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Enzymatic Surface Modification and Functionalization of PET: A Water Contact Angle, FTIR, and Fluorescence Spectroscopy Study

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ABSTRACT: The purpose of this study was to investigate the changes induced by a lypolytic enzyme on the surface properties of polyethylene terephthalate (PET). Changes in surface hydrophilicity were monitored by means of water contact angle (WCA) measurements. Fourier Transform Infrared spectroscopy (FTIR) in the Attenuated Total Reflectance mode (ATR) was used to investigate the structural and conformational changes of the ethylene glycol and benzene moieties of PET. Amorphous and crystalline PET membranes were used as substrate. The lipolytic enzyme displayed higher hydrolytic activity towards the amorphous PET substrate, as demonstrated by the decrease of the WCA values. Minor changes were observed on the crystalline PET membrane. The effect of enzyme adhesion was addressed by applying a protease after-treatment which was able to remove the residual enzyme protein adhering to the surface of PET, as demonstrated by the behavior of WCA values. Significant spectral changes were observed by FTIR–ATR analysis in the spectral regions characteristic of the crystalline and amorphous PET domains. The intensity of the crystalline marker bands increased while that of the amorphous ones decreased. Accordingly, the crystallinity indexes calculated as band intensity ratios (1,341/1,410 cm^{-1} and 1,120/1,100 cm^{-1}) increased. Finally, the free carboxyl groups formed at the surface of PET by enzyme hydrolysis were esterified with a fluorescent alkyl bromide, 2-(bromomethyl)naphthalene (BrNP). WCA measurements confirmed that the reaction proceeded effectively. The fluorescence results indicate that the enzymatically treated PET films are more reactive towards BrNP. FTIR analysis showed that the surface of BrNP-modified PET acquired a more crystalline character.

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Introduction

Polyester (polyethylene terephthalate, PET) is the major synthetic fiber in terms of global production, which accounted for 30.7 million metric tons in 2007 (12.4 staple fibers, 18.3 filament yarn), meaning about 76% of the entire manmade fiber spinning business (Saurer Management AG, 2008). These fibers are used in a great number of application areas such as apparel, home furnishing and interior textiles, hygiene and medical textiles. Despite their outstanding chemical, physical, and mechanical properties, PET textiles suffer of some drawbacks due to the intrinsic hydrophobic and inert nature of the constituent polymer. Other characteristics, such as the low moisture regain (<5% at 20°C and 65% RH) and the poor wettability cause a variety of problems both during manufacturing (e.g., accumulation of static electricity) and consumer use (e.g., clinging to the body, accumulation of fluff, and soil). Conventional finishing techniques for PET textiles are high energy demanding and chemicals consuming. The hydrophilicity of PET fibers can be improved by alkali treatment (Zeronian and Collins, 1989). However, the use of aggressive treatments leads to deterioration of the intrinsic mechanical

and aesthetic properties of PET textiles, with occurrence of faults and overall reduction of the product quality.

Biocatalysis is emerging as an increasingly attractive tool for the surface hydrolysis and functionalization of synthetic polymers. The state of the art in this field has been recently reviewed by Guebitz and Cavaco-Paulo (2008). Polyesters have for a long time been described as resistant to biological attack until it was reported that various lipases can cleave the ester bond in aliphatic polyesters (Tokiwa and Suzuki, 1977). More recently it was discovered that aliphatic-aromatic copolyesters in a certain range of composition were degraded by microorganisms (Müller et al., 2001), and that even aromatic polyesters, such as polyethylene terephthalate and poly(trimethylene terephthalate) can be attacked and modified by lipolytic enzymes (Alisch-Mark et al., 2006; Eberl et al., 2008). Enzymes active on PET substrates include various cutinases, lipases, and esterases (Guebitz and Cavaco-Paulo, 2008). Among them cutinases, that is, the enzymes able to hydrolyze ester bonds in the plant polymer cutin, are under investigation for the bioprocessing of PET textiles on an industrial scale (Silva et al., 2005). Cutinases are able to hydrolyze a large variety of synthetic esters. Contrary to lipases, the activity of which is greatly enhanced in the presence of a lipid–water interface, cutinases do not display, or display little interfacial activation, being active on both soluble and emulsified substrates. Hence, cutinases are potentially applicable for biotransformation of synthetic fibers at different processing stages (Liu et al., 2008; Silva et al., 2005; Vertommen et al., 2005).

The catalytic activity of commercially available cutinases is not sufficiently high to fit the requirements of the textile industry. Present constraints that limit textile application are challenged by the on going research aiming at the development of more efficient enzymes. A thermostable esterase from *Thermobifida fusca* with hydrolyzing activity on PET substrates has been recently identified (Alisch et al., 2004; Alisch-Mark et al., 2006; Müller, 2006). A new hydrolase isolated from *Fusarium oxysporum* strain was more efficient in hydrolyzing PET fibers compared to the well known cutinase enzyme from *Fusarium solani pisi* (Nimchua et al., 2007). Hydrophilicity of PET materials was greatly improved after treatment with enzymes from *Thermobifida fusca*, *Penicillium citrinum*, and *Thermomyces lanuginosus* (Eberl et al., 2008; Liebminger et al., 2007). Cutinase from *Fusarium solani pisi* was genetically modified near the active site, by site-directed mutagenesis, to enhance its activity towards PET and polyamide 6,6 fibers (Araújo et al., 2007; O'Neill et al., 2007). Mutants showed an activity increase for PET oligomers of about four/fivefold when compared with the wild type and may thus also be more active on PET fibers.

These novel esterases with enhanced thermostability and catalytic efficiency are potentially applicable for the functionalization of PET fibers at higher temperatures, closer to the glass transition temperature of PET where the chain mobility is higher, thus resulting in increased hydrolysis rates at the surface of the polymer. It is known that enzymes preferentially attack the amorphous regions of polyesters (Herzog et al., 2006; Müller et al., 2005; Vertommen et al., 2005). Müller (2006) proposed the concept of "chain mobility" as one of the main factors responsible for the biodegradability of polyesters. In the amorphous phase the chain mobility is less restricted than in the crystalline phase, especially at temperatures close to glass transition (T_g), thus allowing a closer contact between the polymer chains and the active site of the enzyme.

The enzymatic modification of PET implies the limited hydrolysis of backbone ester bonds, which generates new free hydroxyl and carboxyl groups at the polymer surface, thus leading to increased hydrophilicity of the PET substrate. From the changes in hydrophilicity as a function of time a peeling effect of the enzymes onto the PET fibers has been suggested to occur upon prolonged hydrolysis, resulting in the exposition of surface areas with fewer free carboxyl/hydroxyl groups due to release of soluble PET fragments into the solution (Alisch-Mark et al., 2006; Heumann et al., 2006). The low molecular weight reaction products produced by enzymatic hydrolysis of PET comprise terephthalic acid and ethylene glycol moieties, along with mono- and bis-(2-hydroxyethyl) terephthalate (Vertommen et al., 2005). The kinetics of formation of the degradation products depends on various factors, including the kind of enzyme used and the chemical-physical structure of the polyester substrate (Eberl et al., 2008; Herzog et al., 2006; Heumann et al., 2006; Liebminger et al., 2007; Vertommen et al., 2005). Titration of the released acids (Herzog et al., 2006), determination of terephthalic acid by fluorescence spectroscopy (O'Neill et al., 2007), quantitative analysis of hydrolysis products by reversed phase HPLC (Vertommen et al., 2005) are some of the analytical techniques used to monitor the kinetics of polyesters hydrolysis by enzymes.

