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Research Article

Engineered *Thermobifida fusca* cutinase with increased activity on polyester substrates

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A bacterial cutinase from *Thermobifida fusca*, named Tfu_0883, was genetically modified by site-directed mutagenesis to enhance its activity on poly(ethylene terephthalate) (PET). The new mutations tailored the catalytic site for PET, increasing the affinity of cutinase to this hydrophobic substrate and the ability to hydrolyze it. The mutation I218A was designed to create space and the double mutation Q132A/T101A was designed both to create space and to increase hydrophobicity. The activity of the double mutant on the soluble substrate *p*-nitrophenyl butyrate increased twofold compared to wild-type cutinase, while on PET both single and double mutants exhibited considerably higher hydrolysis efficiency. The replacement of specific amino acids at the active site was an effective approach for the improvement of the Tfu_0883 cutinase capacity to hydrolyze polyester surfaces. Thus, this study provides valuable insight on how the function and stability of enzymes can be improved by molecular engineering for their application in synthetic fiber biotransformation.

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1 Introduction

Fibers made of poly(ethylene terephthalate) (PET) have numerous favorable properties compared with others; however, they also present substantial disadvantages, such as hydrophobicity, build-up of electrostatic charge, pilling tendency and difficult oil stain release. Different techniques are available to modify the surface of PET fibers, diminishing these disadvantages that are caused by the PET crystallinity and hydrophobicity. Chemicals like acids or alkalis can be used to obtain an acceptable level of polymer hydrolysis and, in that way, increase/create hydrophilic groups. However, the lack of a good control over these chemical reactions frequently leads to high fibers damage [1–3]. Together with these techniques, others like co-polymerization or plasma treatments, can be applied to modify PET surfaces; however, the outcome is still unsatisfying [4].

In the last decade new environmentally benign and specific processes have been developed based on modern enzyme technology. Various research groups have assessed the potential of laccases, lipases, polyesterases (serine esterase) and cutinases in the oxidation or hydrolysis of PET, aiming at the modification and eventual functionalization of this material [3, 5–25].

Nowadays, cutinases from *Fusarium solani pisi*, *Fusarium oxysporum* and *Thermobifida fusca* are the most frequently studied ones regarding PET modification, and their structures and properties

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Abbreviations: PET, poly(ethylene terephthalate); ***p*NPB**, *p*-nitrophenyl butyrate; **TPA**, terephthalic acid

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have been well described [17, 26–29]. Recently, Gross and co-workers studied the degradability of polyester using three different cutinases, from *Humilica insolens*, *Pseudomonas mendocina* and *Fusarium solani* [30]. They showed that the ability of cutinases to hydrolyze PET is negatively affected by the fiber crystallinity.

Several efforts have been made to identify and characterize bacterial cutinases, since until now the most studied ones have come from fungal sources. Müller's group [14] isolated and identified a thermophilic actinomycete, known as *Thermobifida fusca*. This bacterium exhibits a remarkable degradation capability regarding aliphatic–aromatic copolyesters. An extracellular hydrolase (TfH) responsible for the degradation of these copolyesters was purified from the culture broth and initially classified as a serine hydrolase with the highly conserved G-H-S-M-G motif. Afterwards, Chen and co-workers [31] identified TfH as a cutinase and renamed it as Tfu_0883. This enzyme displays very similar enzymatic properties to *Fusarium solani* cutinases. Both belong to the α/β hydrolase fold superfamily, and contain the characteristic G-X_1-S-X_2-G motif as well as a Ser-His-Asp catalytic triad. However, this *T. fusca* cutinase demonstrates remarkable thermostability unmatched by the *F. solani* cutinases [31, 32]. Moreover, Tfu_0883 presents a more open active site when compared to cutinase from *F. Solani pisi* (for illustration, see [31]), which theoretically can better "accommodate" the PET substrate. *T. fusca* cutinase is, therefore, now one of the most promising cutinases regarding PET modification [33, 34]. Altogether, this led researchers to further explore Tfu_0883 in terms of PET modification. Regarding enzymatic activity the attained results between Tfu_0883 and other cutinases were similar, nevertheless the advantage of *T. fusca* cutinase relied on its thermostability, which is a very important feature during a synthetic fiber modification process [32, 33]. It also showed higher stability in the presence of surfactants and organic solvents [14, 32, 34, 35].

Although wild-type cutinases from both bacteria and fungi are able to modify the surface of PET fibers, turnover rates are still very low. The hydrophobicity of the heterogeneous substrate and the natural constrictions of the enzyme near the active site can be the underlying factors. Araújo et al. [20] performed a genetic modification of *F. solani* cutinase, modifying and enlarging the active site to improve the fit between enzyme and the PET chains. Indeed, they succeeded in increasing the cutinase activity on PET, obtaining new variants with an five-fold improved activity relative to the wild-type enzyme.

The work reported here describes in the improvement of *T. fusca* cutinase (Tfu_0883) activity on PET substrates by genetic manipulation of specific amino acids in the active site of the enzyme. The mutations were designed to promote the active site enlargement and to increase its hydrophobic character. The new engineered cutinase is expected to better "accommodate" the synthetic substrate, leading to an increase of hydrolysis efficiency, as obtained for *F. solani* cutinase [20], allying this improvement to the native thermal stability of Tfu_0883. In this work, the different cutinase mutants were tested on a PET fabric and the hydrolysis efficiency was further assessed by quantification of hydrolysis products and evaluation of fiber surface modification.

