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Streamlining biological recycling of poly(ethylene terephthalate) via pre-treatment methods

Katarzyna E. Kosiorowska [a], Antonio D. Moreno [b], Raquel Iglesias [b], Piotr Biniarz [a,1], Aleksandra M. Mirończuk [a,*]

[a] Wrocław University of Environmental and Life Sciences, Laboratory for Biosustainability, Institute of Environmental Biology, Kożuchowska 5b, Wrocław, 51-631, Poland

[b] Advanced Biofuels and Bioproducts Unit, Department of Energy, Research Centre for Energy, Environment and Technology (CIEMAT), Avda. Complutense 40, 28040, Madrid, Spain

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ABSTRACT

In this study, we validated various methods of pre-treatment of poly(ethylene terephthalate) (PET) by the engineered yeast *Yarrowia lipolytica*. This research compares both the effect of the type of plastic used, the processing method and enzymes with different mechanisms of action (PETase and cutinase). The investigation demonstrated that the degradation efficiency varies depending on the type of plastic used, the processing methods and the applied enzyme. Moreover, it indicated that during prolonged yeast culture under the applied conditions, enzyme activity is not impaired. Among all the methods tested, the artificial aging process had the greatest impact on the degradation level by PETase, where the amount of TPA released from commercial PET film was the highest, and yielded over 2 gL^{-1}. The maximum yield of TPA (0.59 gL^{-1}), for the *Y. lipolytica* strain overexpressing cutinase, was observed during the process with recycled PET bottles shredded into 1 mm fragments. The maximum recorded weight loss of plastic film is over 70% for commercial PET film subjected to artificial ageing process.

1. Introduction

Global production of plastics, both virgin and recycled, is increasing progressively every year. According to a recent European report on the worldwide amount of virgin plastic produced, 400.3 million tonnes (Mt) of plastics were produced in 2022. 58.2 million tonnes were generated in Europe, which constitutes 14% of the global total (Plastics Europe Market Research Group (PEMRG), 2023). Unfortunately, due to the uncontrolled littering of plastics into rivers, seas and oceans, nowadays we can observe phenomena such as the Great Pacific Garbage Patch (Lebreton et al., 2018). The widespread use of plastics in every area of life and industry has resulted in massive pollution of the planet. The products of plastic fragmentation resulted as nano- and microplastics (particles less than 1 mm and 5 mm in size, respectively), have been detected in every environment explored by scientists, including the Mariana Trench, Antarctica and the Arctic Ocean (Urbanek et al., 2021).

In 2022, production of polyethylene terephthalate (PET) exceeded 24.8 Mt (Plastics Europe Market Research Group (PEMRG), 2023). This polymer is used on a wide scale to produce a broad range of daily commodities (packaging, bottles, clothing). In Europe, in 2021, over 80 Mt of packaging waste was generated, plastic packaging accounted for 16.1 Mt which is more than 19% of waste produced (Eurostat, 2023). In worldwide scale in 2019, PET plastic waste generated represented 24.81 Mt (OECD, 2022). Unfortunately, only 17% of the raw material obtained returns to the market, e.g. in the form of new PET bottles; however, the rest is used in products which may contain this low-quality plastic (Grant et al., 2022). Growing presence of the recycled PET material in the environment year by year due to the circularity of this polymer indicates the importance of exploring new, efficient methods of degrading this compound, including post-recycled forms.

Current plastic recycling methods are divided into mechanical, thermal, chemical and biocatalytic methods (Maurya et al., 2020). During physical process-based recycling, methods are used that fragment plastic by shredding or cutting; it is then used in an extrusion

* Corresponding author.
E-mail addresses: katarzyna.kosiorowska@upwr.edu.pl (K.E. Kosiorowska), david.moreno@ciemat.es (A.D. Moreno), raquel.iglesias@ciemat.es (R. Iglesias), p.biniarz@gmail.com (P. Biniarz), aleksandra.mironczuk@upwr.edu.pl (A.M. Mirończuk).
[1] Current address: Winnica Alvarium, Ostaszów 23a, 59–170 Przemków.