A challenge in the study of the enzymatic modification of PET substrates is to precisely quantify the changes of surface properties from the chemical, physical, and structural point of view. To this aim, several direct and indirect analytical approaches have been adopted, including measurement of water contact angle (WCA) (Eberl et al., 2008), rising height (Alisch-Mark et al., 2006; Müller, 2006), water drop absorption (Fischer-Colbrie et al., 2004; Liu et al., 2008), staining and dyeing followed by determination of K/S values (Alisch-Mark et al., 2006; Eberl et al., 2008; O'Neill et al., 2007), X-ray photoelectron spectroscopy (Vertommen et al., 2005), and others. A key issue in the determination of surface properties of PET after enzymatic hydrolysis is to avoid any undesired interference caused by the protein adsorbed onto the polymer surface. Being the reaction carried out under heterogeneous conditions an adsorption step is a pre-requisite for the biocatalysis to occur (O'Neill et al., 2007). However, it is necessary to remove any residual enzyme from the fiber surface before the measurement of surface properties and before further treatments. To this aim, more or less severe washing and/or extraction procedures have been adopted, including the use

of solvents, high temperatures, alkali, detergents, ultrasound, and various combinations of the above ones, etc.

The purpose of the present work is to investigate the chemical and structural changes induced by enzyme-assisted surface hydrolysis of PET substrates by Attenuated Total Reflectance (ATR)–Fourier Transform Infrared spectroscopy (FTIR). FTIR analyses were correlated with WCA measurements in order to investigate the change in surface hydrophilicity of the same substrates. Appropriate blank samples were prepared and measured at the same time. The issue of enzyme adsorption onto the PET surface was addressed by applying suitable after-treatments based on proteolytic enzymes and studying their effect by WCA measurements. The free carboxyl groups formed at the surface of PET by enzyme hydrolysis were specifically esterified with a fluorescent alkyl bromide (2-(bromomethyl)naphthalene) and the reaction was followed by WCA, FTIR, and fluorescence measurements. We believe that the results reported in this study will bring new insights into the surface properties of PET substrates after enzyme modification.

Experimental Section

Enzymes, Chemicals, and PET Substrates

The TEXAZYM EM commercial enzyme formulation was obtained from inoTEX Ltd, Dvur Kralove, Czech Republic. Before use, the commercial formulation was diluted 1:1 with water and dialyzed in cellulose tube with molecular cut-off of 12 kDa (Sigma–Aldrich, Milan, Italy) against distilled water. After dialysis, the aqueous enzymatic solution was centrifuged at 10,000 rpm for 25 min, freeze-dried and stored in a refrigerator prior to use. The aim was to remove low molecular weight additives whose presence was found to modify the PET surface properties, interfering with the measurement of hydrophilicity (data not shown). The enzyme was characterized by SDS–PAGE performed as described by Laemmli (1970). The electrophoretic profiles showed an intense band at MW $\cong$ 22 kDa, and a couple of weak bands at lower MWs (data not shown). A value of 22 kDa was reported for the cutinase from *Fusarium solani pisi* (Carvalho et al., 1998).

Analytical grade chemicals (sodium hydroxide, buffer salts, SDS–PAGE reagents, enzyme assay reagents, 2-(bromomethyl)naphthalene, potassium fluoride, *N,N*-dimethylformamide) and the protease Alcalase were purchased from Sigma–Aldrich and used without further purification.

Two different polyethylene terephthalate (PET) membrane substrates were used: crystalline/oriented PET, obtained from Fait Plast S.p.A. (Cellatica, Brescia, Italy) and amorphous PET, obtained from University of Twente (The Netherlands), abbreviated as PET-Cr and PET-Am, respectively. The PET-Cr thickness was about 70 μm, while that of PET-Am was 200 μm. The degree of crystallinity of PET-Cr and PET-Am was 34.8% and 9.8%, respectively, as determined by DSC run (Vertommen et al., 2005).

Activity Assay

Esterase activity (EC 3.1.1.1) was measured using 4-nitrophenyl butyrate as a substrate (Sigma–Aldrich assay). The increase of the absorbance at 405 nm was measured at room temperature using a Schimadzu UV 1601 spectrophotometer. The increase of absorbance at 405 nm indicates an increase of 4-nitrophenolate due to hydrolysis of the substrate. The activity was calculated in units, where 1 unit is the amount of enzyme required to hydrolyze 1 μmol of substrate per minute under the given assay conditions (pH 7.5; 25°C).

Enzyme Treatment of PET Membranes

The PET membranes were cut into pieces of 10 cm × 1.5 cm, washed with 1% w/v SDS solution for 30 min, at 50/60°C, and then thoroughly rinsed with distilled water. PET samples were incubated with the enzyme in phosphate buffer 50 mM, at pH 8.0 into 14 mL Falcon tubes. The enzyme-to-substrate ratio was 20 U/mg and the material-to-liquor ratio 1:85 (g/mL). Incubation time and temperature were 120 min and 40°C, respectively. To ensure effective temperature control and agitation (O'Neill et al., 2007), the Falcon tubes were closed into the beakers of a Linitest Heraeus apparatus set at 40°C and at 40 rpm/min rotating speed. Appropriate blank samples without enzyme and with enzyme deactivated by heat were run under the same experimental conditions. After incubation, the samples were washed with 1% w/v SDS solution for 30 min at 50/60°C and then with distilled water. Samples were dried at room temperature overnight.

To evaluate the effectiveness of a protease after-treatment for the removal of adhered proteins from PET membranes, samples were incubated in 14 mL of 10 mM sodium acetate buffer containing 5 mM calcium acetate, 5 mg/mL Alcalase, at pH 7.5, at 55°C, for 240 min. Final washing and drying was made as previously described.

Alkali Treatment of PET Membranes

For the alkaline treatment, the protocol reported by Maekawa et al. (2006) was adopted. Briefly, PET membranes were immersed in a 1 M NaOH aqueous solution at 40°C, for 2 h, under agitation on a shaking bath (Isco SBH/D, 110 opm/min). Afterwards, membranes were extensively washed with distilled water and dried at room temperature overnight.