2 Materials and methods

2.1 Materials

Wild-type Tfu_0883 [31] and its mutations were produced by recombinant DNA technology in the State Key Laboratory of Food Science and Technology, Wuxi, China. The fabric used in this work was 100% polyester (107 g/m^2, 18 yarns/cm warp and weft, 29 Tex) obtained from Rhodia, Switzerland. The dye used for coloration was Reactive Black 5 (RB5) from Ciba, Switzerland. Lutensol AT25, a non-ionic surfactant, was obtained from BASF, Spain. All other chemicals used were laboratory grade reagents from Sigma, Spain.

2.2 Mutation design

Since there was no 3-D structure for Tfu_0883, a Tfu_0883 3-D model was created. It was predicted by the SWISSMODEL, an automated comparative protein modeling server (http://swissmodel.expasy.org/workspace/index.php?func=modelling_simple1). The template was SeL (PDB code 1jfr), which shares 63% sequence identity with Tfu_0883. This model (Fig. 1) was used to study the hypothetic active site 3-D structure of Tfu_0883, and to design mutations that could make the enzyme more hydrophobic and/or wider, to redirect and/or better "accommodate" a larger and more hydrophobic substrate like PET, respectively.

The active site of serine hydrolases typically contains a highly conserved -G-X_1-S-X_2-G- sequence, which has been found to be the -G-H-S-M-G- sequence in TfH (named Tfu_0883 by Chen and co-workers) [31, 35]. Comparing the Tfu_0883 se-

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Figure 1. Homology modeling of Tfu_0883. Cartoon diagram of a predicted Tfu_0883 model, using SWISSMODEL. The –**G**-H-**S**-M-**G**-motif is shown in light gray. The residues from the catalytic triad are represented as spheres (H248 and D216, in gray; S170 from G-H-S-M-G motif, in light gray). The resulting diagram was obtained with the VMD 1.8.7.

quence and the Tfu_0883 3D model, the catalytic triad seems to be formed by Ser170, His248, and Asp216 [31].

Regarding the mutations design, three residues located in the active site opening edges (I218, on the left and Q132/T101, on the right) were selected (Figs. 2A and C). Since the goals were to increase the hydrophobicity and/or create space, the strategy was based on changing those residues by a smaller and/or a hydrophobic one. Therefore, alanine was the chosen residue. Isoleucine is a larger aliphatic hydrophobic residue than alanine, its substitution with alanine is expected to only enlarge the active site opening; glutamine and threonine are polar hydrophilic large residues, hence their substitution is considered to both create space as well as to increase the hydrophobicity of the active site opening. Given this, two Tfu_0883 mutants were considered: I218A (Fig. 2B) and Q132A/T101A (Fig. 2D).

2.3 Generation of cutinase mutants

Site-directed mutagenesis was used in the wild-type recombinant Tfu_0883 to make the following amino acid substitutions: I218A and Q132A/T101A. They were generated by the standard QuikChange mutagenesis methodology using complementary primers. The mutant cutinase genes were generated using pET20b-Tfu_0883 as template, and the presence of each specific mutation was confirmed by sequencing [31].

For I218A, the forward primer was GCCGACC TCGACACGGCCGCGCCGTCGCCACG, and the reverse primer was CGTGGCGACCGGCGCGGC CGTGTCGAGGTC. For Q132A, the forward primer was CATCACCACCCTCGACGCGCCGGACAGCC GGGCAG, and the reverse primer was CTGCCCG GCTGTCCGGCGCGTCGAGGGTGGTGATG. The double mutant Q132A/T101A was generated using pET20b-Q132A as template. The forward primer was GATCTCCCCCGGCTACGCCGGCACTGAGG CTTC, and the reverse primer was GAAGCCTCAG TGCCGGCGTAGCCGGGGAGATC.

