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Biodegradation of aromatic-aliphatic copolyesters and polyesteramides by esterase activity-producing microorganisms

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ABSTRACT

Biodegradability of polyesteramides prepared by anionic ring-opening copolymerization of ε-caprolactone and ε-caprolactam and of aromatic-aliphatic co-polyesters (PETP/LA) synthesized by solvolysis of poly(ethyleneterephthalate) with water solutions of lactic acid by microorganisms producing extracellular esterase and lipase was investigated. The bacterium *Pseudomonas aeruginosa*, yeast *Candida guilliermondii* and micromycete *Aspergillus fumigatus* exhibiting strong esterase and lipase activities on agar plates with artificial nitrophenyl substrates were selected to be used in six-week degradation experiments at 28°C. PETP/LA samples exhibited mass reductions of up to 5-10% in both the presence of the three microorganisms and in abiotic controls where the polymers were exposed only to Nutrient Broth or malt extract-glucose medium. Scanning electron microscopy revealed breaks in PETP/LA fibres when polymers were exposed to *A. fumigatus* and *C. guilliermondii*, attributable to microbial action. Polyesteramides were resistant to biodegradation. Degradation of both polymer types resulted in a 5-10-fold increase of toxicity of culture supernatans measured with *Vibrio fischeri* bioluminescence test and *Sinapis alba* germination plant test, as compared to the biotic and abiotic controls. No genetic toxicity was detected with *Salmonella typhimurium* His⁻ test. The study suggests that ecotoxicity of compounds produced by biodegradation of polymers should be monitored.

Introduction

Increasing problems with durable plastic polymer waste accumulated in the environment resulted in extensive research focused on the synthesis of biodegradable polymers. As a result, biodegradation becomes a real option of coping with this ecological problem (Lucas et al., 2008; Bordes et al., 2009). Biodegradability of plastics requires a biological attack to the polymer and metabolization of the macromolecular substrate by the microorganisms (German standard DIN, 1998). Our study focused on two types of biodegradable polymers: aliphatic polyesteramides (PEA) represented by poly(ε-caprolactone-*co*-ε-caprolactam) (CLO/CLA) co-polymers and aliphatic-aromatic co-polyesters based on poly(ethyleneterephthalate).

PEA are biodegradable polymers exhibiting remarkable strength, with ester bonds present in the main chain that reduce crystallinity of the materials and make them biodegradable. Ester links are hydrolyzed by esterases/lipases and proteases. So far, however, most degradation studies have been carried out with isolated enzymes but not with the corresponding microorganisms (Fan et al., 2000; Cakir et al., 2011, etc.). An enhanced hydrophilicity by introduction of amide groups increases biodegradability by lipase and in soil (Park et al., 2003). Poly(ε-caprolactone) was shown to be degraded by lipases and esterases of molds (Tokiwa and Suzuki, 1977; Ghosh et al., 2013). Composting process including a high-temperature enzymatic attack effectively degraded CLO/CLA co-polymers and a higher CLO content in random co-polymers resulted in a faster degradation (Brožek et al., 2009; Michell et al., 2009).

Abbreviations: Aromatic-aliphatic copolyesters, PETP/LA; ε-caprolactam, CLA; ε-caprolactone, CLO; lactic acid, LA; malt extract-glucose medium, MEG; Nutrient Broth, NB; p-nitrophenyl acetate, PNAc; p-nitrophenyl butyrate, PNBut; p-nitrophenyl palmitate, PNPal; polyamide, PA; polyesteramides, PEA; poly(ethyleneterephthalate), PETP; polyurethane foam, PUF; Reactive Black 5, RB5; Remazol Brilliant Blue R, RBBR; scanning electron microscopy, SEM.