Esterification of Free Carboxyl Groups

PET membranes were dried under vacuum (0.18 mmHg) at room temperature for 2 h. The esterification with 2-(bromomethyl)naphthalene (BrNP) was performed according to the method reported by Maekawa et al. (2006). The chemistry of the reaction is shown in Figure 1. PET samples

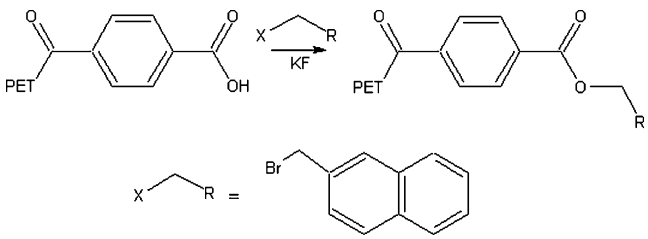

Figure 1. Esterification of free carboxyl groups of PET with BrNP and catalytic KF.

were immersed into a suspension of potassium fluoride (KF; 23.2 mg; 0.4 mmol) in a solution of BrNP (221 mg; 1 mmol) in 20 mL of *N*,*N*-dimethylformamide (DMF) in a beaker shaking for 2 h at room temperature. Membranes were then washed with DMF to remove reagents and dried at room temperature. Afterwards, before contact angles measurements, samples were washed with distilled water and dried at room temperature.

Water Contact Angle Measurements (WCA)

WCA was measured at room temperature using a FTA188 Contact Angle and Surface Tension Analyzer (First 10 Å). Spot WCA measurements made on different areas of PET-Cr and PET-Am membranes showed a high degree of variability, probably attributable to the industrial processing technology used for manufacturing these materials. Thus, the intrinsic variability of PET membranes was preliminarily investigated by means of statistical tools in order to optimize the analytical protocol and to reduce the influence of the variability of surface properties on the results of enzyme treatments. Six different samples of untreated PET-Cr and PET-Am were analyzed by the WCA technique and the results were subjected to the Analysis of Variance (ANOVA). Each PET sample, consisting of a 10 cm × 1.5 cm strip, was fixed to the support of the WCA analyzer with sticky tape and 10 measurements were made along the sample length (Fig. 2). Thus a WCA profile was obtained. Then the WCA results were subjected to the ANOVA test. The results listed in Table I shows that the samples are significantly different because the *P* values of both PET-Cr and PET-Am are smaller than the significant value (0.05) and the *F* values are larger than *F* critical. On the basis of this preliminary investigation it was decided to determine the WCA profiles for each sample under study according to the procedure detailed in Figure 2. An average of 8–10 spot measurements per sample were made and the WCA profiles obtained before and after any treatment were compared. For an accurate determination of the WCA value the sample surface has to be flat. This was not the case of alkali-treated PET-Am,

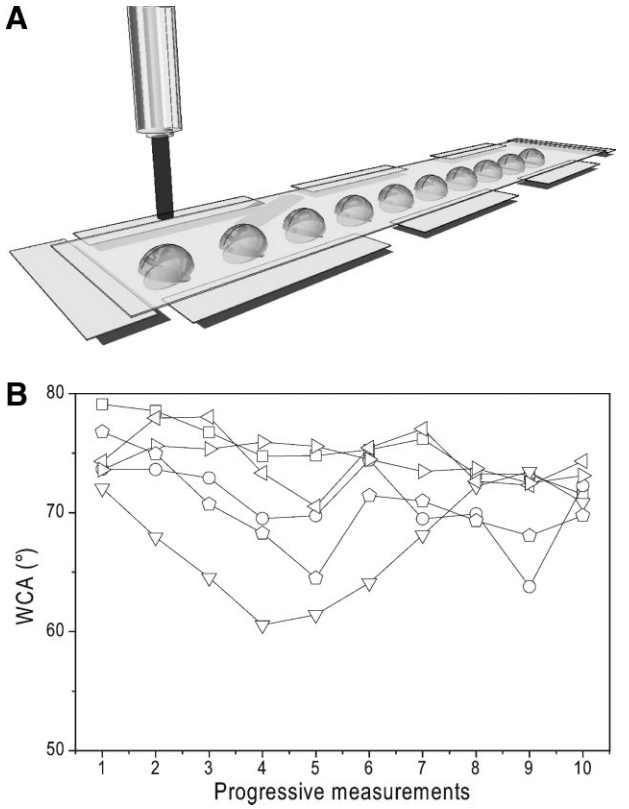

Figure 2. **A**: Progressive WCA measurements on a 10 cm × 1.5 cm PET strip. **B**: WCA profiles measured on different areas of PET-Cr membrane. The profiles are significantly different based on the ANOVA test (Table I).

for which a lower number of spot measurements was made (see Fig. 3B).

Fourier Transform-Infrared Spectroscopy (FTIR)

Measurements were performed on a NEXUS Thermo Nicolet FTIR spectrometer employing an Attenuated Total Reflectance (ATR) accessory mod. Smart Performer. All spectra were obtained with a Ge crystal cell (maximum depth 0.8 μm). Spectra were normalized to the 1,410 cm^{-1} peak before any data processing. Each spectrum reported is the average of at least three spectra measured in different areas of the membrane. Due to the intrinsic orientation of PET-Cr, the spectra were recorded by positioning the samples along one specific direction (conventionally termed MD, "machine direction"). The same procedure was utilized also for the non-oriented PET-Am.

Scanning Electron Microscopy (SEM)

Surface morphology was examined with a SEM model JEOL JSM 6380 LV at 20 kV acceleration voltage, after gold coating (Med 020 Coating System BAL-TEC).

Table I. Analysis of variance of WCA measurements on PET membranes.

PET-Cr	N	Sum	Mean	Variance
Sample 1	10	753.44	75.34	5.68
Sample 2	10	709.26	70.93	10.02
Sample 3	10	675.22	67.52	21.76
Sample 4	10	745.94	74.59	6.35
Sample 5	10	743.31	74.33	1.48
Sample 6	10	704.78	70.48	12.15

Source	SQ	DoF	MQ	*F*-value	*P*-value	*F* critical
Model	466.26	5	93.25	9.74	1.13×10^{-6}	2.39
Error	516.98	54	9.57			
Total	983.24	59				

PET-Am	N	Sum	Mean	Variance
Sample 1	10	725.01	72.50	1.58
Sample 2	10	708.99	70.90	4.43
Sample 3	10	738.47	73.85	7.35
Sample 4	10	705.61	70.56	8.29
Sample 5	10	729.16	72.92	4.44
Sample 6	10	699.66	69.96	4.57

Source	SQ	DoF	MQ	*F*-value	*P*-value	*F* critical
Model	116.34	5	23.27	4.55	1.54×10^{-3}	2.39
Error	275.82	54	5.11			
Total	392.16	59				

Fluorescence Spectroscopy

Photoluminescence emission and excitation spectra were recorded using a FS920 fluorescence spectrometer (Edinburgh Instruments, Livingston, UK) equipped with a Hamamatsu R928P red-sensitive photomultiplier (wavelength range from 200 to 850 nm). The emission and excitation spectra of the surface modified PET films were measured with excitation and emission wavelengths of 350 and 440 nm respectively. The focused excitation beam fell onto sample under an angle of 45° (1 by 3 mm^2), and the emission was monitored under an angle of 45°.

To evaluate the effect of the increased opacity and the scattering effects of the amorphous PET films as a result of the enzymatic treatment compared to the other treatments, the total photoluminescence intensity was measured using an integrating sphere and an Ocean Optics QE65000 fiber optic coupled CCD spectrometer. The excitation spot (wavelength of 350 nm) having a diameter of 2 mm was positioned in the center of round cut samples with a diameter of 5 mm. The samples were put inside the integrating sphere.

