

# Functionalization of Cellulose Acetate Fibers with Engineered Cutinases

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*In the present work, we describe for the first time the specific role of cutinase on surface modification of cellulose acetate fibers. Cutinase exhibits acetyl esterase activity on diacetate and triacetate of 0.010 U and 0.007 U, respectively. An increase on the hydroxyl groups at the fiber surface of 25% for diacetate and 317% for triacetate, after a 24 h treatment, is estimated by an indirect assay. Aiming at further improvement of cutinase affinity toward cellulose acetate, chimeric cutinases are genetically engineered by fusing the 3'-end coding sequence with a bacterial or a fungal carbohydrate-binding module and varying the linker DNA sequence. A comparative analysis of these genetic constructions is presented showing that, the superficial regeneration of cellulose hydrophilicity and reactivity on highly substituted cellulose acetates is achieved by chimeric cutinases. © 2009 American Institute of Chemical Engineers *Biotechnol. Prog.*, 26: 636–643, 2010*

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## Introduction

Cellulose is the most abundant natural polymer and it is a valuable renewable resource (Steinmann, 1998). Since the end of the 19th century, the attained development on cellulose chemistry allowed new processes and a continuous expansion of the applications of cellulose derivatives, in particular, the cellulose esters such as cellulose acetate which has been used as a raw material for plastics, textiles, filter tows, films, or membranes (Rustemeyer, 2004). In the textile field, cellulose acetates are characterized by a combination of desirable and unusual properties like soft and silk-like hand, good textile processing performance and higher hydrophilicity than synthetic fibers, thus being more comfortable to use (Law, 2004; Steinmann, 1998).

The chemical hydrolysis of the acetyl groups on cellulose esters partially recovered the desirable native cellulose properties demonstrating that, the modification of fiber surface is important to improve its reactivity and hydrophilic character without affecting significantly the tensile properties of the polymer (Braun and Kadia, 2005). The major advantages of using enzymes in polymer modification compared with chemical approaches are the milder reaction conditions and

easier control, being environmental friendly processes and performing specific nondestructive transformations on polymer surfaces (Gübitz and Cavaco-Paulo, 2003, 2008). In this work, we report for the first time the hydrolysis of acetate surface groups of cellulose diacetate (CDA) and cellulose triacetate (CTA) fabrics using cutinase (EC 3.1.1.74) from *Fusarium solani pisi*. Cutinase is a small ellipsoid protein that belongs to the class of serine esterases and to the superfamily of  $\alpha/\beta$ -hydrolases (Longhi and Cambillau, 1999). The *F. solani pisi* cutinase also belongs to the family 5 of carbohydrate esterases ([www.cazy.org/fam/CE5.html](http://www.cazy.org/fam/CE5.html)), sharing a similar 3D-structure with two other members with known structure: the acetylxylyl esterase (EC 3.1.1.72) from *Trichoderma reesei* and the acetylxylyl esterase II from *Penicillium purpurogenum* (Ghosh et al., 2001; Hakulinen et al., 2000). However, at the level of the 3D conformation of the active center *F. solani pisi* cutinase reflects a preference for hydrophobic substrates, a suitable and versatile feature to be chosen for surface modification of highly substituted cellulose acetates (Ghosh et al., 2001).

In this work, we present a novel approach to increase cutinase adsorption on cellulose acetate fibers, a reaction that occurs on a heterogeneous medium. Using molecular genetic tools we constructed chimeric cutinases by fusing the catalytic domain with a carbohydrate-binding module (CBM). This strategy resulted in an increase of enzyme concentration

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at the substrate surface (Boraston et al., 2004; Linder and Teeri, 1997). Four constructions were obtained using two distinct CBMs, fused independently to the C-terminal of cutinase, and varying the linker DNA sequence. CBMs were selected on the basis of ligand affinity, as the two cellulose acetate fibers used in this work are structurally distinct from cellulose (the native ligand) and between themselves, with different overall crystallinities. CBM type A, of the Cellobiohydrolase I (CBHI) from *T. reesei* belongs to the family CBM1 and has preference for crystalline or microcrystalline regions of cellulose whereas CBM type B, of the Endoglucanase C (CenC) from *Cellulomonas fimi*, which belongs to the family CBM4, is able to bind amorphous cellulose (Boraston et al., 2004). The results presented here demonstrate that hydrolysis of surface acetyl groups from CDA and CTA with a chimeric cutinase-CBM constitutes a promising approach to increase reactivity and hydrophilicity in these fibers, exhibiting an enhanced reactive dye uptake of treated fabrics compared with the native cutinase.

## Methods

### Reagents and enzymes

The CDA and CTA plain woven fabrics used were kindly supplied by Mitsubishi Rayon Co. Ltd., Tokyo, Japan. The CDA fabric has 41/27 ends/picks per centimeter and 64 g m<sup>-2</sup>. The CTA fabric has 45/31 ends/picks per centimeter and 98 g m<sup>-2</sup>.