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process to manufacture a new product with deteriorated properties relative to the original plastic (Hopewell et al., 2009; Grigore, 2017). Thermal (pyrolysis) and chemical recycling in which monomers are extracted for subsequent re-polymerization belong to tertiary recycling (Singh et al., 2017). Regarding solvolysis, these processes have not been fully developed to date due to high costs, the need for qualified staff as well as the large amounts of toxic waste being generated during the procedure (Grigore, 2017; Kaabel et al., 2021). Biocatalytic recycling is based on the use of hydrolytic enzymes that have the ability to break down bonds present in polymers (Zimmermann, 2020). Previous studies indicate that the ability to hydrolyse ester bonds present in PET is exhibited by enzymes from the esterase subclass such as cutinase from *Fusarium solani*, *F. oxysporum*, and *Thermobifida fusca* (Nimchua et al., 2007; Groβ et al., 2017; Furukawa et al., 2019; Kosiorowska et al., 2022a), PETase and MHETase from *Ideonella sakaiensis* (Tanasupawat et al., 2016; Yoshida et al., 2016; Kosiorowska et al., 2022b) and lipase from *Candida cylindracea* (Ma et al., 2012). Studies conducted to date also indicate the possibility of using new protein engineering methods to increase the efficiency of hydrolytic enzymes, employing protein chimeras or taking advantage of the synergism of action of two different enzymes (Urbanek et al., 2021).

Currently, most studies exploring degradation of plastics such as poly (ethylene terephthalate) use as a substrate an amorphous, low-crystalline PET powder or PET films and well-characterized purified hydrolase(s), possessing a high ability to degrade PET. Those enzymes have enhanced thermostability, which allows the degradation process to be conducted at high temperatures, enabling better chain mobility of the plastic molecules, resulting in higher degradation rates (Austin et al., 2018; Liu et al., 2019; Shirke et al., 2018). Unfortunately, there is still a lack of research focused on the viable material that remains to be degraded.

In this study, biodegradation of plastic from recycled PET film or PET bottles in different forms (films/melts) was carried out. The effects of various processing methods including shredding, alkaline treatment, surfactant treatment and one-month artificial aging of several forms of PET plastic were tested. The biodegradation was conducted directly in the culture media of the engineered *Yarrowia lipolytica* yeast, which extracellularly produces cutinase from *Fusarium solani* (*Y. lipolytica* AJD pAD-CUT_Fs) or PETase from *Ideonella sakaiensis* (*Y. lipolytica* AJD pAD-PET_Is), which have the ability to degrade PET powder (Kosiorowska et al., 2022a, 2022b). We investigated the effect of polymer pre-treatment methods on its degradation efficiency using engineered *Y. lipolytica* strains during flask shake cultures conducted at 28 °C. Here, the plastic material used was recycled PET in the form of PET film, ground PET bottles and a mixture of PET film with pellets (melts). The control material used for the comparison of degradation performance with post-recycled types of PET was low-crystallinity commercially available PET film.

2. Materials and methods

2.1. Microorganisms, media, and culture conditions

Microorganisms used in this study were *Yarrowia lipolytica* strains AJD 2 pAD CUT_FS and AJD 2 pAD PET_IS (Kosiorowska et al., 2021, 2022b). The AJD 2 strain was used as a control. The inoculation medium used in the experiments was YPD (1% yeast extract, 2% peptone, 2% glucose). Cultivation of *Y. lipolytica* strains for derivation of the supernatant were prepared in 30 mL of YPD medium with 50 gL^{-1} glucose content in 0.3 L Erlenmeyer flasks at 28 °C and 200 RPM. Tests of terephthalic acid (TPA) (Sigma-Aldrich), 2-hydroxyethyl-terephthalic acid (MHET) (AChemBlock) and ethylene glycol (EG) (Sigma Aldrich) uptake by *Y. lipolytic*a were performed by adding an appropriate amount of the above-mentioned compounds into YPD media containing 50 gL^{-1} of glucose. Previously prepared stocks of TPA dissolved in 99.9% DMSO (Sigma Aldrich) and MHET dissolved in 99.9% methanol (P. P. H. STENLAB) were sterilised using 0.22 μm syringe filters (Merck Millipore) and added to the medium before inoculation to achieve the final concentration of 100 mgL^{-1}. EG was added directly to the media and sterilised by autoclaving and the concentration of this compound was 10 gL^{-1}. The long-term cultures were performed in 0.3 L Erlenmeyer flasks using 50 mL of YPD medium containing 50 gL^{-1} of glucose at 28 °C and 200 RPM. Each time yeast cultures were standardised to starting OD_{600} 0.5. All cultures carried out in this study were performed in three biological replicates.