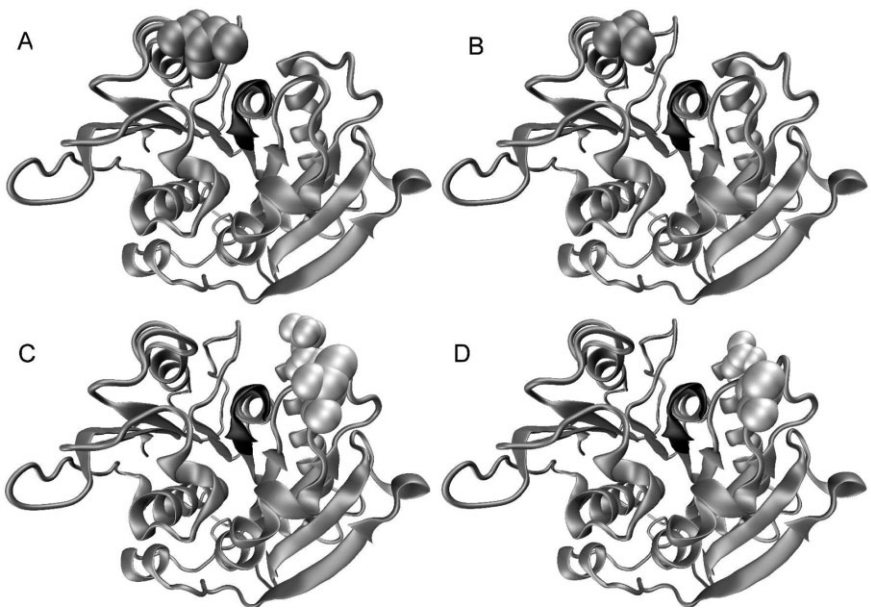

Figure 2. Comparison of the 3-D representations of wild-type, single mutant (I218A) and double mutant (Q132A/T101A) enzymes. (A) Wild-type Tfu_0883 (I218 in gray spheres); (B) I218A mutant of Tfu_0883 (A218 in gray spheres); (C) wild-type Tfu_0883 (Q132/T101 in light gray spheres); (D) Q132A/T101A mutant of Tfu_0883 (A132/A101 in light gray spheres); the black part refers to the –**G**-H-**S**-M-**G**-motif.

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2.4 Protein expression and purification

The recombinant Tfu_0883 mutants were expressed and purified according to the procedures already described by Chen and co-workers for the wild-type recombinant Tfu_0883 [31], except the expression host. The expression vectors carrying the genetically modified Tfu_0883 cutinases were used to transform chemically competent T7 expression host strain *Escherichia coli* BL21(DE3).

2.5 Esterase activity

Esterase activity in solution was determined by a continuous spectrophotometric assay using *p*-nitrophenyl butyrate (*p*NPB) as substrate. One unit of enzyme activity is defined as the production of 1 µmol *p*-nitrophenol per minute. The standard assay was performed at 20°C in a final volume of 1 mL containing *p*NPB (1 mM), the enzyme, and the assay buffer (20 mM Tris-HCl, 10 mM NaCl and 50 mM sodium taurodeoxycholate, pH 8.0). The reaction was initiated by the addition of *p*NPB. The hydrolysis of *p*NPB was monitored by the formation of the *p*-nitrophenol at 405 nm [36].

2.6 Lipase activity

Lipase activity was measured as reported by Jaeger et al. [37] with the following modification: triolein served as substrate and the reaction solution contained 2.5 mL 25 mM potassium phosphate buffer (pH 8) and 2 mL emulsified triolein (3% polyvinyl alcohol:triolein, 3:1). The reaction was initiated by adding the enzyme to the reaction solution and quenched by adding 7.5 mL ethanol. The released fatty acids were quantified via titration using 0.05 N NaOH. One unit of lipase activity was defined as the release of 1 µmol fatty acid per min.

2.7 Hydrolysis of PET with wild-type and genetically modified cutinases

For the enzymatic treatments of PET fabrics, a pretreatment was performed by washing the fabric with 1 g/L Lutensol at 60°C for 60 min and dried in an oven at 40°C for 24 h. Then, the PET fabric samples, 0.4 g each, were incubated at 60°C with 50 mg_{prot}/L of each enzyme (wild type, Q132A/T101A and I218A) in a 100 mL 0.2 M Tris-HCl buffer, pH 7.5. These treatments were performed in Erlenmeyer flasks (120 cm^3 capacity) in a shaking water bath at 75 rpm. For coloration assays, contact angle measurements and FTIR analysis, the samples were washed after the enzymatic treatment to remove adsorbed protein. Washing was performed at 40°C, first, with 2 g/L sodium carbonate for 1 h and then with a nonionic surfactant, Lutensol AT25, for 30 min.

2.8 Terephthalic acid determination

For quantifying the amount of terephthalic acid (TPA), one of the soluble products of PET hydrolysis, flask liquor samples were taken after certain periods of incubation (0, 0.5, 1, 2, 3, 4, 8, 24, and 48 h). A scan was made with a luminescence spectrophotometer between 300 and 600 nm after the reaction of TPA solution with hydrogen peroxide at 90°C, using an excitation wavelength of 315 nm. The solution (1 mL) was added to 2 mL of hydrogen peroxide (30%) and heated at 90°C for 30 min. After cooling to room temperature, the fluorescence was measured and the intensity of the peak at 425 nm was used to calculate TPA concentration [13]. All measurements were done in duplicate.

2.9 Kinetic studies

The classical Michaelis-Menten kinetic model, derived for homogeneous reactions, is based on enzyme-limited conditions. However, in the case of polymer enzymatic hydrolysis, where the substrate is usually insoluble and the reaction is limited to the surface of the substrate (substrate-limited), the Michaelis-Menten model do not apply. Alternative heterogeneous kinetic models based on substrate-limited conditions have therefore been developed for the hydrolysis of different materials like poly(β-hydroxybuterate), cellulose and PET films. In this work the approach of Ronkvist et al. was used [30]. They compared the catalytic activities of cutinases using PET films as model substrates, and found that the enzymatic degradation could be expressed by a two-step kinetic model according to the following initial reaction rate (V_0) equation:

$$V_0 = k_2[S_0]\frac{K[E_0]}{K+[E_0]} \quad (1)$$

Where $[S_0]$ is the initial surface concentration, $[E_0]$ is the initial enzyme concentration, k_2 is the hydrolysis rate constant and K is the adsorption equilibrium constant defined as the ratio of the rate constant for adsorption to that of desorption (k_1/k_{-1}).