All other reagents were laboratory grade reagents from Sigma-Aldrich, St. Louis, USA, unless stated otherwise. The buffer used throughout the work was phosphate buffer at pH 8 (50 mM); the exceptions are specified in the text. The cutinase from *F. solani pisi* used in this work was expressed and purified as previously reported (Araújo et al., 2007). Restriction enzymes were purchased from MBI Fermentas (Vilnius, Lithuania) and from Roche Diagnostics GmbH (Penzberg, Germany). Accuzyme™ DNA polymerase was purchased from Bioline GmbH (Luckenwalde, Germany) and recombinant *Taq* DNA polymerase was purchased from MBI Fermentas. T4 DNA ligase was purchased from Roche Diagnostics GmbH (Penzberg, Germany).

### Esterase activity assay in solution

Esterase activity was determined following the product initial release (*p*-nitrophenol) continuously through the increase in the absorbance at 400 nm at 30°C. The assay conditions for the determination of cutinase activity were described previously (Matamá et al., 2006). All the assays were performed at least in triplicate. Standard solutions of *p*-nitrophenol were used to obtain the calibration curve. One unit of esterase activity was defined as 1 μmol of *p*-nitrophenol released per minute.

### Treatment of cellulose di- and triacetate fabric with cutinase

All samples of cellulose acetate fabric were washed before use to remove possible impurities from manufacture and from human handling. Washing was performed at 35°C and 20 rpm, in stainless steel pots of 450 mL in capacity and housed in a laboratory scale machine, the Rotawash MKIII (vertical agitation simulating European washing machines, from SDL International Ltd.). The fabric was washed twice

for 30 min in Lutensol AT25 (40 mg L<sup>-1</sup>, nonionic detergent; BASF, Ludwigshafen, Germany), then rinsed four times with distilled water for 30 min each and left to dry at room temperature. Several sets of experiments were carried out taking into account the amounts of enzyme, fabric, and time of incubation. For all experiments, the treatment of cellulose acetate fabric was performed in the Rotawash machine, operating at 30°C and 20 rpm. To evaluate the effect of enzyme concentration, samples of CDA and CTA fabric (0.1 g) were incubated in duplicate for 8 h with 5 mL buffered solution of cutinase (0, 25, 50, 75, and 100 U mL<sup>-1</sup>). In another treatment, the average weight of both type of fabric was increased to 0.5 g and the incubation extended to 24 h. The initial activity of cutinase was 25 U mL<sup>-1</sup> in a final volume of 25 mL. For each sample, a control was run in parallel without the enzyme. After enzymatic treatment, all fabric samples were washed at 35°C, in the Rotawash machine, to remove the adsorbed protein, following the order: Lutensol AT25 (250 mg L<sup>-1</sup>) for 30 min, ethanol (70%) for 20 min, isopropanol (15%) for 15 min, three steps of increasing concentrations of sodium chloride for 10 min each, and three steps in distilled water for 20 min each. Between the detergent/alcohol and alcohol/salt steps, the fabric was rinsed under running cold tap water for 5 min.

### Determination of acetic acid concentration in the treatment solutions

Detection of acetic acid in the reaction media was performed with the acetic acid UV test from Roche (Darmstadt, Germany). Protein was previously precipitated using perchloric acid according to the manufacture instructions. The pH of the samples was neutralized using potassium hydroxide (1 M) and the subsequent salts were removed by centrifugation.

### Quantification of total protein

Total protein in solution was quantified following Bradford methodology using BSA as standard (Bradford, 1976). All samples were measured at least in duplicate.

### Coloration of cellulose acetate fabric with a reactive dye

After enzymatic treatment, samples were competitively colored, in duplicate, with Remazol Brilliant Blue R, C.I. 61200 (2% o.w.f.), in phosphate buffer. The coloration was performed at 50°C, for 90 min at 20 rpm, in sealed stainless steel beakers of 140 mL in capacity and housed on a lab-scale dyeing machine (AHIBA Spectradye, from Datacolor International). After coloration, all samples were washed once with Lutensol AT25 (0.25 g L<sup>-1</sup>) and several times with distilled water in Rotawash, at 55°C, until no more dye could be detected in the solution. The color measurements (five for each sample) were carried out with a reflectance spectrophotometer having a standard illuminant D65 (Spectraflash 600 Plus, from Datacolor International). The color strength was evaluated as *K/S* at the maximum absorption wavelength (590 nm) which is proportional to the dye concentration in the samples. The ratio between absorption (*K*) and scattering (*S*) is related to reflectance (*R*) data by applying Kubelka-Munk's law at each wavelength (Kuehni, 1997).

### Fluorescein isothiocyanate labeling

Enzymes were incubated with Fluorescein isothiocyanate (FITC; 33:1 w/w) in sodium carbonate buffer pH 9.5 (0.5 M) for 1 h at room temperature. The unconjugated FITC was removed with HiTrap Desalting 5 mL columns (GE Healthcare Bio-Sciences Europe GmbH, Munich, Germany) whereas the carbonate buffer was exchanged by the phosphate buffer pH 8 (50 mM).