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Pure aromatic polyesters like poly(ethyleneterephthalate) (PET) widely used for food packing resist hydrolysis at mild conditions including the microbial or enzymatic attack. The effectivity of an enzymatic attack can be enhanced by introducing aliphatic components into the aromatic polyester chains (Witt et al., 1997; Müller et al., 2001). As shows the example of Ecoflex®, an aliphatic-aromatic co-polyester of 1,4-butanediol, adipic acid and terephthalic acid, the most efficient biodegradation method seems to be the composting (Kleeberg et al., 1998; Witt et al., 2001). It is a two step process where a depolymerization of co-polyesters by extracellular hydrolases such as the thermophilic, lipase-like serine hydrolase of *Thermobifida fusca* takes place followed by metabolization of the aliphatic and aromatic oligomers and monomers by the compost biota (Müller et al., 2005; Kleeberg et al., 2005). Bacterial genera *Pseudomonas* and *Bacillus* and their lipase- esterase- and protease activities are involved in the latter step (Cerda-Cuellar et al., 2004; Hu et al., 2008). The catalytic properties of *T. fusca* hydrolase are unique compared to bacterial and yeast lipases whose degradation efficiency is much lower. Marten et al. (2005) however demonstrated that aliphatic-aromatic polyesters can be degraded by the action of a bacterial lipase but under specific conditions that keep the polyesters chain mobile enough to reach the enzyme active site.

Screening for bacteria, yeasts and fungi capable to degrade aliphatic-aromatic polyesters under mesophilic temperatures showed that a number of soil microoganisms could degrade these polymers, among others the genera *Bacillus*, *Pseudomonas*, *Aspergillus* and lipolytic yeasts producing extracellular lipase/esterase activities (Tan et al., 2008). This group of enzymes that includes also cutinase-type polyesterases can be screened using p-nitrophenyl acyl esters. On the other hand, poor biodegradation of co-polyester films has been obtained with *Phanerochaete chrysosporium* and *Trametes versicolor*, which suggested that a machinery of oxidative enzymes capable to degrade lignin polymer was not efficient in degradation of aliphatic-aromatic copolyesters (Tan et al., 2008).

Toxicity of end products should always be checked in any biodegradation process but such studies are scarce in case of biodegradation of polyesters. Products of polyester hydrolysis, adipic acid, mandelic acid, terephthalic acid, 1,4-cyclohexane dimethanol etc. exhibited phytotoxicity and cytotoxicity in radish-seed germination test and HeLa cells test, respectively, the aromatic compounds being more harmful (Kim et al., 2001). In contrast, 99.9% degradation of Ecoflex® with *T. fusca* providing monoesters of adipic acid and terephthalic acid with 1,4-butanediol and monomers of the co-polyesters (1,4-butanediol, terephthalate and adipate) as the end products did not produce a significant ecotoxicological effect when measured with the acute toxicity *Photobacterium phosphoreum* and *Daphnia magna* tests.

Our aim was to investigate biodegradability of PEA and of an aliphatic-aromatic co-polyester by bacterial and fungal microorganisms producing high levels of extracellular esterase or lipase and assess the ecotoxicity of degradation products. Poly(ε-caprolactone-*co*-ε-caprolactam) copolymers with various ratio of ε-caprolactone and ε-caprolactam monomers and an aliphatic-aromatic co-polyester containing PETP and lactic acid were synthesized and their degradability by selected microorganisms tested in liquid medium cultures at 28 °C. A lipolytic bacterium, a yeast and a micromycete were screened on C2–C16 p-nitrophenyl alkanoates for high esterase/lipase activities and used in the biodegradation experiments. Biological toxicity of end products was evaluated by standard acute toxicity tests with *Vibrio fischeri* and *Sinapis alba* and the genetic toxicity by *Salmonella typhimurium* His$^-$ test.

Material and methods

Materials

Polyesteramides (PEA) were prepared by anionic ring-opening co-polymerization of ε-caprolactone (CLO) and ε-caprolactam (CLA) at various ratios of structural units by a procedure described by Chromcová et al. (2008). PEA fibre layers were prepared by electrospinning from solution of PEA in mixed solvent formic/acetic acid 1/2 (V/V) at Technical University of Liberec (applied voltage-30 kV, distance between the electrode and collector-10 cm, laboratory temperature) (Malinová et al., 2013).

