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**ORIGINAL ARTICLE**

# Enzymatic surface hydrolysis of PET enhances bonding in PVC coating

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**Abstract**

The effect of polyesterase preparations from *Thermomyces lanuginosus* and *Beauveria brongniartii* on the hydrophilicity of PET materials was assessed. As a result of polyesterase treatment the hydrophilicity of PET fabrics was increased by up to 8 cm in terms of rising height with increases in surface tension from 6.2 mNm (heat-inactivated control samples) to above 8 mNm. Both enzymes were able to increase the amount of hydroxyl groups on PET from 90 to a maximum of 182 mmol $kg^{-1}$, while only the *B. brongniartii* polyesterase released significant amounts of terephthalic acid from PET. Enzymatic surface hydrolysis of PET increased the bonding strength in PVC coating to 13.40 daN $5cm^{-1}$ using 0.5% adhesive compared to 11.5 daN $5cm^{-1}$ obtained without enzyme pretreatment and 6% of adhesive.

**Keywords:** Beauveria brongniartii, *polyesterase, PVC, PET*

## Introduction

PET is the most important polymer used for the production of synthetic textile fibres (Guebitz & Cavaco-Paulo 2008), and also has many other applications ranging from bulk products (plastic bottles) to medical or electronic devices. It has been already reported that enzymes can be used for the hydrolysis and recycling of PET (Mueller 2006). In domestic washing processes of PET based fabrics, polyesterases have shown potential for depilling (Yoon et al. 2002). More recently, targeted surface hydrolysis for functionalization of PET fabrics has been reported and PET hydrolysing enzymes were described among cutinases, lipases and esterases. Increased hydrophilicity obtained due to enzymatic surface hydrolysis can improve properties, such as water permeability and wear comfort of PET fabrics. An increased number of hydroxyl and carboxyl groups on the PET surface serve as anchor groups for bonding of molecules that can make finishing processes such as dyeing more efficient (Guebitz & Cavaco-Paulo 2008).

Similarly, an increased amount of functional groups on the PET surface could improve coating processes such as in the production of flexible electronic devices (Laskarakis et al. 2008) or with biomaterials in general (Goddard et al. 2007). In coating of PET fabrics with PVC, the bonding strength depends on chemical cross-linking with functional groups on the PET surface. Currently, high amounts of adhesives (up to 10%) are used to obtain a sufficiently high bonding strength suitable for the production of tarpaulins. However, adhesives are both toxic and expensive.

In this study, we assessed the potential of two polyesterases, from *Thermomyces lanuginosus* and *Beauveria brongniartii,* to insert additional functional groups into PET materials, thereby enhancing bonding strength in PVC coating.

## Materials and methods

### *Enzymes*

*Beauveria brongniartii* was grown on potato dextrose agar (PDA) plates (42 g $l^{-1}$) for 2 days at 30°C then stored at 4°C. To maintain the viability of the fungus, it was replated every 3 months. The culture medium contained yeast extract (1.5 g $l^{-1}$), glucose (1.0 g $l^{-1}$), $MgSO_4.7H_2O$ (4.0 g $l^{-1}$), $(NH_4)_2SO_4$ (1.0 g $l^{-1}$) $KH_2PO_4$ and $Na_2HPO_4$ (2.5 g $l^{-1}$ each)

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in tap water. The pH was adjusted to 6.5 using HCl. Wheat bran (10.0 g $l^{-1}$) was added as an additional carbon source. Erlenmeyer flasks filled with 400 mL medium were inoculated with 1% v/v of the fungus pre-culture and incubated on a rotary shaker at 30°C and 130 rpm for 5 days. The biomass was separated using a sieve and the supernatant centrifuged at 7000 g for clarification of the medium. The extracellular enzymes were stored at −20°C for further use.

The enzyme preparation from *Thermomyces lanuginosus* was provided by inoTEX s.r.o. (Czech Republic).

*Protein concentration.* Protein concentrations were determined following Bradford's method using a Bio-rad kit. 100 l enzyme solution were added to 700 l buffer (pH 6.0) and 200 l Bio-Rad reagent, and the change in absorption at 595 nm was recorded using a Hitachi U-2001 spectrophotometer. The method was calibrated using bovine serum albumin (BSA) as reference.