2.2. Plastic pre-treatment methods

Plastic material used in this study was: commercial, amorphous, 0.25 mm thickness PET film (GoodFellow ES301445/7, England), industrially recycled PET film, recycled bottles and a mixture of the recycled film and melts (PROINVEST, Poland). The films are shown in Fig. 1. PET films were pre-treated using Triton X-100 (Sigma-Aldrich) by 15 min of incubation at 28 °C with continuous orbital shaking at 500 RPM. Subsequently, the films were rinsed 3 times in Milli-Q water and dried at room temperature. PET films were also incubated in 50% NaOH (Sigma-Aldrich) for 15 min and then washed three times with Milli-Q water and dried at 70 °C for 2 h in the oven. Artificial ageing pre-treatment was performed using a solution of 35‰ sea salts (NutriSelect Basic, Sigma Aldrich). PET films were incubated for 1 month in total immersion with cyclic irradiation for 8 h per day using a 125 W high-pressure mercury lamp (BELLIGHT, Poland). Artificial ageing was carried out at room temperature. Following incubation, the PET material was flushed three times with Milli-Q water and dried at room temperature. The PET grinding process was carried out using a ZM 200 (Bio-Meta, Spain) grinder with 1 mm, 2 mm and 6 mm diameter screens. The PET film milling process was carried out with consideration of temperature rise during friction to avoid melting of the plastic. PET material after pre-treatment is shown in Supplementary Fig. 7. The microscope images were taken with a Delta Optical MET-200-TRF metallographic microscope, using the EPI (transmitted light) observation technique. Lens used Plan 4×, total magnification 40×. DLT-Cam PRO 6.3 MP USB 3.0 microscope camera, DLTCamViewer software.

2.3. Enzyme activity and kinetic characteristics

Esterase activity assay was used to determine enzyme activity in the supernatants of AJD 2, AJD 2 pAD CUT_FS and AJD 2 pAD PET_IS strains and was performed using 4-nitrophenyl acetate (Sigma Aldrich). The experiments were conducted in 50 mM phosphate buffer at pH 8.5 at 28 °C and 37 °C. One unit of enzyme leads to hydrolysis of 1 μM 4-nitrophenyl acetate (4-NP) resulting in 4-nitrophenol release in 1 min at pH 8.5 and the appropriate set temperature. Enzyme kinetic characteristics were determined by enzymatic analysis carried out at 37 °C in 96-h supernatant using a various substrate (4-NP) concentration range (10–100 mM) in the conditions described above. To determine the enzyme's actual activity during culture studies, in the enzyme activity assay, the temperature of 28 °C, corresponding to the condition used in this study, was also verified. The enzymes present in the post-culture supernatants were characterized in terms of the affinity of the enzyme for the substrate used (K_m) and the maximum rate of reaction (v_{max}). Protein concentration was determined with Lowry's method, and a standard protein curve was prepared using the bovine serum albumin (BSA) (Sigma Aldrich) standard (0.025–2 mgL^{-1}). Absorbance was determined using Synergy H1 Microtiter Plate Reader (BioTek, USA) at $\lambda = 405$ nm (esterase activity assay) and $\lambda = 750$ nm (protein concentration measurement).