### Fluorescence microscopy

Thin strips of CDA and CTA fabric samples were embedded in an epoxy resin (Epoxy kit, Struers, Copenhagen, Denmark) and cross sections were cut with 20–25  $\mu\text{m}$  thickness. The samples were observed under a Leica Microsystems DM5000 B epifluorescence microscope equipped with a 100 W Hg lamp and an appropriate filter setting. Digital images were acquired with Leica DFC350 FX digital Camera and Leica Microsystems LAS AF software, version 2.0 (Leica Microsystems GmbH, Wetzlar, Germany).

### Fourier transformed infrared spectroscopy (FT-IR)

The diffuse reflectance (DRIFT) technique was used to collect the infrared spectra of CDA and CTA fabric samples treated during 24 h with cutinase and respective controls. The spectra were recorded in a Michelson FT-IR spectrometer MB100 (Bomem, Inc., Quebec, Canada) with a DRIFT accessory. The fabric pieces were placed and hold on top of the sample cup, previously filled with potassium bromide powder that was used to collect the background. All the spectra were obtained under a nitrogen atmosphere in the range 4000–800  $\text{cm}^{-1}$  at 8  $\text{cm}^{-1}$  resolution and at the ratio of 32 scans to the same number of background scans. The spectra were acquired in Kubelka-Munk units and baseline corrections were made using Bomem Grams/32R software, version 4.04.

### Bacteria, plasmids, and genes

The bacterial hosts used for cloning and expression of cutinase fusion genes were the *Escherichia coli* strain XL1-Blue and strain BL21 (DE3), respectively. The plasmid pGEM<sup>®</sup>-T Easy (Promega Corporation, Madison, USA) was used to clone and sequence the PCR products. The plasmid pCWT [pET25b (+) carrying native cutinase gene from *F. solani pisi*] was used to insert the genes coding for the CBMs at the 3'-end of the cutinase gene and to express the fusion proteins (Araújo et al., 2007). The DNA coding the wild-type linker and a CBM similar to the CBM of *T. reesei* CBH I, wtCBM<sub>CBHI</sub>, was synthesized and purchased from Epoch Biolabs (Missouri City, USA), as well as, the DNA fragment coding for a smaller linker and the CBM, sCBM<sub>CBHI</sub>. The plasmid pTugN1 containing the sequence of CBM<sub>N1</sub> from *C. fimi* CenC was kindly provided by Professor Anthony Warren (Department of Microbiology, University of British Columbia, Vancouver, Canada; Johnson et al., 1996).

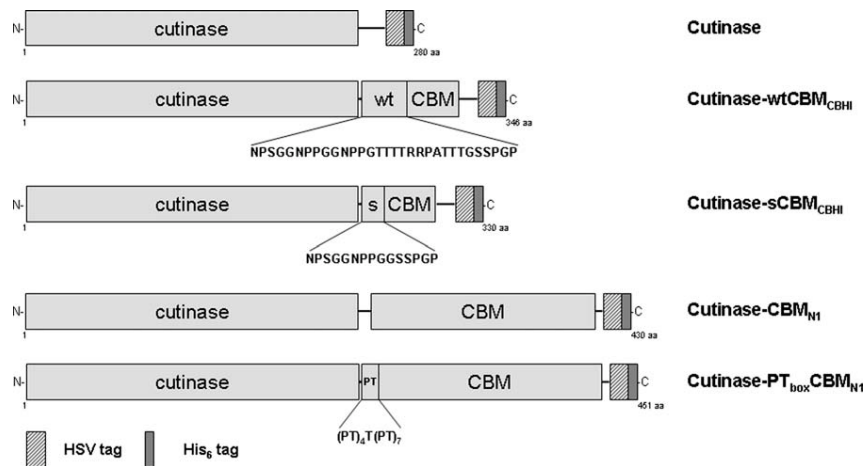
### Plasmid construction

Standard techniques were used for all the DNA manipulations. The wtCBM<sub>CBHI</sub> and sCBM<sub>CBHI</sub> were amplified by PCR, using the primers supplied by Epoch Biolabs, and cloned directly into pGEM<sup>®</sup>-T Easy. Transformants were