*Enzyme activity.* Esterase activity was determined at 25°C monitoring the hydrolysis of *p*-nitrophenyl-propionate as substrate spectrophotometrically at 405 nm as described previously (Liebminger et al. 2007). One nkat is defined as the amount of enzyme cleaving 1 nmol of ester $s^{-1}$ under the given assay conditions. Lipase activity was determined similarly using *p*-nitrophenyl palmitate as substrate (Heumann et al. 2006). Cutinase activity was estimated qualitatively by incubating 1 mL of the pure enzyme preparation with 25 mg cutin isolated from apple peel following a slightly modified procedure as previously described (Murphy et al. 1996) at 25°C with gentle shaking for 24 h. The cutin was subsequently removed by filtration, and the incubation mixture acidified and subsequently extracted with chloroform. The extract was applied on a silica gel TLC plate and developed with 2:1 chloroform/ethyl acetate. Spots were compared with two blanks, one consisting of the extract from a substrate-free enzyme preparation, and one from an incubation assay using a 100 mM Tris buffer instead of enzyme. Results were compared with a cutinase from *Optimyces sp.*, obtained from VTT (Helsinki, Finland).

*Enzymatic modification of synthetic fibres.* PET fabrics with densities of 62 and 177 g $m^{-2}$ were obtained from CIBA and labelled as PET1 and PET3. They were washed with $Na_2CO_3$ (1 g $L^{-1}$) for 30 min to remove surface finishes and subsequently rinsed with water for 10 min. During the PET production process, shorter polymer chains may remain on the fibre surfaces. To ensure an enzymatic reaction with the polymers and not with the residual oligomers, the fibres were extracted with $CH_2Cl_2$ overnight and afterwards dried at room temperature. PET fabrics were cut into pieces of $2 \times 20$ cm and treated in 50 mL of diluted enzyme preparations with final activities as indicated below in 250 mL flasks on a shaker (PET3: 120 rpm, PET1: 80 rpm). Control samples with heat denatured enzyme were treated identically. After the treatment, fabrics were washed with 1 g $mL^{-1}$ $Na_2CO_3$ (pH 10) and distilled water (four times) to remove residual enzyme or other media impurities. Thereafter, fabrics were dried at room temperature overnight

*Measurement of surface hydrophilicity*

A slightly modified method from DIN 53924 was applied for the measurement of rising height. It consisted of hanging the fabrics (cm) on a glass rod and suspending the lower part in a bath of distilled water. Afterwards the level of the adsorbed water on the fabric was measured.

The drop dissipation measurements were carried out as described by Heumann et al. (2006). A drop of distilled water (20 L) was placed on the surface of the fabric. The time for the fabric to take up the drop was measured. All measurements were carried out in triplicate. For surface tensiometry the K 100 apparatus from Krüss (Hamburg, Germany) was used. The surface tension s in mNm of the samples was determined in the Wilhelmy-type automatic surface balance mode at 25°C.

*Hydroxyl group determination.* The determination of the amount of hydroxyl groups in the PET samples was based on esterification of the hydroxyl groups with sulphobenzoic acid anhydride, following a method modified from Zimmermann & Kolbig (1967). Samples 0.5 g PET and 0.9 g sulphobenzoic acid anhydride were immersed in 10 mL dry nitrobenzene. The reaction mixture was heated with stirring in a round bottomed flask on a reflux cooler to 170°C for 10 min or until complete dissolution of the PET material. After complete dissolution, the reaction mixture was cooled and kept at a constant temperature of 130°C. After 60 min, the reaction was cooled to room temperature and 500 μL of a 0.1 M NaOH solution was added to the mixture in order to hydrolyse the residual sulphobenzoic acid anhydride to the sulphobenzoic acid. 50 mL of an acetone/water (9/1 v/v) mixture were added under strong stirring in order to precipitate the esterified PET. Precipitation was allowed to complete by putting on ice for 15 min. The reaction mixture was subsequently filtered using a ceramic filter device. The content

of sulphobenzoic acid in the filtrate was determined by HPLC. Samples were diluted 1:10 and applied on a reversed-phase $C_{18}$ column (Hypersil® ODS 5 μm 250 × 4.6 mm, Chrom) and eluted with 50 mM $H_2SO_4$ containing 5% methanol (solvents obtained from Sigma-Aldrich were HPLC gradient grade) on a Dionex HPLC. Peaks were detected at 215 nm on a photodiode array detector. The amount of OH groups was calculated using the following equation:

$$\text{OH end groups} = [(SBAA_{init} - SBA_{end}) \times 10^6/\text{PET}] \text{ mmol kg}^{-1}$$

where $SBAA_{init}$ is the total sulphobenzoic acid anhydride used for the reaction (mol), $SBA_{end}$ is the total sulphobenzoic acid determined as unreacted (mol) and PET is the total of PET used for the reaction (g).