For hydrolysis rate determination (V_0), several concentrations of cutinase (WT and Q132A/T101A) were used to hydrolyze PET fabric samples at 60°C for 48 h. The hydrolysis rates for each concentration were determined from the initial linear slopes of [TPA] versus time data.

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2.10 Protein adsorption

The protein in solution was measured at different time points to indirectly calculate the percentage of adsorption. For this, samples were taken from the supernatant at 0, 0.5, 1, 2, 3, 4, 8, 24, and 48 h of incubation. The percentage of adsorption was calculated according to the method described previously [13].

2.11 Surface evaluation by reactive dyeing

To evaluate the modifications at the fabric surface catalyzed by the tested cutinases, a coloration process was applied based on the chemical reaction between the hydrolysis products and a reactive dye. The extension of hydrolysis is directly proportional to the intensity of fabric color after reactive dyeing. The coloration process was performed at 60°C, below the glass transition temperature (T_g) of PET fibers. Polyester fabric samples after enzymatic treatment (4 h and 48 h) were stained together in the same sealed, stainless steel dye pot of 120 mL capacity in an Ahiba machine (laboratory scale dyeing machine). The coloration was performed with Reactive Black 5 (RB5) (2% on weight of fabric [o.w.f.], bath ratio of 100:2, 100 mL liquor for 2 g fabric), at pH 11.0 using sodium carbonate (20 g/L) and sodium sulfate (60 g/L), at 60°C for 90 min with agitation (30 rpm). After the coloration process, samples were all extensively washed for 1 h at 50°C in water and then dried at room temperature.

2.12 Scanning electron microscopy (SEM)

The hydrolyzed samples were scanned using the electron microscopy at different places of the fabric using 10 000× magnification. PET fabric samples were coated with a thick layer of gold before scanning and all images were carefully taken using the same sample position on weft and warp directions.

2.13 Contact angle measurements

Water contact angles were measured with deionized water droplet on the treated and control fabrics at room temperature. Contact angles were measured after 15 s and the data were obtained from the averages of measurements taken from ten different points on the surface of each sample. The volume of the water droplet was set as 15 µL using a Hamilton 500-µL syringe type. The Ellipse fitting was the model selected for the contact angle measurement.

3 Results and discussion

3.1 Mutations design

The mutation design studies were performed with a 3-D model of Tfu_0883 using the SWISSMODEL modeling server. The server retrieved an inferred 3-D structure sharing 63% of homology with SeL (PDB code 1jfr) (Fig. 1). The highly conserved –G-X_1-S-X_2-G- sequence, typically found in serine hydrolases, showed 100% homology between SeL and Tfu_0883 (-G-H-S-M-G- sequence); comparing the Tfu_0883 sequence and the 3D structure of SeL, the Tfu_0883 catalytic triad seems to be formed by Ser170, His248, and Asp216 [31]. All these data led us to use the SeL model to devise the Tfu_0883 mutations.

The mutations were designed to increase the hydrophobicity and/or to create more space in the active site, so that the hydrophobic PET polymer could be better fitted.

Structural analysis on the inferred active site suggested that the replacement of the bulky side chain of I218, Q132 and T101 by a smaller residue such as alanine (the I218A and Q132A/T101A mutants, respectively) would provide a less-restrained active site, allowing a better accommodation of a larger substrate (Fig. 2) like PET, ultimately improving the enzyme activity. Moreover, replacement of polar hydrophilic residues like Q132 and T101 by aliphatic hydrophobic ones, like alanine (Q132A/T101A mutant), gives a more hydrophobic active site than the wild-type one. Our experimental data have corroborated this hypothesis (see data below).

3.2 Activity study on different substrates

Since the fungal cutinase from *F. solani* is able to hydrolyze both insoluble and soluble esters, we first characterized the ability of the bacterial cutinase from *T. fusca* to hydrolyze triolein and *p*NPB. As shown in Fig. 3, both the wild-type and the mutant cutinases exhibit activity towards *p*NPB substrate. The Q132A/T101A mutant presented the highest value of cutinase activity compared with the wild-type.

Regarding the lipase activity, all the enzymes showed similar abilities to use triolein as a substrate, although both mutants appeared to show a slight improvement over the wild type. The highest efficiency in hydrolyzing triolein was shown by the I218A mutant (Fig. 4). The hydrophobicity and the increase in space in the active site, due to the amino acid replacements, may be responsible for the higher catalytic activity on both *p*NPB and triolein

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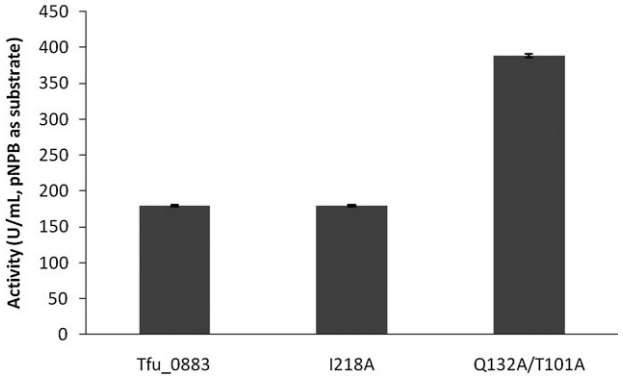

Figure 3. Esterase activity of wild-type and mutant cutinases [one unit of activity was defined as the amount of enzyme required to convert 1 μmol *p*NPB to *p*-nitrophenol (*p*-NP) per minute]. The experiments were done in triplicate and the error bars correspond to the SD.

seen for the mutant cutinases. All three enzymes showed a preference to hydrolyze the shorter carbon chain of *p*NPB, in accordance with the fact that cutinase is an esterase and not a true lipase.