Results and Discussion

Enzymatic Hydrolysis of PET Membranes: WCA Measurement of Surface Hydrophilicity

The hydrolysis of backbone ester bonds at the surface of PET generates new free hydroxyl and carboxyl groups, thus leading to increased hydrophilicity that can be quantitatively assessed by WCA measurements. Alkaline hydrolysis of PET by immersion in an aqueous solution of sodium hydroxide was first performed as model reaction for determining the increase of hydrophilicity by conventional way (Maekawa et al., 2006). To evaluate the extent of surface modification induced by alkali, WCA measurements were carried out on the samples before and after treatment. Upon alkali treatment the WCA profiles of both crystalline and amorphous PET membranes shifted to lower values, indicating that a higher degree of surface hydrophilicity was achieved (Fig. 3). The average WCA values decreased of about 19° and 30° for PET-Cr and PET-Am, respectively (Table II). The effect of alkali was stronger on the amorphous membrane, whereas the variability along the membrane surface was higher in the case of the crystalline membrane.

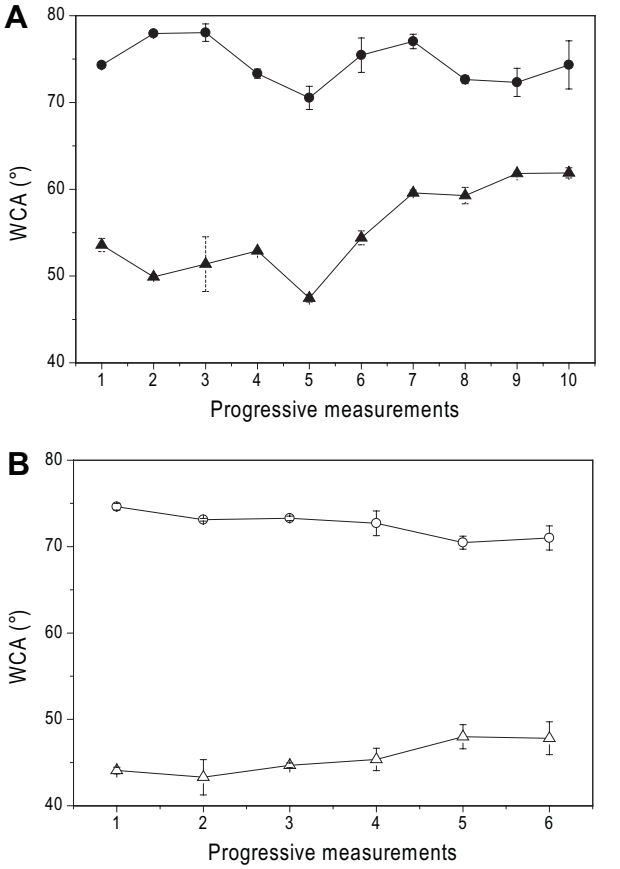

Figure 3. WCA profiles of PET-Cr (**A**) and PET-Am (**B**) untreated (-○-) and treated with 1 M NaOH (-△-).

When the crystalline and amorphous PET membranes were incubated with the lipolytic enzyme the WCA profiles shifted downwards indicating a net increase of surface hydrophilicity (Figs. 2B and 4A). The effect was more pronounced for the amorphous membrane, whose average WCA value decreased of about 19°. A decrease of only 10°

Table II. Average WCA values of crystalline and amorphous PET membranes subjected to alkali or enzyme treatment.[a]

Samples	PET-Cr	PET-Am
Untreated	74.6 ± 1.1	75.2 ± 0.8
Alkali	55.2 ± 0.7	45.5 ± 1.2
Lipolytic enzyme	64.2 ± 0.8	53.2 ± 2.4
Lipolytic enzyme + Alcalase	68.2 ± 1.2	58.3 ± 2.5

[a]For each sample, the individual WCA values collected along the profile were averaged (±SD).

was measured for the crystalline membrane. The amorphous nature of the PET-Am favored the hydrolytic attack of the lipolytic enzyme, in good agreement with previously reported findings (Herzog et al., 2006; Müller, 2006; Vertommen et al., 2005).

To ascertain whether the increase of surface hydrophilicity was influenced by the presence of enzyme proteins still sticking onto the surface of PET after washing, the membranes were further incubated with an alkaline protease (Alcalase). A PET membrane previously treated with a solution of deactivated lypolitic enzyme was also incubated with the protease. This protease was chosen because it showed very low levels of non-specific binding to the PET substrates under the experimental conditions adopted (Fig. 4D). Moreover, it proved effective in removing other non-enzymatic proteins, that is, egg albumin, which displayed non-specific adhesion to the PET membrane (data not shown). Upon incubation with protease the WCA profile of the PET membranes shifted upwards. The membrane previously treated with the deactivated enzyme recovered its initial level of hydrophobicity (Fig. 4C), while both PET-Cr and PET-Am previously treated with active lypolitic enzyme showed a partial recovery of surface hydrophobicity (Fig. 4A and B). However, the average WCA values remained lower that those of the corresponding untreated samples, with PET-Am still displaying a greater extent of surface hydrophilicity than PET-Cr (Table II).

The surface morphology of alkali and enzyme treated PET membranes was examined by SEM (Fig. 5). The enzyme treatment did not induce any detectable change of surface morphology of the PET substrate, whereas alkali caused the formation of holes, in good agreement with previously reported results (Kim and Song, 2006). Therefore, it can be concluded that the lipolytic enzyme was able to increase the hydrophilic character of the PET membranes, especially of the amorphous one, without altering the surface and bulk properties of the substrate (no weight loss was detected). This is unanimously considered a big advantage of the