### *Detection of terephthalic acid*

Terephthalic acid released from PET during enzyme treatment was reacted with 30% hydrogen peroxide at boiling temperature to give hydroxy-terephthalic acid, which was monitored using fluorescence spectroscopy as described previously (O'Neill et al. 2007).

### *PVC coating*

PET samples for truck tarpaulin production were obtained from Sattler AG (Graz, Austria; material number 6602 PET endless multifilament, 500 dtex). Treated and blank samples for truck tarpaulin production were investigated by measuring the adhesiveness of a PVC coating paste. Samples (about 30 × 42 cm) were coated on a Mathis laboratory size machine. The material was coated twice with a PVC paste (Sattler No. 161004). The first coating was applied at 180°C (150 g $m^{-2}$) containing 0.5–2% of an adhesive agent ('Haftvermittler 2007', Bayer Leverkusen, Germany), the second coating was done at 160°C using 100 g $m^{-2}$ PVC paste.

Adhesiveness of the coating was tested according to DIN EN ISO 2411. Two pieces of the PVC-coated PET were welded by high frequency inductive welding. The power needed to destroy the welding seam, which was exactly 5 cm, was measured and expressed as daN (5 cm)$^{-1}$.

## Results and discussion

*Beauveria brongniartii* was grown on a complex cultivation medium containing wheat bran. Under these conditions this organism produced 1.0 nKat $mL^{-1}$ of esterase activity on *p*-nitrophenyl propionate and lipase activity on *p*-nitrophenyl decanoate (0.8 nKat $mL^{-1}$) and palmitate (0.8 nKat $mL^{-1}$). In a preliminary step, the potential of this esterase preparation to increase the hydrophilicity of PET fabrics was assessed.

The higher the *p*-nitrophenyl propionate activity of the *B. brongniartii* esterase dosed, the higher the observed rising height, until a plateau value was reached at 5 cm at an esterase activity of 1.0 nKat $mL^{-1}$ (Figure 1). In agreement with this data, the minimum drop dissipation time was also reached at an esterase activity of 1.0 nKat $mL^{-1}$ (Figure 2). A similar behaviour was observed for fabrics with 177 and 62g $m^{-2}$ fabrics. It has previously been reported that even after extensive washing considerable amounts of protein (i.e. enzyme) adsorb to PET which can also increase the rising height (Vertommen et al. 2005). Thus, all results represent the difference between samples treated with active enzyme and heat denaturated controls. In addition, esterases have been shown to hydrolyse PET oligomers originating from the fibre production process (Hooker J. et al. 2002). To ensure that hydrolysis of PET oligomers did not contribute to results from PET hydrolysis, fibres were washed with dichloromethane overnight and dried at room temperature. However, the rising height of enzyme treated samples did not decrease upon extraction of oligomers with dichloromethane.

The effect of the *B. brongniartii* polyesterase on PET was compared with the polyesterase from *T. lanuginosus*. This enzyme preparation had lipase activity on *p*-nitrophenyl decanoate and palmitate as well as esterase activity with *p*-nitrophenyl propionate. Other enzymes capable of hydrolyzing PET such as those from *Humicola* sp., *Candida* sp., *Pseudomonas* sp. have been reported to be typical lipases (Andersen et al. 1999; Kellis et al. 2001; Yoon et al. 2002; Heumann et al. 2006). Like the *B. brongniartii* polyesterase, no cutinase activity was detected for polyesterase from *T. lanuginosus* based on a qualitative comparison of products released from cutin by an *Optimyces sp.* cutinase. In the literature, however, several cutinases including that from *Pencillium citrinum* (Liebminger et al. 2007), and from *Fusarium solani* and *F. oxysporum* (Silva et al. 2005; Vertommen et al. 2005; Araujo et al. 2007; Nimchua et al. 2007) have been reported to hydrolyse PET.

