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Mineralisation of ^{14}C-labelled polystyrene plastics by *Penicillium variabile* after ozonation pre-treatment

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ABSTRACT

Large amounts of polystyrene (PS), one of the most widely used plastics in the world, end up in the environment through industrial discharge and littering, becoming one of the major components of plastic debris. Such plastics, especially the small-sized microplastics and nanoplastics, have received increasing concerns in terms of their potential environmental risks. Feasible approaches for the degradation of PS in waste materials and in the environment are highly desirable. Physicochemical pretreatments of PS may be applied to enhance biological degradation. In the present study, we synthesized ^{14}C-labelled PS polymers, either uniformly labelled on the ring ([U-ring-^{14}C]-PS) or labelled at the β-carbon position of the alkyl chain ([β-^{14}C]-PS), and investigated the mineralisation of the ^{14}C-PS polymers by the fungus *Penicillium variabile* CCF3219 as well as the effect of ozonation as a physico-chemical pre-treatment on the mineralisation by the fungi. Biodegradation of the ^{14}C-PS polymers was studied in liquid medium (pH 7.5, without additional carbon substrate) with *P. variabile* for 16 weeks. During the incubation time, $^{14}CO_2$ was captured to calculate the mineralisation of ^{14}C-PS and the remaining polymers were analysed by means of scanning electron microscopy (SEM), Fourier transform infrared (FT-IR) spectrometry and gel-permeation chromatography (GPC). The results showed that the fungi mineralised both labelled polymers, and that the [U-ring-^{14}C]-PS with a lower molecular weight led to a higher mineralisation rate. Ozonation pre-treatment strongly enhanced mineralisation of [β-^{14}C]-PS. SEM analysis showed that the surface of the ozonated [β-^{14}C]-PS became uneven and rough after the incubation, indicating an attack on the polymer by *P. variabile*. FT-IR analysis showed that ozonation generated carbonyl groups on the [β-^{14}C]-PS and the amount of the carbonyl groups decreased after incubation of the [β-^{14}C]-PS with *P. variabile*. GPC analysis showed that the molecular weights of the ozonated [β-^{14}C]-PS decreased after incubation. The present data suggest that ozonation pretreatment could be a potential approach for degradation of PS waste and remediation of PS-contaminated sites.

1. Introduction

Global plastic production amounted to 311 million tons in 2014 [1] and about 4.8–12.7 million tons of plastic waste entered the marine environment [2]. In the environment, plastic debris may be broken down and become small sized entities (e.g., microplastics and nanoplastics) by mechanical fragmentation, photochemical and biological degradation [3–5]. Recently, the plastic particles, especially the small sized microplastics (<5 mm) has become an international issue of increasing concern for global environmental pollution, both for marine and terrestrial ecosystems [6–9]. Polystyrene (PS) comprises 7.1% of the entire plastic products worldwide [1] and is one of the major plastic debris in the marine environment [10]. PS particles may be taken up by copepods as food [11,12], transferring via he food web [13], and causing negative effects on animal reproduction and hepatotoxicity [14], [15], especially when the particles are small (micrometer range). It is therefore important to find effective degradation approaches to mitigate environmental risks related to PS. In general, plastics can be degraded via thermal degradation, catalytic degradation and biodegradation [16]. Of the three, biodegradation is thought to be the most promising way to remediation of plastic pollution [3].

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Owing to the recalcitrance of PS to microbial degradation, its biodegradation in the environment is considered very slow and traditional analytical methods, such as gravimetry, may fail to detect slight reduction. A few studies have applied a ^{14}C-tracer to investigate PS biodegradation. Guillet et al. [17], showed that PS was mineralised to 0.01% after two months incubation in garden soil. Kaplan et al. [18] investigated the biodegradation of ^{14}C-PS with 17 species of fungi in axenic cultures, 5 groups of soil invertebrates, and a variety of mixed microbial communities from sludge, soils, manures, garbage and decaying plastics. In the different microbial systems, total decomposition of PS over 5 or 11 weeks ranged from 0.04 to 0.57% and the addition of cellulose and minerals did not significantly increase decomposition rates. In a landfill soil, as much as 3% of PS was mineralised after four months of incubation. Using ^{13}C-labelled PS, Yang et al. [19] demonstrated that gut microorganisms of mealworms could mineralize Styrofoam (PS foam).

Biodegradation of plastics is governed by polymer characteristics, the type of organism, and the nature of pretreatment [20]. Fungi are probably the predominant microorganisms responsible for environmental biodegradation of plastics [21]. While the brown-rot fungus *Gloeophyllum trabeum* substantially degraded the water-soluble PS sulfonate, it appeared to fail to attack solid PS [22]. Castiglia et al. [23] tried to apply the fungal strain *Aureobasidium pullullans* var. *melanogenum*, capable of producing a large number of hydrolytic enzymes, to degrade expanded-polystyrene (EPS), but EPS beads were resistant.

Penicillium is a genus of ascomycete fungi of major importance in the natural environment [24]. Yamada-Onodera et al. [25] isolated a strain of *Penicillium simplicissimum* and reported that this fungus could degrade polyethylene in liquid medium. Within the research consortium of the EU FP7 project BIOCLEAN, it was also found that the *P. variabile* CCF3219 strain is a promising PS degrader (Dr. Cenek Novotny, personal communication).