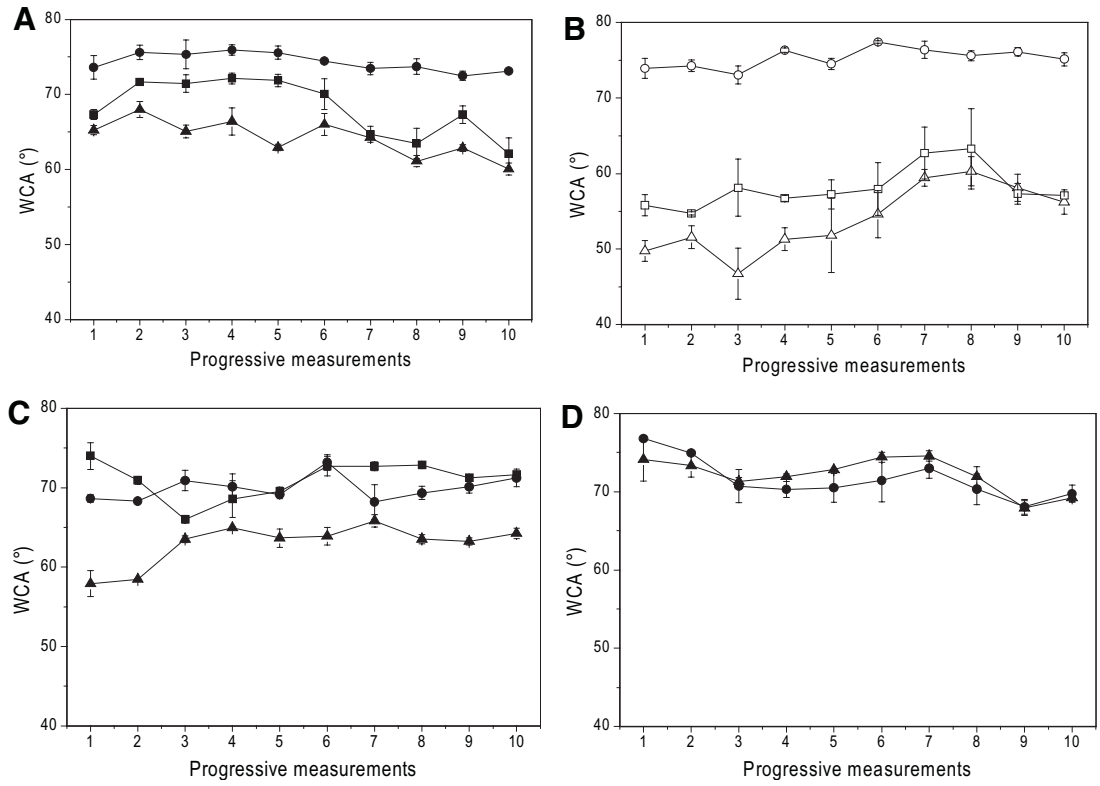

Figure 4. WCA profiles of PET-Cr (**A**) and PET-Am (**B**) untreated (-○-), after treatment with lipolytic enzyme (-△-), and subsequently treated with protease (-□-). **C**: WCA profiles of PET-Cr untreated (-○-), incubated with deactivated lipolytic enzyme (-△-), and subsequently treated with protease (-□-). **D**: WCA profiles of PET-Cr untreated (-○-) and incubated with protease (-△-).

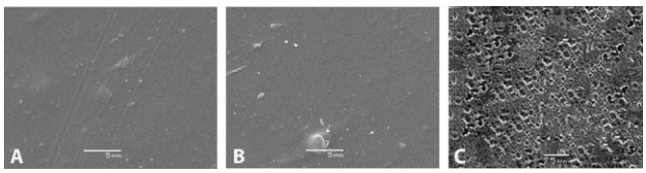

Figure 5. SEM photographs of PET-Cr untreated (**A**), treated with lipolytic enzyme (**B**), and treated with 1 M NaOH.

biocatalytic approach compared to the conventional alkali treatment.

The overall results here reported address a couple of key topics related to the surface modification of PET substrates by lipolytic enzymes. We specifically refer to (i) the effect of the crystalline structure of PET and (ii) to the artefacts sometimes caused by the hydrophobically driven adsorption of enzyme proteins onto the PET surface. It is widely recognized that the higher the crystallinity of the PET polymer the lower the ability of the enzyme to hydrolyze it. Herzog et al. (2006) extensively investigated the mechanism of the enzymatic hydrolysis of aliphatic and aromatic polyesters and found that the amorphous nature of the polymer substrate enhanced the rate of the hydrolytic degradation of ester bonds. As stated earlier in this paper Müller (2006) proposed the chain mobility concept as one of the most relevant factors controlling the rate and extent of polyester hydrolysis. Chain mobility is higher in the amorphous regions and is enhanced as the temperature approaches the T_g of the polymer (Müller et al., 2005). Accordingly, a thermophilic lipolytic enzyme from *T. fusca* displayed increased ability to cleave the ester bonds of aromatic polyesters like PET at 70°C than at 50°C (Alisch et al., 2004). The results obtained in this study confirm those previously reported by Vertommen et al. (2005) about the higher accessibility of amorphous PET substrates to the action of lipolytic enzymes. The nature of the changes induced by the enzyme at the surface of PET will be addressed in the next paragraph.

Enzyme protein adsorption is a key topic for the biocatalytic modification of polymers (Guebitz and Cavaco-Paulo, 2008). On the one hand, adsorption of the enzyme to the surface of solid PET substrates is required for the effective hydrolysis of the polymer chains. This is a prerequisite for any heterogeneous reaction system such as that under investigation (O'Neill et al., 2007). On the other hand, effective removal of the enzyme after reaction is needed to avoid undesired interferences with the measurement of surface properties, in particular with the determination of surface hydrophilicity by contact angle or other techniques, which may lead to misleading results. Thus, it is very important to adopt suitable after-treatments capable to completely remove the enzyme (Liu et al., 2008). This topic was clearly addressed by one of the authors in a previous study, where protein layer thicknesses up to about 3 nm were measured by XPS (Vertommen et al., 2005). In the present study, the use of a protease for the effective removal of residual proteins from the surface of PET membranes was attempted to clean the samples before further surface analysis. Indeed, the findings here reported may open the way to develop suitable protocols for the enzyme processing of PET fabrics, comprising lipolytic and proteolytic enzyme treatments which may leave a clean and activated fabric surface with accessible hydroxyl and carboxyl groups for further functionalization and finishing. Complete removal of residual protein layers adhering to the fabric surface will be beneficial to improve the fastness of finishing treatments.

Surface Structural Changes of Enzyme-Treated PET Membranes: FTIR Measurements

FTIR spectroscopy is a powerful method for the structural and conformational characterization of polymers because of the sensitivity of molecular vibrations to bond strengths and configurations. This technique has been widely applied to probe the conformational changes of PET polymers involving either the ethylene glycol moieties, or the benzene rings, or the carbonyl groups (Štokr et al., 1982). Thus, systematic FTIR measurements of PET-Cr and PET-Am membranes before and after enzyme or alkali treatments were performed with the aim to highlight surface chemical, structural, and conformational changes. In this study, a spectroscopic characterization of untreated amorphous and crystalline PET membranes is presented first. Then, the main spectral changes observed on amorphous PET membrane upon incubation with the lipolytic enzyme are discussed in more detail. A complete spectroscopic characterization of PET membranes, including enzyme-treated crystalline membranes and alkali-treated membranes, will be reported elsewhere.