It can be clearly seen that different amounts of the two enzyme preparations were necessary to reach a rising height of 5 cm (Figure 1). To reach this rising height, about 5 nKat $mL^{-1}$ of the *T. lanuginosus* polyesterase were necessary compared to only 1 nKat $mL^{-1}$ of the *B. brongniartii* preparation. In agreement with these data, it has previously been demonstrated that activity on *p*-nitrophenyl

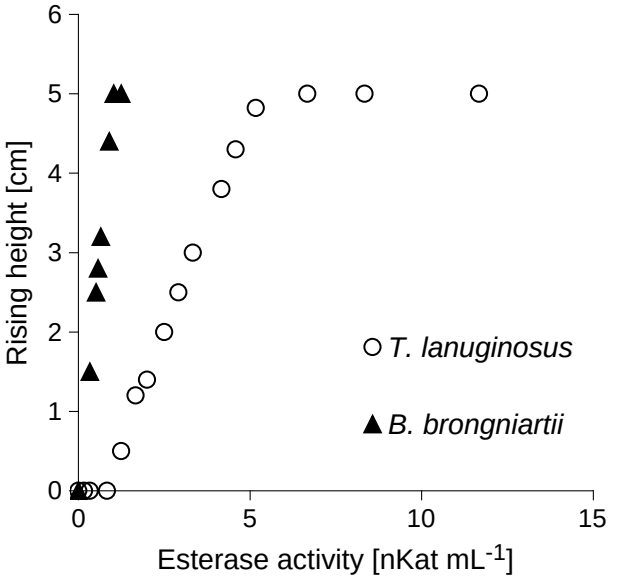


Figure 1. Hydrophilicity of PET samples (177g $m^{-2}$) treated with enzyme preparations from *B. brongniartii* and *T. lanuginosus*.

propionate or on *p*-nitrophenyl propionate does not correlate with hydrolysis activity on PET (Liebminger *et al.* 2007). Nevertheless, the spectrophotmetric esterase assay using *p*-nitrophenyl propionate as substrate is easier to use than HPLC quantification of the hydrolysis of PET oligomers if enzyme activity needs to be checked before dosing for PET treatment. Thus, for all further experiments on PET (e.g. PVC coating) the *B. brongniartii* and *T. lanuginosus* enzyme preparations were dosed according to this activity.

Release of terephthalic acid during treatment of PET was observed for the *B. brongniartii* polyesterase,

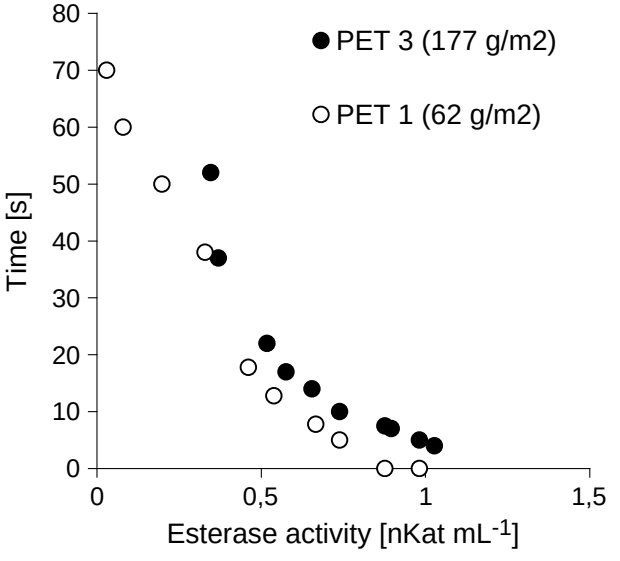


Figure 2. Hydrophilicity increase of different PET samples (drop dissipation time) depending on activity dosed from an enzyme preparation from *B. brongniartii*.

while the *T. lanuginosus* polyesterase did not release significant amounts of terephthalic acid (Figure 3). This indicates different mechanisms of hydrolysis of PET either more exo-wise (*B. brongniartii*) or more endo-wise (*T. lanuginosus*). Previously, it has been shown that a *Fusarim oxysporum* cutinase released more terephthalic acid from PET and increased the hydrophilicity to a greater extent than a *F. solani* cutinase (Nimchua et al. 2007).

There was a slight synergism when the two enzymes were used together for PET hydrolysis (Figure 3). Previously, several enzymes have been reported to increase the hydrophilicity of PET (Alisch et al. 2004, 2006; Fischer-Colbrie et al. 2004; Nimchua et al. 2007). However, apart from the release of terephthalic acid, no correlation with changes in the chemical composition of PET has been reported. Thus, in this study the hydrophilicity results were corroborated by the analysis of the amount of hydroxyl groups before and after enzyme treatment. With the *T. lanuginosus* enzyme preparation, an optimum hydroxyl group content was found at an enzyme activity of 4 nKat $mL^{-1}$ (Figure 4), which is slightly lower than that determined for reaching the maximum rising height (5 nKat $mL^{-1}$).