Aromatic-aliphatic co-polyesters (PETP/LA) were synthesized by solvolysis of poly(ethyleneterephthalate) (PETP) with water solutions of lactic acid (LA) and a subsequent polycondensation of the reactive products by a procedure described in (Prokopová et al., 2008). Fibre layers were prepared by melt-blown technology at Technical University of Liberec (die temperature 215 °C; air temperature 255 °C; pressure 0.25 MPa).

Malt extract (Oxoid, UK), p-nitrophenyl acetate (PNAc), p-nitrophenyl butyrate (PNBut), p-nitrophenyl palmitate (PNPal), Remazol Brilliant Blue R (RBBR), Reactive Black 5 (RB5) (all Sigma–Aldrich, Czech Republic). All the other chemicals were of analytical grade.

The dyeing liquor was obtained from INOTEX a.s., Czech Republic and contained a mix of textile disperse dyes Itosperse Yellow RAP dye mix (5.47 g l^{-1}), Itosperse Red RAP dye mix (3.75 g l^{-1}), Itosperse Blue RAP dye mix (2.47 g l^{-1}), the disperging agent Nicca Sunsolt™ RF-557 (1 g l^{-1}) and acetic acid (0.3 ml.l^{-1}).

Microorganisms

Bacteria: *Pseudomonas aeruginosa* DSM 1253, *Pseudomonas* sp. DSM 9959, *Pseudomonas fluorescens* DSM 9958, *Pseudomonas putida* DSM 12735 (all from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany); yeasts: *Rhodotorula glutinis* CCY 20-2-41, *Candida guilliermondii* CCY 29-4-31, *Candida tropicalis* CCY 29-7-60, *Candida rugosa* CCY 29-15-2, *Yarrowia lipolytica* CCY 29-26-46 (all from Culture Collection of Yeasts, Slovak Republic); micromycetes: *Aureobasidium pullulans* CCF 3129, *Botrytis cinerea* CCF 917, *Aspergillus niger* CCF 3433, *Aspergillus terreus* CCF 3389, *Aspergillus oryzae* CCF 1602, *Aspergillus fumigatus* CCF 3430, *Emericella nidulans* CCF3379, *Fusarium oxysporum* CCF3428, *Fusarium solani* CCF 3465, *Fusarium verticillioides* CCF 3501, *Humicola grisea* CCF 3257, *Mucor hiemalis* CCF 2698, *Neosartoria fischeri* CCF 3544, *Neurospora sitophila* CCF 3485, *Penicillium roqueforti* CCF 1741, *Penicillium funiculosum* CCF 1994, *Penicillium viridicatum* CCF 3214, *Rhizopus arrhizus* CCF 1669, *Xylaria* sp. CCF E58, (all from Culture Collection of Fungi, Czech Republic); ligninolytic fungi: *Irpex lacteus* 238, *Pleurotus ostreatus* 3004, *Dichomitus squalens* 750 (all from Culture Collection of Basidiomycetes, Czech Republic). The strains were maintained at 4 °C on agar slopes prepared with complex media mentioned below.

Screening and biodegradation

The yeasts and micromycetes were pre-screened for extracellular esterase and lipase activities using PNAc, PNBut or PNPal at 0.25 mmol l^{-1} on plates with malt extract (5 g l^{-1}) and glucose (1 g l^{-1}) (MEG) medium pH 5 solidified with agar (20 g l^{-1}) (Gilham and Lehner, 2005). The amount of extracellular enzyme produced was evaluated by reading the intensity of halo formed around the colonies. Similar screening was carried out with the bacteria on Nutrient Broth (NB, Difco, USA) pH 7 plates. The most active strains were used in biodegradation experiments.