Plastic with hydrophobicity and large molecular dimensions is biodegraded if it undegoes abiotic degradation before biotic attack [25]. Nam et al. [26] found that ozonation followed by bacterial degradation significantly increased the degradation of polycyclic aromatic hydrocarbons (PAHs) in soil, suggesting that pretreatment with ozone could be an approach to enhance biodegradation of compounds with similar structures, such as PS. In the present study, we implemented a ^{14}C tracer approach in order to investigate the degradation of PS by the fungus *P. variabile* CCF3219 and evaluate the potential of ozonation pretreatment to enhance biodegradation efficiency.

2. Materials and methods

2.1. Chemicals

Two ^{14}C-labelled PS polymers, labelled either uniformly on the ring ([U-ring-^{14}C]-PS, 480 Bq/mg) or on the β-carbon of the chain ([β-^{14}C]-PS, 3200 Bq/mg), were synthesised by polymerisation of styrene monomer ^{14}C-labelled on the ring or the β-carbon, respectively. For [U-ring-^{14}C]-PS, two polymers with molecular weight (MW) of 15 and 29 kDa, respectively were prepared. Details of the ^{14}C-PS polymers are shown in Table 1. Other chemicals were of analytical grade or high-performance liquid chromatography (HPLC) grade and were purchased from Sigma–Aldrich (Buchs, Switzerland) or J. T. Baker (Munich, Germany).

2.2. Preparation of ^{14}C-PS films

Chloroform solutions containing [U-ring-^{14}C]-PS (1.5×10^4 Bq and 2.5×10^3 Bq for 29 and 15 kDa PS polymer, respectively, Table 1) and [β-^{14}C]-PS (1.6×10^4 Bq) were added to 40 mL vessels and after the evaporation of the chloroform solvent, PS films (ca. 5 cm^2) were formed at the bottom of the vessels.

2.3. Ozonation of ^{14}C-PS films

Ozonation of the [β-^{14}C]-PS films was conducted using an ozone generator (Ozonia LAB2B; Degrémont Technologies–Triogen, Switzerland). The films were put into a glass column (31×3 cm) and O_3 gas introduced into the column at a rate of 5 L/min for 3 h, which was equivalent to approximately 17 g of ozone in total. Some of the ozonated films were resuspended three times in 1 mL methanol to separate the ozonation products from the PS films. The radioactivity of methanolic extracts was determined by liquid scintillation counting (LSC, see below).

2.4. Incubation of ^{14}C-PS films with Penicillium variabile

P. variabile was cultivated in 1000 mL of sterilised (121 °C, 20 min) mineral Czapek-Dox medium (3 g of $NaNO_3$, 1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 0.01 g of $FeSO_4 \cdot 7H_2O$, pH value adjusted to 7.5) amended with 30 g sucrose, on a rotary shaker (KS 4000i control, IKA Technology, Germany) at 24 °C and 120 rpm for 7d. The biomass of *P. variabile* was washed with sugar-free Czapek-Dox medium by repeated (three times) suspension and centrifugation. The washed *P. variabile* cells were added to culture vessels containing ^{14}C-PS films. The final volume of medium in each vessel was 6 mL and the dry biomass of *P. variabile* was approximately 4 mg. The vessels were sealed with silicon stoppers from which vials were hung containing 0.8 mL of 1 M NaOH to trap $^{14}CO_2$ released during the incubation. The vessels were incubated at 24 °C on a rotary shaker (120 rpm). During the incubation, the vessels were opened for approximately 1 min each day to allow the headspace to be replenished with fresh air. Blank treatments without ^{14}C-PS and abiotic control treatment without *P. variabile* were also prepared. All treatments were performed in triplicate.

2.5. Extraction and separation of ^{14}C-PS

At the end of incubation, the ^{14}C-PS films were removed from the medium. Small pieces of the films (1×1 mm) were cut and soaked in 2% sodium dodecyl sulfate (SDS) for 4 h, washed with 75% ethanol to remove the biofilm adherent to the films and analysed by scanning electronic microscopy (SEM) (S–3400 N II, Hitachi, Japan) to monitor alteration of the plastic surface. Before SEM analysis, PS films were coated with gold powder. Some other small pieces of PS films were analysed by Fourier transform infrared spectrometry (FT-IR) (Nexus 870, Nicolet, USA) to determine variations in functional groups on the film (Resolution: 1 cm^{-1}; range: 400–3600 cm^{-1}).

The remaining films were dissolved in chloroform to analyse the MW distribution of the ^{14}C-PS by gel-permeation chromatography (GPC) using a PL-GPC 50 (Agilent technologies, USA) chromatograph equipped with a refractive index detector. Two coupled polystyrene gel columns, PL gel-MIXED B (10 μm, 300×7.5 mm) and PL gel-MIXED C (5 μm, 300×7.5 mm) were used to separate the molecules using tetrahydrofuran (THF) as eluent at a flow rate of 1.0 mL/min at 40 °C. The columns were calibrated using MW standards of polystyrene ranging from 588 Da to 858 kDa (Agilent technologies, USA). The eluent was collected every minute into 5 mL vials for radioactivity determination using LSC (see below).

2.6. Determination of radioactivity

The CO_2 traps were replaced at weeks 1, 2, 4, 8, 12, and 16. Both NaOH solution and methanol extracts, respectively were mixed

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