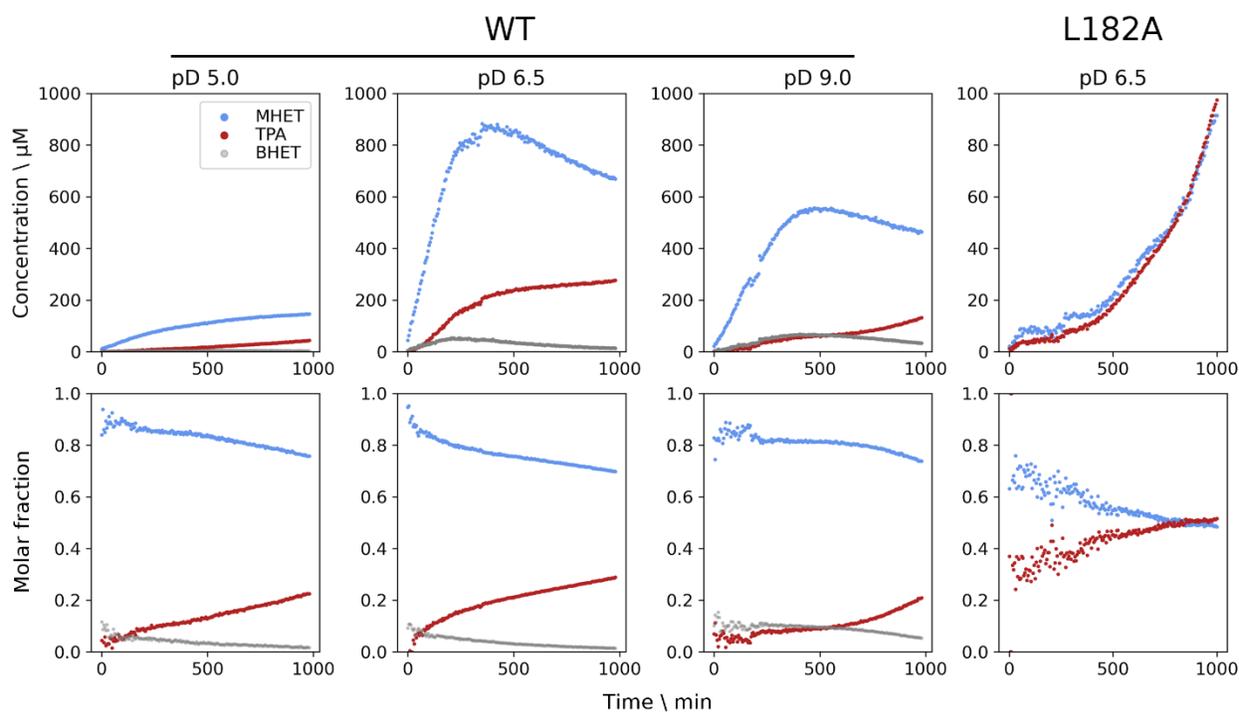
260 Suspension-based assays on microplates require manual sampling over long time periods to  
261 obtain kinetic data. This drawback of discontinuous assays has recently been addressed by the  
262 development of a continuous UV-based assay<sup>28</sup>. Here we demonstrate the applicability of a  
263 continuous assay based on time-resolved NMR spectroscopy. An advantage of time-resolved  
264 NMR is that the technique allows direct observation of all intermediates and products  
265 simultaneously (Figure S5), providing direct insights into the reaction progress. However, NMR  
266 signals can be affected by other factors than product formation, such as line broadening due to  
267 inhomogeneities in the magnetic field caused by the presence of insoluble substrate in the NMR  
268 tube. Even though caution should be taken when comparing NMR-derived activity profiles  
269 between samples, trends can be appreciated in the activity profiles (Figure 3). In all conditions  
270 only lesser amounts of BHET were seen in the activity profiles, suggesting that BHET is

271 hydrolyzed at a faster rate than PET. The main product from PET hydrolysis at all pD values is  
272 MHET, which comprises about 80% of the products (Figure 3; WT bottom panels). After about  
273 400 minutes, at pD 6.5 and 9.0, the concentration of MHET decreases linearly at a slower rate  
274 and is accompanied by an increase in the TPA concentration. The slower hydrolysis of MHET  
275 hydrolysis to TPA suggests that MHET is not a preferred substrate for FsC.