2.4. Plastic degradation conditions

The conditions used in the study to determine the effect of PET processing methods on degradation performance were applied in a two-

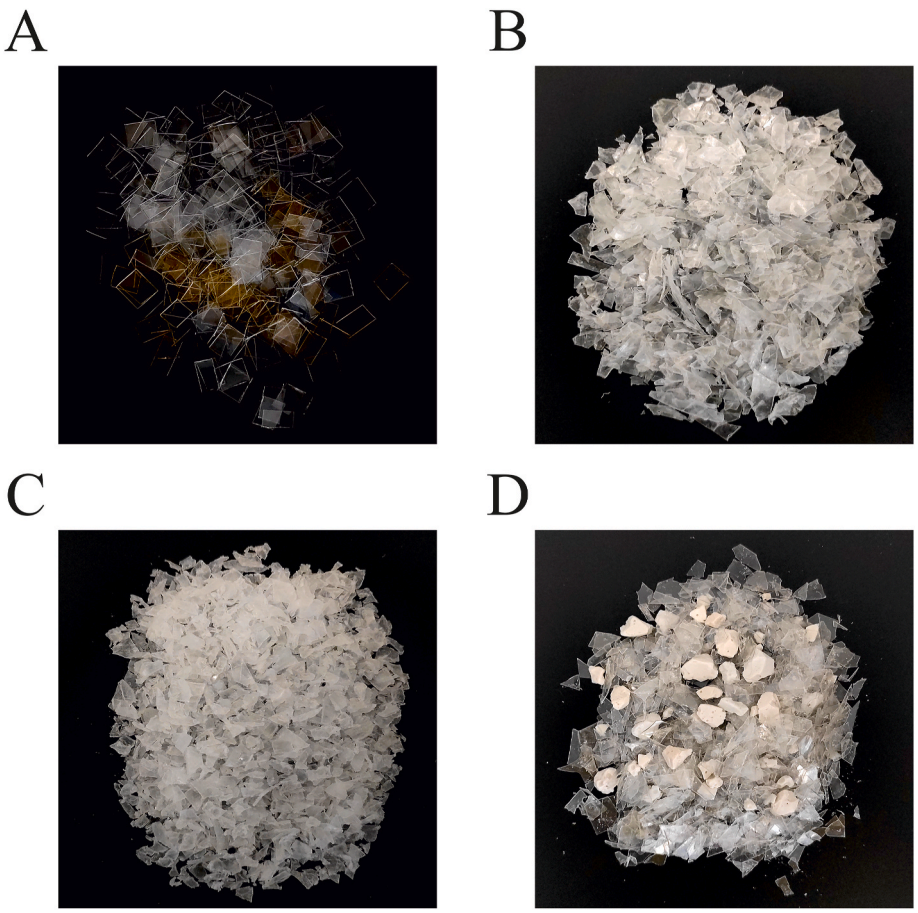


Fig. 1. Plastic material used in this study. A- Commercial, amorphous PET film, B- Recycled PET films, C- Recycled PET bottles, D- Recycled PET films with melts.

fold approach. Due to the time-consuming process of PET hydrolysis, screening of pre-treatment methods was performed using post-culture supernatant containing enzymes capable of degrading PET. Enzymatic hydrolysis reactions were carried out using 2 mL of supernatant derived from 96-h culture of AJD 2 pAD CUT_FS, AJD 2 pAD PET_IS. Supernatant derived from the AJD 2 control strain was used to exclude spontaneous release of PET degradation products such as TPA, MHET, BHET and EG as a consequence of the pre-treatment method applied. All post-culture fluids were pre-sterilised using 0.22 μm syringe filters (Merck Millipore). Incubation was carried out at 37 °C with constant agitation at 200 RPM in 2 mL Eppendorf tubes and 0.1 g content of PET material. Long-term cultures of genetically modified *Y. lipolytica* yeast strains and the AJD 2 control strain were carried out in a 0.3 L Erlenmeyer flask at 28 °C with constant agitation at 200 RPM and 1 g addition of PET plastic film. Plastic material was not previously sterilised. The screening process was carried out for 14 days. The direct PET degradation process in the shake flask culture was carried out for 28 days, and samples were taken weekly.