selected and the gene sequences were confirmed by DNA sequencing, following the method of Sanger (1977). The constructs pGEM::wtCBM<sub>CBHI</sub> and pGEM::sCBM<sub>CBHI</sub> were digested with *SacI* and *SalI*, the DNA fragments were extracted and purified from agarose gels and cloned into the *SacI/SalI* restricted and dephosphorilated pCWT, resulting in the final pCWT::wtCBM<sub>CBHI</sub> and pCWT::sCBM<sub>CBHI</sub> vectors. The CBM<sub>N1</sub> sequence was PCR-amplified from pTug, with the primers CBM N1 for (5'-ataagaat**g**cgccgctagcccgatcggg-gaggaacgt) and CBM N1 rev (5'-accgctc**g**agctcgacctcg-gagtgcagcgc) containing the *NotI* and *XhoI* sites (in bold). The PCR product was cloned into pGEM<sup>®</sup>-T Easy and a positive clone was selected and confirmed by DNA sequencing. The construct pGEM::CBM<sub>N1</sub> was restricted with *NotI* and *XhoI*; the DNA fragment was extracted and purified from agarose gel and cloned into the *NotI/XhoI* restricted and dephosphorilated pCWT, resulting in the final pCWT::CBM<sub>N1</sub> construct. The DNA coding the linker PT<sub>box</sub> of *C. fimi* CenA was obtained by PCR amplification of two overlapping primers (underlined sequence) containing the *SalI* and *NotI* sites (in bold): PTbox for (5'-ctcgagctc**g**tcgacccgacccaaccccgacgcctaca**ctc**gactccgacgccc**g**acccgactc) and PTbox rev (5'-gagggactc**g**ctc**g**ggc**g**cgctaggggctcgtgttg-gagtcgggctc**g**ctc**g**gagtcggagttg; Shen et al., 1991). The PCR amplification conditions consisted in 30 cycles of 20 s at 94°C and 20 s at 72°C for Accuzyme extension. The PCR product was cloned into pGEM<sup>®</sup>-T Easy and a positive clone was selected and confirmed by DNA sequencing. The plasmid pGEM::PT<sub>box</sub> was restricted with *SalI* and *NotI*, the DNA fragment was extracted and purified from agarose and cloned into the *SalI/NotI* restricted and dephosphorilated pCWT::CBM<sub>N1</sub>, resulting in the final pCWT::PT<sub>box</sub>::CBM<sub>N1</sub> vector.

### Expression and purification of chimeric cutinases

The constructs pCWT::wtCBM<sub>CBHI</sub>, pCWT::sCBM<sub>CBHI</sub>, pCWT::CBM<sub>N1</sub>, and pCWT::PT<sub>box</sub>::CBM<sub>N1</sub> were first established in *E. coli* strain XL1-Blue. Medium-scale purifications of plasmid DNA were made and used to transform the *E. coli* strain BL21 (DE3). Clones harboring the constructs were grown, at 15°C and 200 rpm, in 2.5 L Luria-Broth medium supplemented with ampicillin (100  $\mu\text{g mL}^{-1}$ ) until an absorbance A600 nm of 0.3–0.5 was reached. Cells were induced with isopropyl-1-thio- $\beta$ -D-galactopyranoside, IPTG (0.7 mM), and further incubated for 16 h at 15°C. Cells were harvested by centrifugation at 4°C (7500g, 10 min), washed with PBS pH 7.4, and frozen at  $-80^\circ\text{C}$ . Ultrasonic disruption of the bacterial cells was accomplished on ice with a 25.4 mm probe in an Ultrasonic Processor VCX-400 Watt (Cole-Parmer Instrument Company, Illinois, USA). The lysate was centrifuged for 30 min at 16,000g and 4°C. The supernatant was collected, pH was adjusted to 7.6, and imidazole was added to a final concentration of 25 mM. Protein purification was performed with the affinity chromatography system HiTrap Chelating HP (GE Healthcare Bio-Sciences Europe GmbH, Munich, Germany) coupled to a peristaltic pump. The 5 mL column was loaded with nickel II (100 mM) and equilibrated with the binding buffer (20 mM phosphate buffer pH 7.6, 500 mM sodium chloride, 25 mM imidazole). The samples were loaded and washed with 10 column volumes of binding buffer followed by buffers with 50 and 100 mM imidazole. The fusion proteins (Scheme 1)



**Scheme 1.** Schematic representation of the recombinant wild-type cutinase from *F. solani pisi* and its fusion proteins with a carbohydrate-binding module based on the one of CBH I, from *T. reesei*, and the bacterial carbohydrate-binding module N1 of CenC, from *C. fimi*. The primary sequences of the linkers are specified in the figure.

were eluted with imidazole buffer (20 mM phosphate buffer pH 7.6, 500 mM sodium chloride, 550 mM imidazole).

The fractions obtained were monitored by SDS-PAGE with Coomassie Brilliant Blue staining. The elution buffer was changed to phosphate buffer with HiTrap Desalting 5 mL columns (GE Healthcare Bio-Sciences Europe GmbH, Munich, Germany). Before the 2.5 L culture scale up, Western blotting was performed with monoclonal Anti-polyHistidine-Peroxidase Conjugate from mouse (Sigma-Aldrich, St. Louis, USA) to confirm the expression of the fusion proteins. The detection was made with ECL Western blotting reagents and analysis system (Amersham Biosciences Europe GmbH, Freiburg, Germany).