Figure 6 shows the FTIR spectra of PET-Am and PET-Cr samples. The spectra were normalized to the area of the 1,410 cm^{-1} band (ring CH in plane bending and CC stretching), which has already proved its suitability as internal reference band (Cole et al., 1994; Walls, 1991). One of the most important characteristics of the molecular conformation of PET concerns the existence of *trans* and *gauche* rotational conformers for the ethylene glycol moiety (Boerio et al., 1976; Cunningham et al., 1974; D'Esposito and Koenig, 1976; Štokr et al., 1982; Ward, 1985; Ward and Wilding, 1977). Both types of conformers are present in the amorphous phase, but only the *trans* conformer is present in the crystalline phase (Ward and Wilding, 1977). The marker bands attributed to crystalline PET with the ethylene glycol moiety in the *trans* conformation fall at 1,471 cm^{-1} (CH_2 bending), 1,341 cm^{-1} (CH_2 wagging), 1,123 cm^{-1} (O–CH_2 and ring CC stretching, ring CH in plane bending), 972 cm^{-1} (O–CH_2 and C(=O)–O stretching), and 849 cm^{-1} (various bending modes of the benzene ring) (Dadsetan et al., 1999; Dunn and Ouderkirk, 1990; Holland

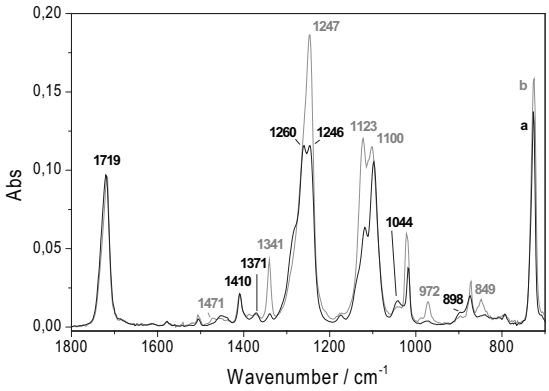

Figure 6. FTIR–ATR spectra of untreated PET-Am (**a**) and PET-Cr (**b**). Spectra were recorded along the same direction (MD).

and Hay, 2002; Krimm, 1960; Liang and Krimm, 1959; Liu et al., 2000). All these bands are present in the spectrum of PET-Cr, whereas they are sensibly weaker or absent in the spectrum of PET-Am. On the contrary, PET-Am showed the presence of bands at 1,371 cm^{-1} (CH_2 wagging), 1,044 cm^{-1} (C–O stretching), and 898 cm^{-1} (CH_2 rocking), which are attributed to the *gauche*/amorphous conformation of PET.

Other spectral features confirm the higher crystallinity of PET-Cr, such as the position of the strong carbonyl stretching band at 1,719 cm^{-1} and the value of full width at half maximum (FWHM; Table III). In PET-Am this band moved to lower wavenumbers (1,717 cm^{-1}) and the FWHM increased (Table III). A similar behavior was observed by Cole et al. (1994) going from an amorphous PET sample with <5% crystal content to a crystalline PET with 28% crystal content. This spectral pattern is determined by the more ordered structure of crystalline PET in which the carbonyl groups are coplanar with the benzene rings.

Another important spectral range significantly influenced by the amorphous/crystalline state of PET polymers is the doublet near 1,100 cm^{-1} (C–O stretching and other vibrations). Going from amorphous to crystalline PET, the bands slightly shifted to lower wavenumbers and the intensity of the 1,120 cm^{-1} component decreased. This behavior is in agreement with the assignment reported for these bands. In fact, the component at about 1,100 cm^{-1} has been attributed to *gauche* and *trans* conformers (Cole et al., 1994; Ward and Wilding, 1977) or to the amorphous conformation (Dunn and Ouderkirk, 1990; Krimm, 1960), whereas the component at about 1,120 cm^{-1} has been assigned to crystalline PET (Liang and Krimm, 1959; Liu et al., 2000; Štokr et al., 1982). The strong absorptions falling at about 1,250 cm^{-1} (C(=O)O stretching, ring-ester CC stretching, C=O in plane bending) are not conformationally sensitive but are helpful for the identification of the structural state of PET. In fact, a couple of bands with maxima at 1,260 and 1,246 cm^{-1} and quite similar intensity characterize the amorphous PET, whereas in crystalline PET a single intense band appears at 1,247 cm^{-1}. For the quantitative assessment of crystallinity, the A_{1341}/A_{1410} ratio (based on peak areas) and the I_{1120}/I_{1100} ratio (based on peak heights) were calculated (Table III). Both absorbance ratios were significantly higher in PET-Cr than in PET-Am. These results are in agreement with the degree of crystallinity determined by DSC runs, which gave values of 34.8% and 9.8% for PET-Cr and PET-Am, respectively (Vertommen et al., 2005).

Table III. Intensity ratios (±SD) calculated from the normalized spectra of PET-Cr and PET-Am.

Samples	A_{1341}/A_{1410}	I_{1120}/A_{1100}	$FWHM_{1715}$
PET-Cr	1.87 ± 0.05	1.03 ± 0.02	18.6 ± 0.9
PET-Am	0.21 ± 0.02	0.59 ± 0.01	22.1 ± 0.2
PET-Am + enzyme	0.37 ± 0.06	0.66 ± 0.02	21.8 ± 0.1
PET-Am + enzyme + BrNP	1.40 ± 0.06	0.91 ± 0.02	20.0 ± 0.2

Each value represents the average obtained from three spectra recorded along the MD direction.

Upon incubation with the lipolytic enzyme, PET membranes underwent surface chemical and structural changes which were detected by FTIR spectroscopy. Here we report the results obtained on the amorphous PET membrane where spectral changes were more evident. Figure 7 shows typical FTIR spectra of PET-Am before and after enzyme treatment, with enlargements of the spectral ranges changed by the enzyme reaction. The carbonyl stretching band at 1,720 cm^{-1} decreased in intensity upon enzyme reaction (Fig. 7A) but its position and FWHM value remained almost unchanged (Table III). In the 1,250 and 1,100 cm^{-1} regions (Fig. 7B and C, respectively), where strong carbonyl vibrations fall, the bands changed in shape and relative intensity. In particular, in the 1,250 cm^{-1} range the intensity of the component at 1,246 cm^{-1} decreased and the shoulder at higher wavenumbers increased. The doublet near 1,100 cm^{-1} also displayed a decrease of the component at lower wavenumbers, attributed to the amorphous phase, and an increase of the shoulder at higher wavenumbers. These changes confirm that the ester bonds of PET were the main target of the enzyme cleavage activity. As a consequence of these spectral changes, the I_{1120}/I_{1100} absorbance ratio increased after enzyme treatment, suggesting an increase of crystallinity (Table III). Accordingly, the intensity of the marker band of crystalline PET at 1,341 cm^{-1}, which arises from CH_2 wagging of *trans* conformers of the ethylene glycol unit, significantly increased (Fig. 7D) and became stronger than the band at 1,370 cm^{-1} (*gauche* conformers). It can be inferred from these results that upon enzyme treatment the *trans* conformers were enriched either by selective hydrolysis and removal of the *gauche* conformers or by *gauche* → *trans* conversion. Therefore, it is confirmed that the lipolytic enzyme displayed a noticeable level of hydrolytic activity towards the amorphous regions of PET, resulting in an increase of crystallinity and/or in conformational changes leading to a more ordered state of the polymer chains at the PET surface.