With the enzyme preparation from *B. brongniartii* (0.5 nKat $mL^{-1}$) an increase in hydroxyl groups from 20 (control with heat inactivated enzyme) to 182 mmol $kg^{-1}$ was measured. Considering the fact that the hydrolysis of the PET surface results in an increase in carboxylic acid groups to the same extent as hydroxyl groups, the increase in hydrophilicity can be understood as the introduction of polar groups onto the polymer surface facilitating water uptake, as suggested in the literature (Yoon et al. 2002, Gouda et al. 2002).

The decrease in hydroxyl groups, when higher activities of esterase are used (Figure 4), supports the assumption of PET hydrolysis into small soluble cleavage products that are released into solution. This hypothesis would also explain the plateau reached when rising height was used a parameter for hydrophilicity.

In PVC coating of PET for the production of truck tarpaulins, considerable amounts of toxic and expensive adhesives are needed. The amount of adhesives required are usually optimized to give a certain bonding strength which was 11.5 daN 5 $cm^{-1}$ in our case. Adhesives cross-link PET to the coating material, acting on free hydroxyl and carboxylic acid groups. Thus, limited enzymatic surface hydrolysis of PET could introduce novel hydroxyl and acid groups thus enhancing targets for the adhesives and consequently bonding strength.

Indeed, using only 0.5% adhesive agent and enzyme pretreatment a higher bonding strength

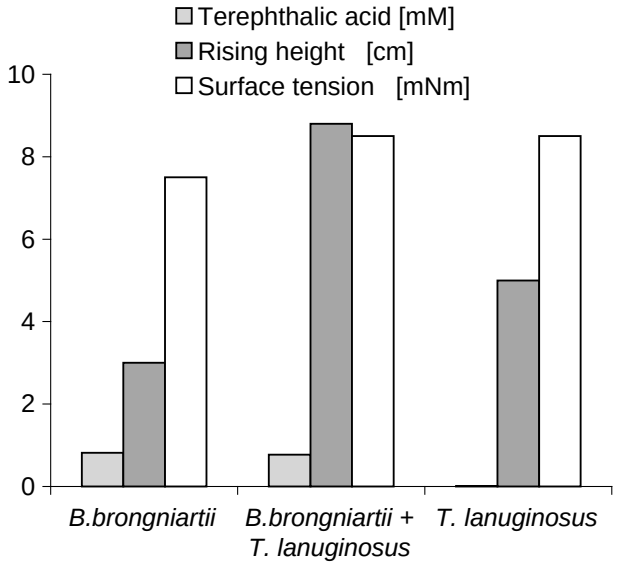


Figure 3. Hydrophilicity of PET samples and concurrent release of terephthalic acid after treatment with enzyme preparations from *B. brongniartii* (0.5 nKat $mL^{-1}$) and *T. lanuginosus* (5 nKat $mL^{-1}$) and a combination of both (0.5 nKat $mL^{-1}$ *B. brongniartii* and 5.0 nKat $mL^{-1}$ *T. lanuginosus*) The rising height and surface tension of control samples were 0 cm and 6.2 mNm, respectively.

was obtained compared to the use of 6% adhesive without enzyme pre-treatment (11.5 daN $5cm^{-1}$). From Figure 5, it can be seen that there is a treatment optimum near 30 min and 11 nKat $mL^{-1}$ of esterase activity when 0.5% adhesive are used. With increasing concentration of adhesive (1%), a bonding strength of 13.40 daN 5 $cm^{-1}$ was reached with an enzyme activity of 6 nKat $mL^{-1}$ after 30 min treatment. These results in terms of treatment time and enzyme activity compare reasonably well with the conditions necessary for the achievement of the hydrophilicity optimum (rising height) and the highest amount of hydroxyl groups.