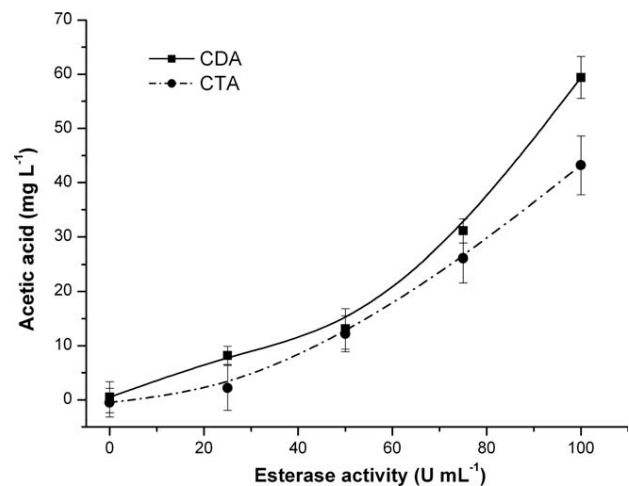
#### Treatment of cellulose di- and triacetate with native cutinase and chimeric cutinases

All samples of cellulose acetate fabric used were previously washed as already described earlier. CDA and CTA fabric samples (0.1 g) were incubated with buffered solutions (10 mL), with 0.01% sodium azide, of cutinase (100 U mL<sup>-1</sup>), cutinase-CBM<sub>N1</sub> (100 U mL<sup>-1</sup>), cutinase-PT<sub>box</sub>CBM<sub>N1</sub> (50 U mL<sup>-1</sup>), cutinase-wtCBM<sub>T.reesei</sub> (50 U mL<sup>-1</sup>), and cutinase-sCBM<sub>T.reesei</sub> (25 U mL<sup>-1</sup>), under continuous vertical agitation at 30°C and 20 rpm, for 18 h. A control for both types of fabric was run in parallel in which the buffer substituted the enzyme. After enzymatic treatment, all fabric samples were washed according to the procedure described previously.

### Results and Discussion

The hydrolysis of acetyl groups on cellulose di- (CDA) and triacetate (CTA) fabrics by recombinant cutinase from *F. solani pisi* was monitored following the effect of enzyme concentration on the release of acetic acid into solution, after 8 h of treatment (Figure 1). The reaction conditions were based on a methodology previously described for cutinase activity (Matamá et al., 2006).

The acetic acid production increased in the range of the tested enzyme concentrations. At the maximum enzyme concentration used, the acetyl esterase activity was of 0.010 U and 0.007 U which corresponds to a release of 0.54% and 0.36% of the acetyl groups from CDA and CTA, respec-



**Figure 1.** Acetic acid formation as a function of cutinase concentration using as substrate CDA and CTA samples.

Assays were carried out during 8 h, at pH 8 and 30°C. Concentration of cutinase is expressed as the initial esterase activity in U mL<sup>-1</sup>, determined as described in the Methods section.

tively. These values were obtained considering a standard degree of substitution (DS) 2.4 for CDA and of 2.7 for CTA commercial fibers (Steinmann, 1998; Zugenmaier, 2004). These data are in accordance with the literature because a higher level of acetic acid released is found for CDA compared with CTA, a more substituted cellulose acetate. Studies reported in literature on the biodegradation of cellulose acetate, using microorganisms or cell-free enzymes, demonstrated the requirement of the concerted action of several enzymes, among which acetyl esterases, providing evidences that the DS is inversely correlated with the degree of deacetylation (Altaner et al., 2001, 2003; Moriyoshi et al., 1999, 2002; Puls et al., 2004; Samios et al., 1997). It was proposed that steric hindrance and crystallinity are important factors on the effectiveness of the adsorbed enzyme to promote the hydrolysis thus favoring CDA over CTA (Lee and Fan, 1982).

Altaner et al. (2001) reported that acetyl esterases from 13 different commercial origins could significantly use cellulose acetates with DS ≤ 1.4 as substrates. Among them only one enzyme from *Humicola insolens* was able to release a small

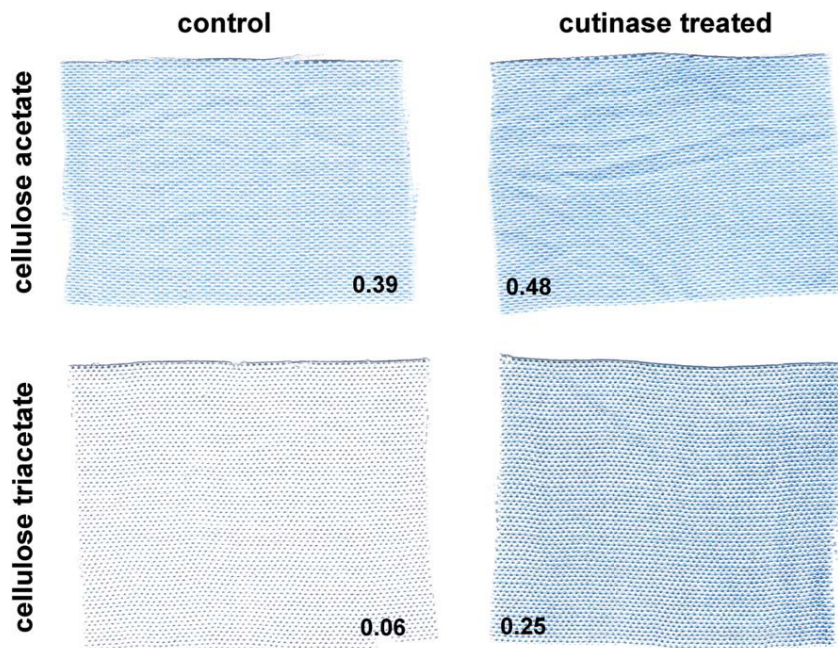


Figure 2. Scanned image of cellulose acetate (1st row) and triacetate (2nd row) controls (1st column) and cutinase treated samples (2nd column), competitively colored with Remazol Brilliant Blue R, C.I. 61200, after a treatment with 1250 U of esterase initial activity per gram of fabric, at pH 8 and 30°C, for 24 h.