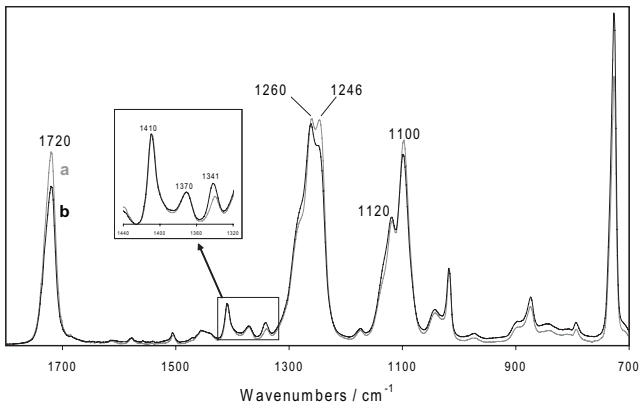

Figure 7. FTIR–ATR spectra in the 1,800–700 cm^{-1} range of PET-Am untreated (**a**) and treated with lipolytic enzyme (**b**). Box: enlarged view of the 1,440–1,320 cm^{-1} spectral range.

Surface Modification of PET Membranes by Esterification of Free Carboxyl Groups

Enzyme hydrolysis of PET membranes causes the formation of free hydroxyl and carboxyl groups on the surface which might provide suitable functionality for subsequent chemical reactions (Guebitz and Cavaco-Paulo, 2008). In this study, the selective esterification of the carboxyl groups using an alkyl bromide (BrNP) with catalytic potassium fluoride (KF) was used as model reaction to evaluate the potential of chemical modification of PET after enzyme hydrolysis (Maekawa et al., 2006). Since the esterification of the hydrolyzed PET surface is expected to make it more hydrophobic, the extent of esterification was first monitored with WCA measurements. The ester groups formed by reaction of BrNP with the carboxylic groups are quite similar to those of the original structure of PET. Accordingly, the restoration of the WCA value close to that of non-hydrolyzed PET should indicate that the reaction proceeded efficiently.

Figure 8 shows the WCA profiles of different PET samples. Esterification of an untreated PET membrane did

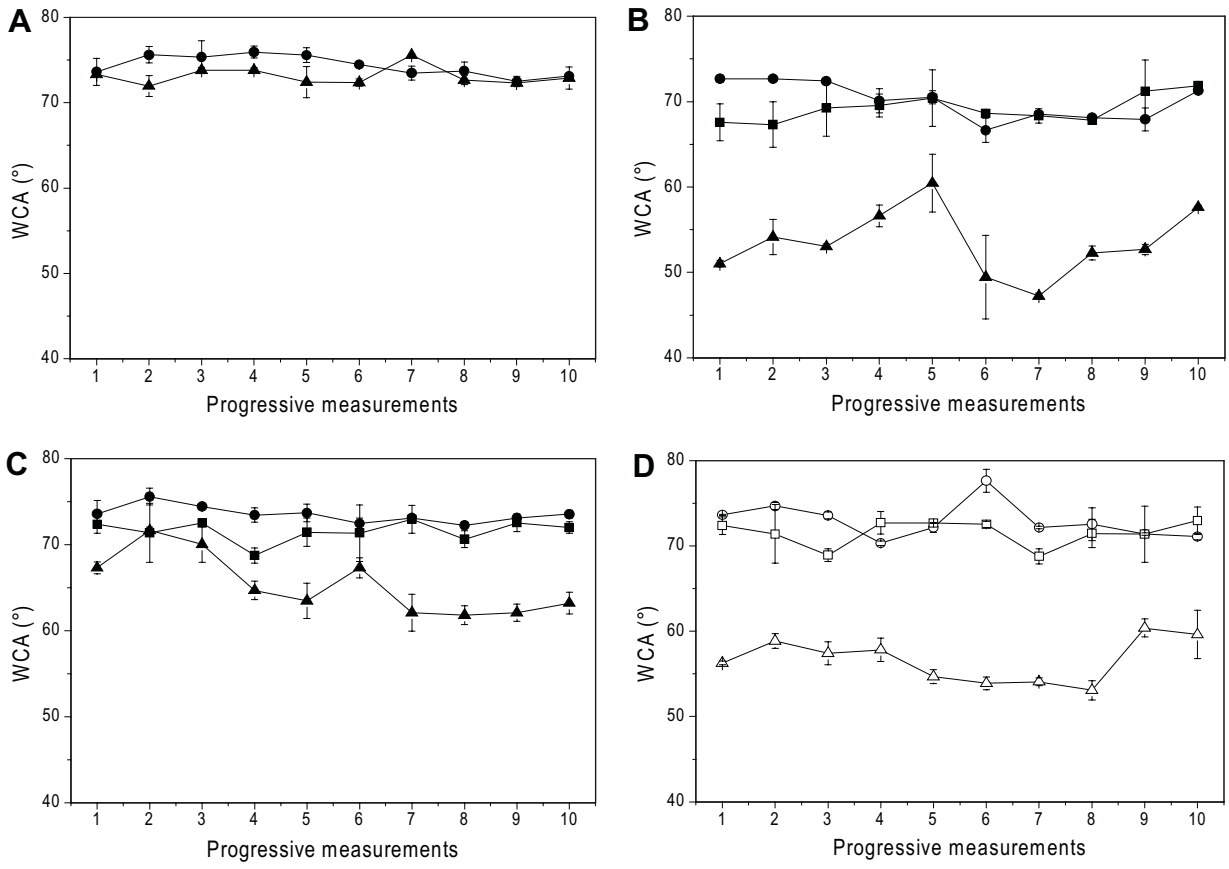

Figure 8. **A**: WCA profiles of PET-Cr untreated (-○-) and after reaction with BrNP (-△-). **B**: WCA profiles of PET-Cr untreated (-○-), treated with 1 M NaOH (-△-), and subsequently alkylated with BrNP (-□-). **C**: WCA profiles of PET-Cr untreated (-○-), treated lipolytic enzyme/protease (-△-), and subsequently alkylated with BrNP (-□-). **D**: WCA profiles of PET-Am untreated (-○-), treated lipolytic enzyme/protease (-△-), and subsequently alkylated with BrNP (-□-).

not change the surface hydrophobicity of the material (Fig. 8A). When the alkali-treated PET membrane was esterified with BrNP, the initial hydrophobic level was restored as shown by overlapping of the WCA profiles before and after treatment (Fig. 8B). Similar results were obtained with PET-Cr and PET-Am membranes treated with the lipolytic enzyme (Figs. 8C and 6D, respectively). These results indicate that the free carboxyl groups made available by surface hydrolysis can be used for further chemical reaction.

Figure 9 shows the excitation and emission spectra for different treatments as well as the relevant blanks. The spectra are identical for all six samples. It is evident from these results that enzymatically treated amorphous polyester films have six times higher photoluminescence than the other samples. The hydroxide treated crystalline film only shows a slight increase. The increased photoluminescence is most likely the consequence of increased esterification with BrNP, which is higher in case of increased surface hydrolysis (sample C and E).

The enzymatically treated PET films show somewhat increased opacity (Vertommen et al., 2005). To evaluate whether the increased photoluminescence is caused by the increased esterification and not the effect of increased opacity the emission spectra were measured using an integrating sphere (Fig. 10). In the standard experimental setup the measurement is only over a limited space angle. By placing the sample inside an integrating sphere, all effects of scattering and light out-coupling are taken into account. Similar to the analysis using the standard setup the enzymatically treated amorphous polyester films have three to four times higher photoluminescence than the other samples.