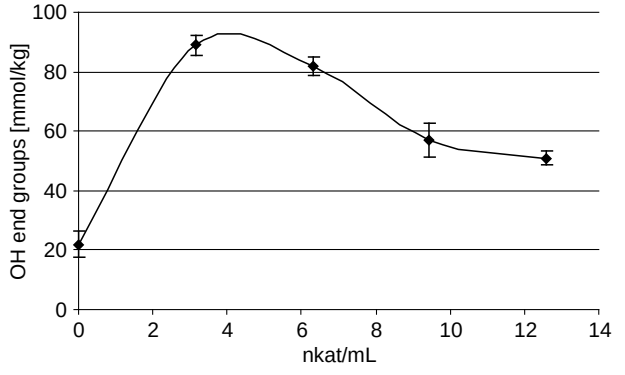


Figure 4. Hydroxyl groups in PET after treatment with different activities of the *T. lanuginosus* enzyme preparation. Error bars show the standard deviation of the measurement carried out in triplicate.

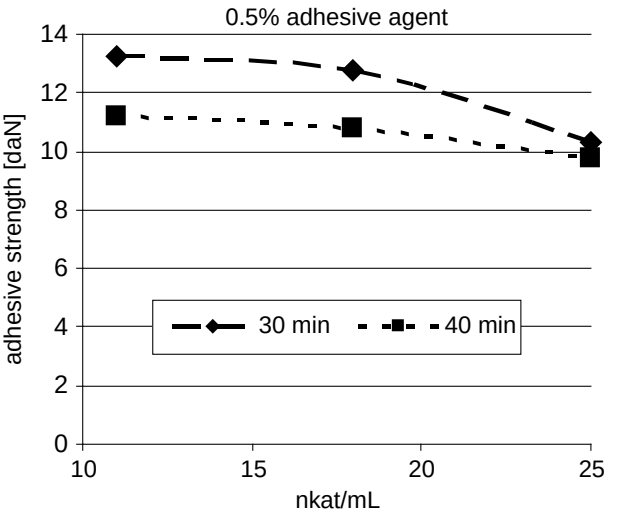


Figure 5. Adhesiveness values for PVC-coated PET material treated at pH 7 for 30 and 40 min with varying activities of the *T. lanuginosus* enzyme preparation using 0.5% of adhesive agent in the first coating layer

At higher enzyme concentration or after prolonged incubation the bonding strength due to enzyme pre-treatment decreased to about 10 daN $5cm^{-1}$ at an enzyme activity dose of 25 nKat $mL^{-1}$. As discussed above for the formation of hydroxyl groups, prolonged incubation or elevated enzyme activities will lead to loosening and eventually solubilization of smaller fragments on the PET surface obviously reducing bonding strength.

The optimum bonding strength of PVC-coated PET after pretreatment with the *B. brongniartii* enzyme preparation was measured at around 1 nKat $mL^{-1}$. The lower activity needed compared with the *T. lanuginosus* enzyme preparation correlates with the results obtained on increasing hydrophilicity, but again shows that activity on small substrates (i.e. *p*-nitrophenyl propionate) does not correspond to the effect on polymers. Finally, the enzyme preparations of *T. lanuginosus* and *B. brongniartii* were applied in combination dosing 5.5 and 0.5 nKat $mL^{-1}$, respectively. Although not dramatic, there was a synergistic effect of both enzymes giving a 24% higher increase in bonding strength. As with the hydrophilicity increase, this slight synergism might indicate that one enzyme is producing a substrate that is more accessible to the other enzyme, i.e. the fragments generated by the more endo-acting *T. lanuginosus* polyesterase could be more easily hydrolysed by the *B. brongniartii* polyesterase.

## Conclusions

Two polyesterase preparations from *T. lanuginosus* and *B. brongniartii* have been compared in terms of

their ability to increase the hydrophilicity of PET materials. Although treatment with both polyesterases led to the same maximum increase of hydrophilicity, this effect was achieved with 10-fold different enzyme activities based on *p*-nitrophenyl propionate hydrolysis. At the same level of hydrophilicity increase, only the *B. brongniartii* polyesterase released significant amounts of terephthalic acid from PET indicating that this enzyme might be more exo-acting than the *T. lanuginosus* polyesterase. Nevertheless, both enzymes were able to increase the amount of hydroxyl groups onto the surface of PET. Pretreatment of PET fabrics with both polyesterases can reduce the amount of adhesive needed in subsequent PVC coating by more than 90%.

Currently only a hand-full of polyesterases have been described in the literature. It will be interesting to compare the mechanisms of these enzymes on PET surface hydrolysis and consequences for PVC coating, once they become available in higher amounts and purified form. Genetic engineering will also play a major role in making polyesterases more efficient for industrial applications as initial results have indicated (Araujo et al. 2007).

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