The  $K/S$  values are specified in the image.

amount (10%) of acetyl groups from a cellulose acetate DS 1.8, after 220 h. Another report describes the application of an acetyl esterase from *Aspergillus niger*, purified from a commercial preparation that was able to hydrolyze 5% of acetyl groups on a cellulose acetate DS 1.8 after 140 h and unable to release acetic acid for a DS 2.3 (Altaner et al., 2003). Considering these values with the ones here reported (Figure 1) for an assay carried out only during 8 h, we demonstrate that recombinant cutinase presents an activity with a great potential as acetyl esterase for CDA and CTA materials than other enzymes previously reported in literature.

Two independent coloration assays were performed on CDA and CTA samples treated with 25 U mL<sup>-1</sup> of recombinant cutinase for 24 h (Figure 2). These are competitive assays in which both cutinase-treated and untreated (control) fabric samples were colored in the same bath. This coloration methodology was proved to be a valuable and a very sensitive approach to qualitatively evaluate the hydroxyl groups at the fiber surface (Matamá et al., 2006, 2007; Silva et al., 2005). The basic principle is the specific reaction between a vinylsulphonic group from the reactive dye Remazol Brilliant Blue R and the hydroxyl group on the fiber. The differences on color strength were measured as  $K/S$  values at 590 nm (described in the Methods section), a parameter proportional to the dye concentration in the fiber comparing the cutinase-treated and -untreated samples. This approach provides evidence that hydroxyl groups at the surface of CDA and CTA increased on enzymatic treatment as the  $K/S$  values increased 25% ± 9% and 317% ± 32%, respectively (Figure 2). This method presents limitations when comparing different materials as no absolute quantification of the hydroxyl groups can be made.

The DRIFT technique was used to collect the infrared spectra to obtain further evidence of the hydrolysis of the ester linkage at the surface of the fibers. Figure 3 shows the

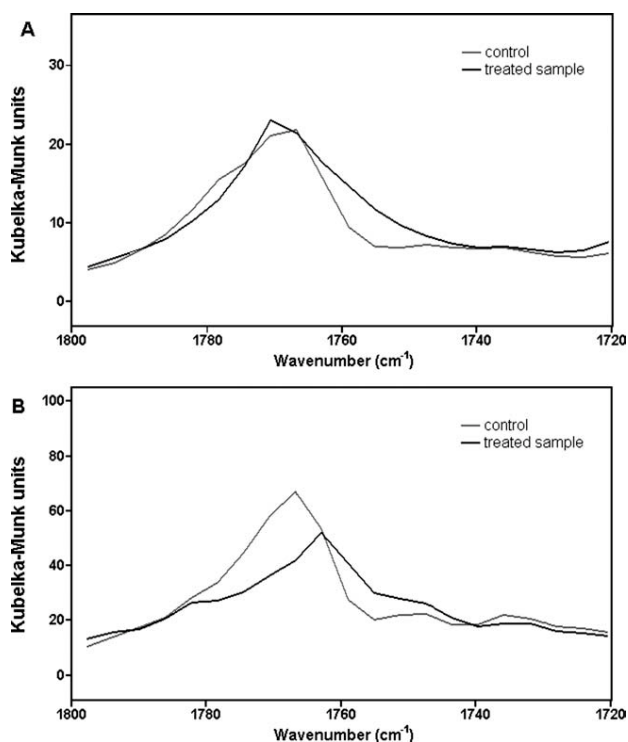
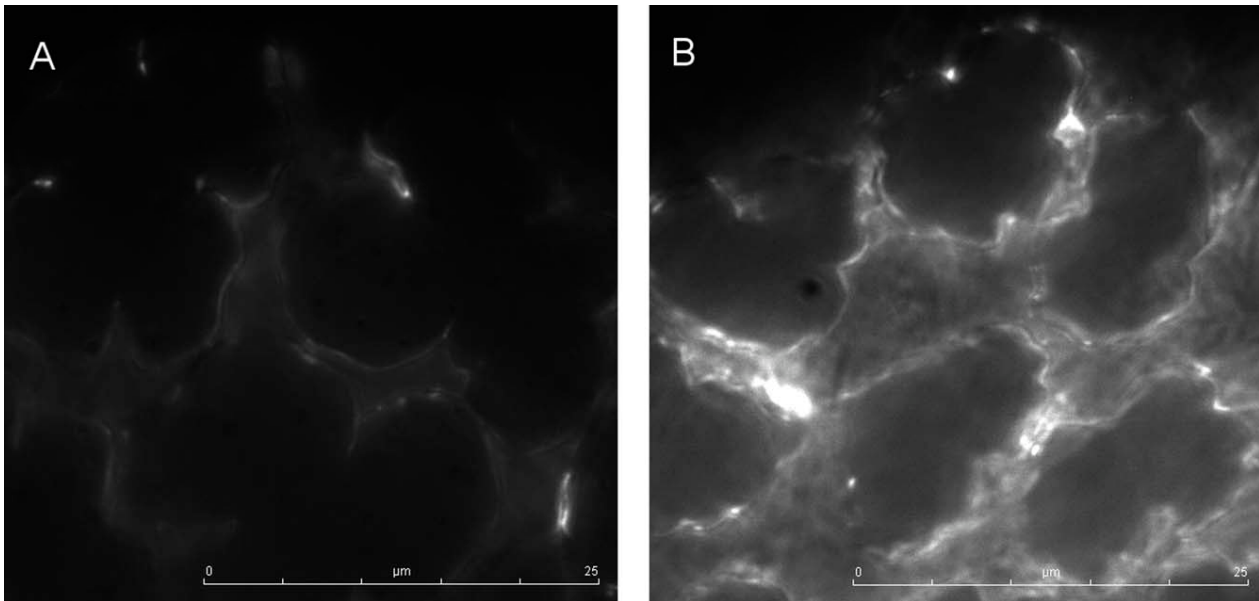