276 Using the same time-resolved NMR method we investigated a FsC-L182A mutant that has  
277 previously<sup>27</sup> been shown to have higher PET hydrolyzing activity. The time courses (Figure 3;  
278 L182A panels) show production of approximately equal amounts of both TPA and MHET, a  
279 markedly different product distribution to the wild-type enzyme. However, in contrast to earlier  
280 findings<sup>27</sup> (see above), the product yields after 1000 minutes were about 8-fold lower than for the  
281 wild-type enzyme. We propose the following reasoning for the difference in relative yields.  
282 Whereas we detected TPA and MHET hydrolysis products directly and by using an enzyme  
283 concentration of 10  $\mu$ M, the experimental conditions used by Araújo et al were markedly  
284 different. The authors used approximately a 100-fold higher enzyme concentration, and a  
285 detection method in which only TPA is observed indirectly via fluorescence detection of  
286 hydroxyterephthalate, which is produced by reacting TPA with hydroxy radicals at 90°C<sup>29</sup>. This  
287 means that only TPA amounts were detected and, since FsC-L182A produces more TPA than the  
288 wild-type enzyme (Figure 3), it is possible that the overall catalytic performance of the mutant  
289 was overestimated by Araújo et al. Moreover, the different enzyme loadings, which are known to  
290 significantly affect the catalytic performance of PET hydrolyzing enzymes<sup>20</sup>, could have further  
291 contributed to the discrepant yields.

292 At pD 9.0, the decrease in MHET (and increase in TPA) concentration after 400 minutes  
293 appears to be slower than at the other pD values (Figure 3). This suggests that, in addition to

294 mutations around the active site, the pH and pD conditions may be used to tune the relative  
295 amounts of degradation products, which could be of interest for optimizing enzymatic synthesis  
296 of MHET by cutinases<sup>30</sup>.  
297



298  
299 **Figure 3. Enzymatic activity measured by time-resolved <sup>1</sup>H NMR for wild-type FsC and**  
300 **L182A-FsC on a PET film at 40 °C and different pD values.** The profiles for each product are  
301 proportional to the integrals of the aromatic proton signals, monitored via 196 individual <sup>1</sup>H  
302 spectra recorded every 5 min for a total of 16.3 hours. The top panels show the product  
303 concentration over time, which was calculated based on the integral ratio to a TSP signal  
304 (corresponding to 400 µM) used as an internal standard. The bottom panels show the molar  
305 fraction of the products. The structures and chemical shift assignments of the degradation  
306 products are shown in Figure S5. Note that the y-scale for the top panel of L182A is different.

307 In contrast with the PET powder assay where pH 9.0 gave highest activity, time-resolved NMR  
308 assays on PET films at different pD values showed that pD 6.5 (and not pD 9.0) resulted in  
309 maximum enzymatic activity (Figure 3). Ronkvist et al<sup>9</sup> have previously observed that FsC  
310 activity on PET varies little from pH 6.5 to 8.5, but it drops sharply at pH 9.0. This observation  
311 has previously been observed to agree with the pH range where FsC is most stable; its maximum  
312 thermostability is found at pH 6 – 8.5 but it decreases sharply at pH values outside the range<sup>24</sup>.  
313 Differences in optimal pH and pD values for maximum enzymatic activity measurements on  
314 PET powder and PET films may thus be explained by FsC having lower thermal stability in the  
315 assays with PET films. The PET powder has both higher crystallinity ( $37.7 \pm 2.6\%^{20}$ ) and surface  
316 area ( $552 \text{ mm}^2$ ) than PET films (crystallinity:  $2.0 \pm 1.6\%^{20}$ ; surface area:  $257 \text{ mm}^2$ ). These  
317 morphological differences likely translate to variations in protein-substrate interactions, which  
318 influence enzymatic activity.

319

## 320 CONCLUSION

321 We have characterized a PET-hydrolyzing cutinase from *F. solani pisi*, FsC, by using a  
322 combination of NMR spectroscopy and kinetic studies at different pH and pD values. In  
323 summary, our results show that continuous time-resolved NMR experiments are a useful tool to  
324 assay enzymatic activity on PET, complementing discontinuous UV-based plate assays. These  
325 assays we show that pD conditions and an amino acid around the active site (i.e., L182)  
326 influence product distribution (i.e. the TPA-to-MHET ratio), and that weak interactions between  
327 FsC and BHET/PET, combined with inefficient hydrolysis of MHET, likely contribute to the  
328 lower catalytic activity of FsC on PET compared to other cutinases (e.g., TfC and LCC). NMR  
329 titration experiments providing insights into the molecular interaction of FsC with BHET can be

330 used for future studies seeking to engineer FsC for use in biocatalytic plastic recycling  
331 applications.

332

### 333 **DATA AVAILABILITY**

334 Data and python scripts used for data processing and making figures are available from  
335 <https://github.com/gcourtade/papers/tree/master/2023/FsC-PET>.

336

### 337 **ACCESSION CODES**

338 *Fusarium solani pisi* cutinase (FsC): P00590

339

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