The ability of cutinase mutants to hydrolyze the solid substrate was compared to the wild-type enzyme. The PET fabric samples were incubated separately for several hours with the wild-type and the mutant cutinases. The hydrolysis products were quantified in terms of TPA formation, which is released during the hydrolysis of most superficial polymer chains of PET.

The results in Table 1 indicate that the TPA formation was faster for the mutants, which caused a two-fold increase in the release of TPA during the first 2 h of incubation compared to the wild type. For both mutants the values of TPA in the samples taken after the longest period of incubation (48 h)

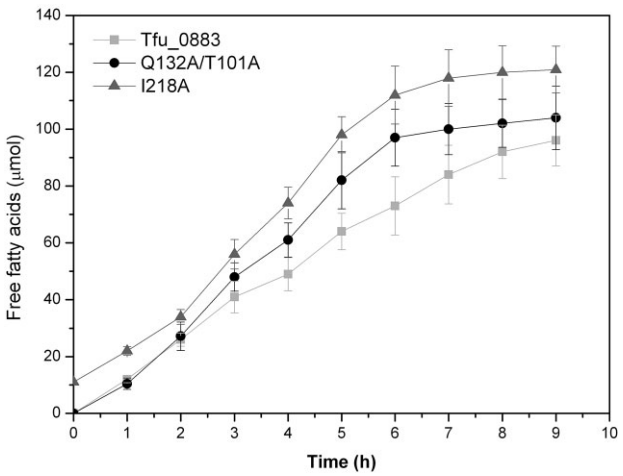

Figure 4. Lipase activity of wild-type and mutant cutinases on triolein substrate, expressed in terms of fatty acid released; experiments were done in triplicate and the error bars correspond to the SD.

are very similar to those obtained for the shortest period (2 h), which indicates a saturation level. However, the mutants presented both a higher catalytic efficiency and higher levels of protein adsorption when compared to the wild-type enzyme. Thus, both mutants were successful in hydrolyzing PET. The space created around the active site may be the main factor for the improved substrate attachment and further hydrolysis. Replacing the bulky side chain of I218 by a smaller residue, such as alanine, provides a less-restrained active site, allowing a better accommodation of the synthetic substrate (PET). This mutation allows the hydrophobic cleft of the enzyme active site to open, providing a better fit of PET substrate. Besides creating more space, the double amino acid substitutions (Q132A/T101A) led to a more hydrophobic binding site, which may be the reason for the slight improvement in both hydorlysis product formation (TPA and *k/s* results) and protein adsorption regarding the single mutant cutinase (Table 1).

3.3 Kinetic study using PET fabric as substrate

Following the preliminary tests, a complete kinetic study was undertaken for the double mutant (Q132A/T101A), comparing its performance with the wild-type cutinase on solid PET substrate. The PET fabric samples were incubated separately for several hours with both enzymes, and the hydrolysis products were used to determine the dependence of the PET fabric hydrolysis rate on cutinase concentration. Concentrations of both cutinases, wild-type and mutant, were varied at a fixed PET fabric surface concentration (13 cm^2/mL). Incubations were performed for 48 h and the quantification of hydrolysis products formation was followed over time (Figs. 5A and B).

From the TPA quantification, we were able to determine that the hydrolysis of PET surface occurred within the first 4 h of incubation for both enzymes assayed. After this point, the substrate was saturated by the enzyme so that hydrolysis could not occur, and hence the TPA level remained stationary. The sites for enzyme attachment on the fabric, where the microenvironment allows the enzymatic reaction to occur, were totally occupied leading to the saturation of the solid substrate.

It was also observed that TPA production was directly related to the enzyme concentration, reaching however different levels for both enzymes. The mutant cutinase showed the highest amount of hydrolysis. The amount of TPA after the first 4 h of incubation with Q132A/T101A was ten-fold higher when compared with the values obtained for the wild type (Figs. 5A and B). The space

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Table 1. TPA release (mM) during incubation of PET with different cutinases Tf_0883 forms; spectral values of PET fabrics after enzymatic treatment (*k/s*); level of protein adsorption at two different incubation periods (%); the experiments were done in triplicate (± SD)

	TPA variation (mM)			*k/s*		Protein adsorption (%)[a]	
	0 h	2 h	48 h	2 h	48 h	2 h	48 h
No enzyme	0.11 ± 0.02	0.11 ± 0.02	5.9 ± 0.1	0.070 ± 0.003	0.090 ± 0.002	–	–
Tfu_0883	5.4 ± 0.1	7.2 ± 0.2	12.3 ± 0.1	0.24 ± 0.06	0.30 ± 0.04	56 ± 3	77 ± 4
I218A	1.73 ± 0.05	15.3 ± 0.2	15.4 ± 0.1	0.25 ± 0.03	0.38 ± 0.07	15 ± 3	48 ± 7
Q132A/T101A	1.1 ± 0.2	16.9 ± 0.2	19.3 ± 0.1	0.26 ± 0.03	0.36 ± 0.02	59 ± 2	84 ± 2

a) The values were calculated with respect to time zero.