Figure 3. DRIFT spectra showing the carbonyl group stretching band of (A) CDA and (B) CTA controls and samples.

The samples were treated with cutinase (1250 U per gram of fabric), at pH 8 and 30°C, for 24 h.

IR spectra in the region of 1800–1720 cm<sup>-1</sup>, the wavenumber region for the stretching vibration of the carbonyl group, in CDA and CTA samples (Krasovskii et al., 1996). For



**Figure 4. Epifluorescent photographs of cross-sections from (A) CDA and (B) CTA samples.**

The samples were treated with FITC-conjugated cutinase (10 mg of protein per gram of fabric), at pH 8 and 30°C, for 15 h. Both images were acquired with a total magnification of  $\times 1000$ .

CTA (Figure 3B), there was a decrease in the intensity after the cutinase treatment and there was also a shift of the band to lower wavenumbers. The decrease in the intensity was correlated to the enzymatic hydrolysis of ester linkages at the surface of the samples. The displacement could be caused either by the formation of intermolecular hydrogen bridges between the remaining carbonyl groups and the newly formed hydroxyl groups or to a preferential hydrolysis of the carbonyl groups at  $C_2$  and  $C_3$  positions (Ilharco and Barros, 2000; Srisodsuk et al., 1993). For CDA (Figure 3A), the observed differences between the control and sample were not significant.

The conditions used in all the assays described earlier were based on time course assays with fixed concentrations of enzyme (25–50 U mL<sup>-1</sup>). It was verified that enzyme adsorption to the fabrics reached a steady state (free vs. bound) after 4 h of incubation (not shown). Our data proved that cutinase was able to modify the surface of the cellulose acetate fabrics, increasing the number of hydroxyl groups (both in CDA and CTA) and lowering the carbonyl groups (detected only in CTA).

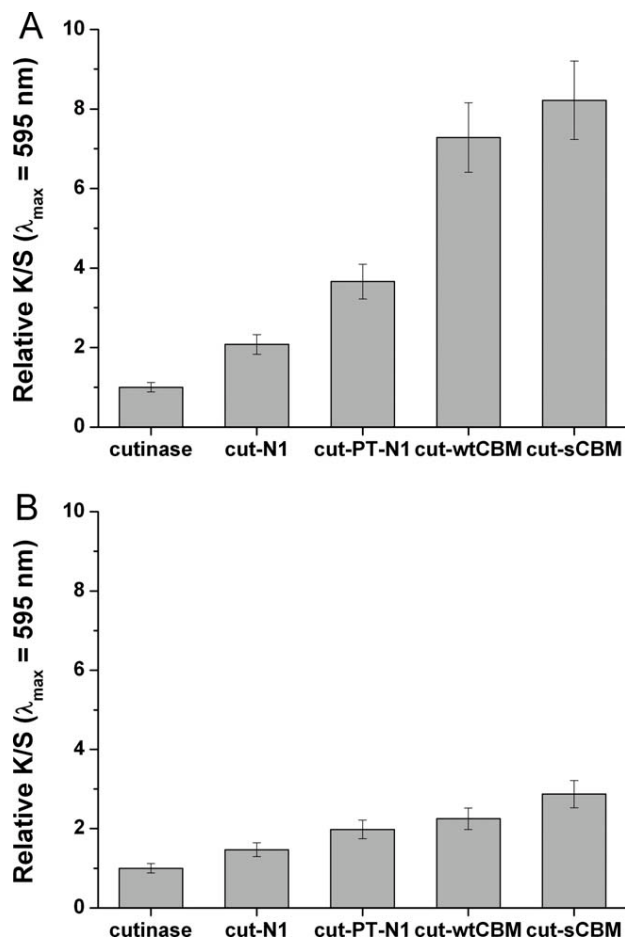
Cross sections of fibers treated with cutinase conjugated with FITC were observed by fluorescence microscopy (Figure 4). The fluorescence signal was located mainly at the surface, the core did not emit fluorescence indicating that the labeled protein did not penetrate inside the fibers, therefore confirming the superficial action of cutinase on them.