The chemical and structural changes induced on PET membranes by esterification were examined by FTIR. Figure 11 compares the spectra of PET-Am treated with the lipolytic enzyme before and after esterification with BrNP. The spectrum of the esterified sample showed significant spectral changes. The FWHM of the carbonyl stretching band at 1,719 cm^{-1} decreased (Table III). The crystalline marker band at 1,341 cm^{-1} significantly increased in intensity and other new components typical of crystalline PET appeared at 1,471, 972, and 849 cm^{-1}. Also, the spectral pattern in the 1,250 cm^{-1} region significantly changed. Some vibrational modes shifted to wavenumber positions closer to the crystalline state of PET. This was the case of the ring CH in-plane bending at 1,017 cm^{-1} which shifted to higher wavenumbers and of the ring mode at 874 cm^{-1} which shifted to lower wavenumbers. Finally, the intensity of the bands at 1,371 and 1,044 cm^{-1} typical of amorphous PET decreased. All these changes seem to support the hypothesis that upon esterification with BrNP the surface of PET achieved a more crystalline character. A quantitative evaluation of this structural transition is provided by the A_{1341}/A_{1410} and I_{1120}/I_{1100} intensity ratios which increased significantly, approaching the values of crystalline PET (Table III).

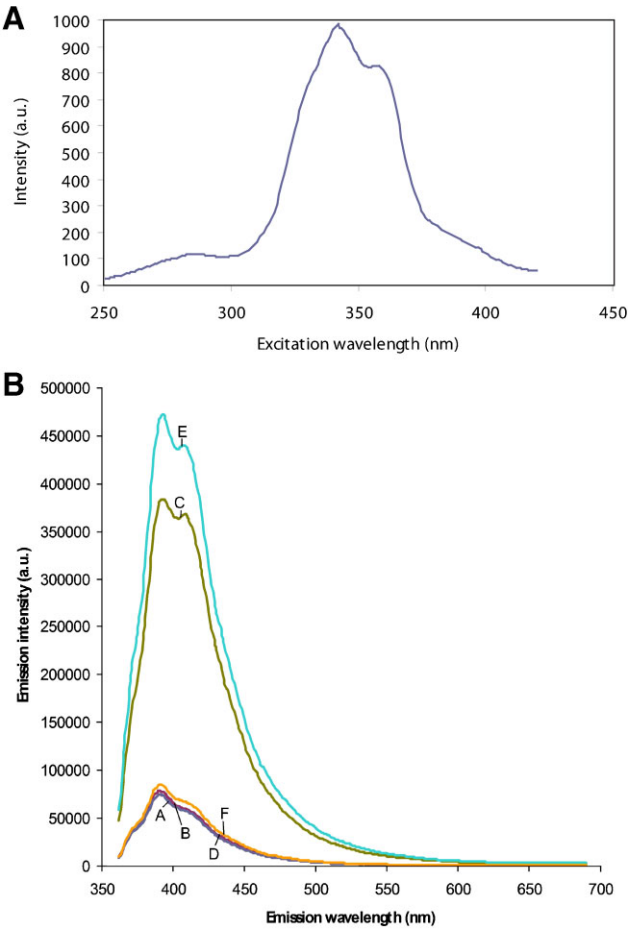

Figure 9. The excitation (**A**) and emission (**B**) spectra of the six samples (the spectra are corrected detector sensitivity). The excitation spectrum is for sample E only. A = PET-Cr + BrNP, B and D = PET-Cr + Enzyme + BrNP (duplicate samples), C and E = PET-Am + Enzyme + BrNP (duplicate samples), and F = PET-Cr + NaOH + BrNP. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

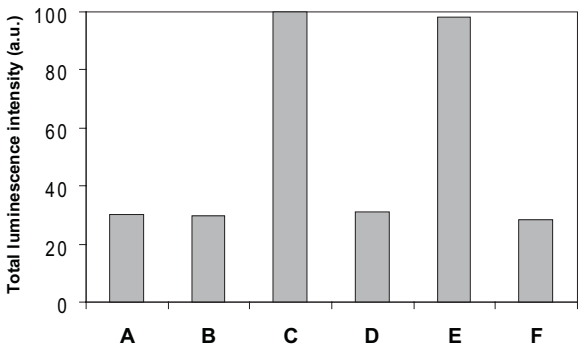

Figure 10. The total emission intensity of the six samples upon excitation at 350 nm as measured with an integrating sphere. **A** = PET-Cr + BrNP, **B** and **D** = PET-Cr + Enzyme + BrNP (duplicate samples), **C** and **E** = PET-Am + Enzyme + BrNP (duplicate samples), and **F** = PET-Cr + NaOH + BrNP.

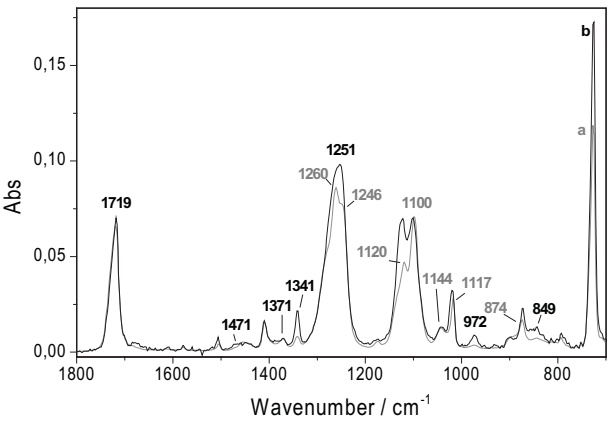

Figure 11. FTIR–ATR spectra of PET-Am treated with lipolytic enzyme/protease (**a**) and subsequently alkylated with BrNP (**b**). Spectra were recorded along the same direction (MD).

Conclusions

The lipolytic enzyme displayed higher hydrolytic activity towards the amorphous PET membrane, as indicated by the significant increase of surface hydrophilicity. Indeed, also the surface of the crystalline PET membrane was attacked by the enzyme, although to a much lower extent.

The protease after-treatment effectively removed the residual enzyme protein adhering to the surface. These results point out that the adsorption of enzyme proteins onto the PET surface is a key topic that must be addressed for both scientific and technological purposes. The effective removal of adhering proteins will avoid artefact when the surface properties of enzyme-treated PET materials are measured, and will provide cleaner and activated surfaces ready for further modification, such as in the case of PET textiles finishing.

FTIR analysis allowed to highlight chemical and structural features involving the effect of the enzyme on the outermost layers of the PET polymer. Further investigations will be aimed at going deeper into the details of the mechanism of the biocatalytic modification of PET and of the enzyme-PET interactions. Fundamental knowledge in this field may not only contribute to the development of bioprocesses for polyester substrates, but also address the genetic engineering of lipolytic enzymes with enhanced hydrolyzing activity with respect to the native ones.

Finally, the free carboxylic groups made available at the surface of PET membranes by enzyme hydrolysis were esterified with a fluorescent alkyl bromide. The reaction proceeded effectively and the enzymatically treated PET films were the most reactive. The obtained results demonstrate that we are not just able to modify PET films through enzymatic surface hydrolysis, but that we can functionalize the enzymatically modified PET surface as well.

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