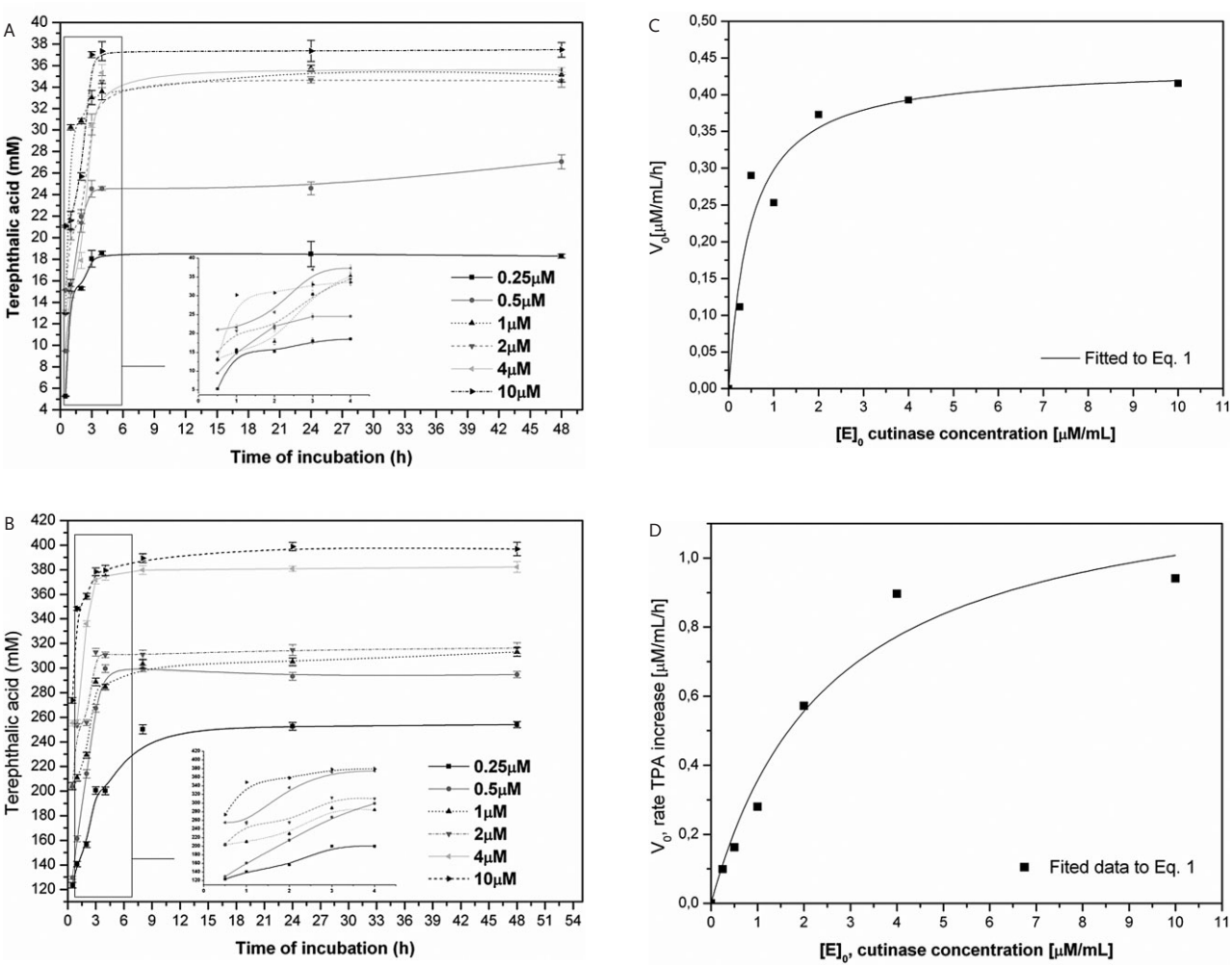

Figure 5. TPA (mM*)* vs. time of incubation for the several concentrations used of (A) wild-type Tfu_0883 and (B) Q132A/T101A Tfu_0883; fitting of initial rate of TPA production as a function of (C) wild-type and (D) double mutant Tfu_0883 concentrations.

created around the active site may be the main factor for the more efficient protein attachment and consequent hydrolysis by the mutant Tfu_0883. Besides creating more space, the higher hydrophobic character due to the double amino acid substitution also contributed to the high level of hydrolysis catalyzed by the mutant Q132A/T101A (Fig. 5B).