For further improvement of cutinase affinity to the modified cellulose fibers, several fusion proteins with carbohydrate binding modules (CBMs), from distinct origins and properties were produced by recombinant DNA methodologies using the same expression system previously adopted for the native cutinase (Araújo et al., 2007). The inclusion of different spacers between the catalytic domain and the CBMs was considered on the genetic constructions (Scheme 1). The importance of these spacers was studied by several authors demonstrating that, linker characteristics influence the synergistic activity of both domains (Poon et al., 2007;

Shen et al., 1991; Srisodsuk et al., 1993). We produced two chimeric enzymes by fusing the C-terminus cutinase with the fungal CBM CBHI using either the wild-type linker sequence or a mutant deleted on the O-glycosylation sites (Scheme 1). Additionally, the C-terminus of cutinase was also fused in-frame with the sequence coding for the N1-CBM domain present on the CenC-endoglucanase of the bacteria *Cellulomonas fimi*. For this approach, two other constructions were considered, one without linker as it occurs in the native CenC protein and another one containing the sequence of the proline-threonine-box present on the CenA-endoglucanase from *C. fimi* (Scheme 1; Shen et al., 1991). All the four constructions were cloned, expressed, and purified as previously described for the native cutinase from *E. coli* cells extracts using affinity chromatography Ni<sup>2+</sup> columns (not shown; Araújo et al., 2007).

An accurate study on each of the constructs regarding binding parameters of these constructions will be subject of another publication. In this work, we present the data of the effect of the chimeric cutinases on the modification of cellulose acetate fabrics surfaces. Figure 5 shows the results obtained in competitive coloring assays, normalizing the  $K/S$  values for the enzyme activity concentration (U mL<sup>-1</sup>). From these results it was found a clear difference for CDA and CTA on the relative  $K/S$  obtained with the chimeric cutinases vs. the native enzyme, with a more pronounced effect for the less substituted acetate, independently on the CBM used.

The cutinase-wtCBM<sub>CBHI</sub> and cutinase-sCBM<sub>CBHI</sub> are the most efficient catalysts under the treatment conditions used for both fibers. On CDA (Figure 5A), the relative  $K/S$  was improved around seven- and eightfold by cutinase-wtCBM<sub>CBHI</sub> and cutinase-sCBM<sub>CBHI</sub>, respectively, regarding native cutinase. On CTA (Figure 5B), the increase in  $K/S$  was improved around two for cutinase-wtCBM<sub>CBHI</sub> and threefold for cutinase-sCBM<sub>CBHI</sub>. The better performance of chimeric cutinases with the fungal CBM could be explained by the difference in size of both CBMs, being more



**Figure 5. Relative increase in  $K/S$  values for the (A) CDA and (B) CTA treated with wild-type cutinase and chimeric cutinases.**

The samples were incubated independently with native cutinase (cutinase), cutinase-CBM<sub>N1</sub> (cut-N1), cutinase-PT<sub>box</sub>CBM<sub>N1</sub> (cut-PT-N1), cutinase-wtCBM<sub>T.reesei</sub> (cut-wtCBM), and cutinase-sCBM<sub>T.reesei</sub> (cut-sCBM), at pH 8 and 30°C. Samples and the control were competitively colored at 60°C. Relative  $K/S$  was calculated as  $\frac{K/S - K/S_{control}}{K/S_{cutinase} - K/S_{control}}$ , where  $K/S$  is the  $K/S$  value for a treated sample,  $K/S_{control}$  is the  $K/S$  value for the control, and  $K/S_{cutinase}$  is the  $K/S$  value for the sample treated with native cutinase.

constrained by the bigger bacterial CBM than by the smaller fungal CBM. Another reason could be the inherent mechanism for ligand recognition displayed by each type of CBM favoring the superficial binding of the fungal CBM toward these unnatural fibers. Again, from a conceptual point of view we would expect that the interactions necessary for the ligand recognition (anhydroglucose units) by the CBM should be more impaired on CTA than on CDA surface. In fact, our results express the presence of stronger steric constraints in the triacetate fiber, due to higher ornamented polymer backbone with acetyl groups, than in CDA.

### Conclusions

In this work, we have demonstrated that cellulose acetate with a high DS can be modified by the enzyme cutinase, bringing novel applications for industry that utilizes these manufactured materials and creating alternative tools toward their functionalization. The acetyl-esterase activity of cutinase was restricted to the surface of the fibers, increasing the

hydroxyl groups and partially regenerating the hydrophobicity characteristics of cellulose, thus creating reactive sites for further modifications. As the cellulose acetates used in this work were insoluble, the enzyme adsorption to the substrate was a prerequisite for the formation of the enzyme-substrate complex. By mimicking nature strategies to overcome these constraints, we have designed and produced chimeric cutinases fused with a CBM which increased the performance of native cutinase.

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