Plots of experimental enzymatic hydrolysis rates versus cutinase concentrations were prepared for both double mutant (Q132A/T101A) and wild-type enzymes. Initial hydrolysis rates were

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Table 2. Kinetic parameters of wild-type and double mutant (Q132A/T101A) Tfu_0883, derived from Eq. (1), determined for PET hydrolysis at 60°C for 48 h; $[S]_0$, the initial surface concentration; *K*, adsorption equilibrium constant; k_2, hydrolysis rate constant

	$[S]_0$ (μM)	K (μM^{-1})	k^2(μmol/cm^2/h)
Wild-type (Tfu_0883)	13	0.48	0.07
Q132A/T101A	13	2.54	0.63

determined from initial linear slopes of TPA production versus time data and V_0 was further calculated for each enzyme concentration. Experimental data points are shown in Figs. 5C and D with the corresponding data fitting. The maximum rates reached by the wild-type enzyme and the Q132A/T101A mutant were 0.4 μmol and 1 μmol, respectively. Using the software Origin 8, values for *K* and k_2 were found by fitting Eq. (1) to experimental data according to a least mean-square regression fit (Table 2). The highest hydrolysis rate k_2 (0.63 μmol/cm^2/h) was obtained for the double mutant, almost ten-fold higher than the k_2 obtained for the wild type. This is in agreement with the experimental findings from the TPA quantification. The adsorption constant *K*, shown in Table 2, is a measure of the enzyme affinity to PET substrate. The double mutant (Q132A/T101A) showed a higher *K* value when compared to the wild-type cutinase. This finding was also confirmed experimentally when the percentage of protein adsorbed during the treatment was quantified (14% for wild type and 58% for Q132A/T101A). The active site of Q132A/T101A enzyme displays a higher hydrophobic character than the native enzyme, since Q132/T101 polar hydrophilic residues were substituted by A132/A101 aliphatic hydrophobic residues. The experimental results, depicted in Table 2, agree well with the expected behavior for the designed mutations, and they support the fact that the enzyme binding to PET involves the residues Q132 and T101 according to the structural analysis based on the predicted 3D model of Tfu_0883.

3.4 Surface quantification of hydrolysis products

The main goal of the work was to increase the hydrophilic groups on fabric's surface, through partial hydrolysis of the polymer chain. Thus, to measure the presence of these groups (carboxylic and hydroxyl groups) at the surface of PET fabrics, a textile evaluation was performed, based on the dyeing of the hydrolyzed samples with a reactive dye able to specifically react with the hydroxyl groups. The color strength after reactive staining should be directly proportional to the number of hydrophilic groups resulting from enzymatic hydrolysis. The

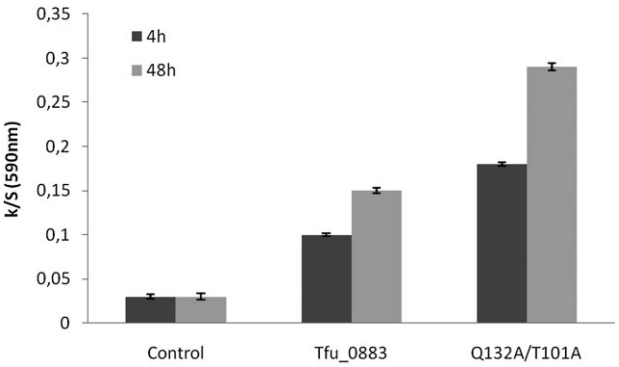

Figure 6. *k/s* values for hydrolyzed fabric samples dyed with Reactive Black 5 (previous enzymatic treatments of 4 and 48 h at 60°C using 10 μM/mL wild-type and Q132AT101A cutinases); the experiments were done in triplicate and the error bars correspond to the SD.

spectral values (*k/s*) obtained after staining are shown in Fig. 6. Comparing with control values, the enzymes used were efficient in modifying the surface of the textile, presenting higher levels of coloration at all periods of incubation. As expected, the samples treated with the double mutant (Q123A/T101A) revealed the highest color levels at both incubation periods. These results corroborate those obtained for TPA quantification, where the increased space and higher hydrophobicity of the active site allowed a higher adsorption to PET, increasing the level of surface modification by the mutant cutinase when compared to the wild type. An increase in the hydrophilic groups at fiber's surface is responsible for a higher hydrophilicity and dye uptake.

3.5 Scanning electron microscopy

Enzymatic hydrolysis of PET fabric surfaces causes several changes, either physicochemical or morphological, that can be detected by different techniques. One of these techniques is the scanning electron microscopy. The results shown in Fig. 7 demonstrate the morphological changes seen in random cuts at the surface of the treated fibers. Enzyme action is constricted to the fiber surface, promoting the formation of hydrolysis products that could improve the intrinsic properties of the textile fibers, like hydrophilicity and dye uptake. The most evident modification was obtained when Q132A/T101A was used.

3.6 Surface wettability – Water contact angle

The polyester surface changes caused by the enzymatic action were quantified in terms of water contact angle. Similar to other studies performed by

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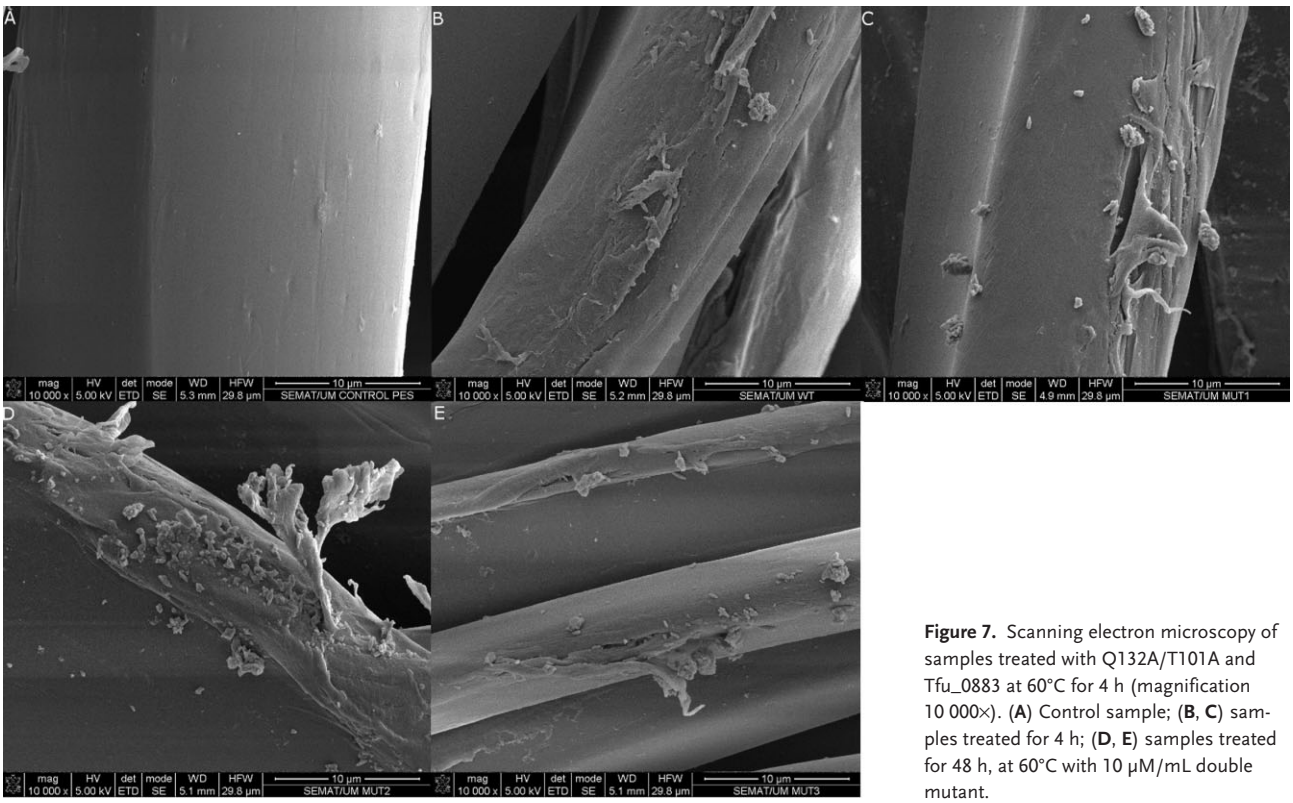

Figure 7. Scanning electron microscopy of samples treated with Q132A/T101A and Tfu_0883 at 60°C for 4 h (magnification 10 000×). (**A**) Control sample; (**B**, **C**) samples treated for 4 h; (**D**, **E**) samples treated for 48 h, at 60°C with 10 μM/mL double mutant.

Feuerhack and co-workers [21], a higher capacity to absorb water by samples hydrolyzed by cutinase was found. Compared to the control, which is hydrophobic (contact angle: 97°), the samples incubated with the Tf_0883 double mutant presented a hydrophilic behavior (contact angle: 25.4°). The increase in hydrophilic groups at the surface of the fabric, resulting from enzymatic action, is the main factor responsible for the significant change in the ability to absorb water. Figure 8 illustrates the behavior of the samples after treatment with both wild-type and mutant enzymes. After hydrolysis, the samples became hydrophilic and it is difficult to capture the exact moment of water drop absorption. Therefore, only one picture is presented as representative of the absorption phenomena.

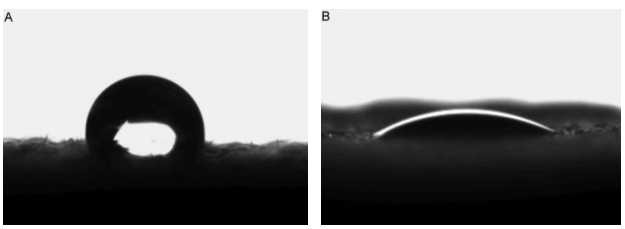

Figure 8. Images of a water droplet (15 μL) on: (**A**) the control, and (**B**) Q132A/T101A-treated sample after 15 s; contact angle values:control: 92.00 ± 3.25°; treated samples: 25.40 ± 7.28°.

4 Conclusions

In summary, we have created cutinases with an enhanced activity on polyester fibers, namely I218A and Q132A/T101A. The increase in activity of these mutants can be attributed to a greater space created at their active sites and to a substrate-binding site that is more hydrophobic (especially in the case of the double mutant). Adsorption levels to the PET surface were affected by the hydrophobic character of the enzyme active site. The mutant I218A was the least hydrophobic and consequently was only slightly adsorbed to PET; the opposite was true for the double mutant Q132A/T101A. The flexibility in substrate recognition by cutinase, which is able to recognize different solid substrates like cutin and polyester, was confirmed in this work. The genetic modification of cutinase from *T. fusca* proved to be a powerful tool for enhancing its activity on insoluble substrates like PET and probably other synthetic fibers.

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The authors have declared no conflict of interest.

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