2.5. Analytical methods and statistical analysis

PET plastic crystallinity was analysed with the use Thermal Analysis System TGA/DSC 3+ (METTLER TOLEDO, USA). Initial temperature of analysis was 25 °C, final temperature was 280 °C, heating rate was 10 °C/min. Results were normalised to sample weight and are shown in Supplementary Fig. 8. Crystallinity was calculated according to Perklin Elmer manual (Sichina, 2000) by equation $\%Crist = ((|\Delta H_m| - |\Delta H_c|)/|\Delta H_m^{\circ}) \cdot 100\%$, where ΔH_m is heat of PET melting, ΔH_c heat of PET cold crystallization, ΔH_m° heat of melting 100% crystalline PET (140.1 Jg^{-1}). The amounts of released degradation products in this study were determined using ultra-performance liquid chromatography (UPLC) Dionex Ultimate 3000 (Thermo Fisher Scientific, UK). TPA, MHET and BHET concentrations were analysed using a Hypersil GOLD C18 column (Thermo Fisher Scientific, UK) with Uniguard Guard Cartridge Holder (Thermo Fisher Scientific, UK). Gradient chromatographic separation was carried out with two mobile phases: acetonitrile with 0.1% trifluoroacetic acid (TFA) (v/v) and Milli-Q water with 0.1% TFA (v/v). Column temperature was 45 °C and the flow rate was 0.8 $mLmin^{-1}$. Compounds were identified with a UV detector (Dionex, Thermo Fisher Scientific, UK) at 243 ± 2 nm. Additionally, absorption spectrum analysis in the range of 150–500 nm was performed. Glucose and ethylene glycol (EG) were analysed using a HyperREZ XP Carbohydrate H+ column (Thermo Scientific, Waltham, MA), and RI (refractive index) detector (Shodex, Ogimachi, Japan) by isocratic chromatographic separation with 0.25 mM TFA used as an eluent. Column temperature was 65 °C and the flow rate 0.6 $mLmin^{-1}$. A detailed description of the programme used in this study was published previously before (Kosiorowska et al., 2022a). Data analysis was conducted using Chromeleon 7.1 software.

PET material after each culture was collected, washed three times with Mili-Q water and dried at room temperature. The samples were then weighed to estimate the weight loss of the PET.

Statistical analysis conducted in this study was performed using Student's t-test. Significance of the influence of pre-treatment methods in efficiency of PET degradation was determined by comparison of the means from cultures with addition of non-pre-treated PET plastic material (labelled as the control) with the cultures containing pre-treated PET (two-tailed, unequal variance). Standard deviation is shown in error bars and was calculated based on results from three biological repetitions.

3. Results

This study emphasizes the development of sustainable and environmentally friendly biodegradation strategies for effective pollution management and remediation. Detailed description about enzymes used in this study providing a comprehensive knowledge of the degradation processes and mechanism of action has been described before (Carvalho et al., 1998; Yoshida et al., 2016; Kosiorowska et al., 2021, 2022b). The research has focused on the identification and quantification of degradation intermediates and by-products, based on the quantities of which PET degradation rate was determined.

3.1. Verification of plastic pre-treatment methods

To establish PET degradation efficiency, PET films were incubated for two weeks in the post-culture supernatant (96 h) of AJD 2 pAD CUT_FS and AJD 2 pAD PET_IS strains. The AJD2 strain was used as a control. PET degradation yield can be relatively quickly estimated using purified proteins under the optimal temperature and pH of the applied enzyme. The optimal temperature condition (37 °C) for enzymatic hydrolysis conducted by cutinase from *Fusarium solani* and PETase from *Ideonella sakaiensis* was selected in accordance with previous studies (Han et al., 2017; Ping et al., 2017). Next, the screening of PET pre-treatment methods was done. Based on the available reports, we selected a few of the most promising plastic pre-treatment methods: NaOH treatment, grinding (1 mm, 2 mm, 6 mm), artificial aging process and Triton X, as a surfactant. We have tested a variety of Tritons, namely X-45, X-102, X-100, X-114, X-165, X-405 and the best results were achieved with Triton X-100 (data not shown), which was consequently used in this study. Artificial ageing was performed using a mixture of sea salts with a concentration corresponding to the average salinity found in the seas and oceans (35‰). This process was studied due to the fact that the environment of the seas and oceans contains a very large amount of plastic waste. In addition to the use of a specific concentration of sea salts in the screening study, we also examined the effect of a short (1 and 2 weeks) incubation of the PET material in an artificial seawater environment, during which we observed more efficient degradation of the PET plastic after a longer incubation (data not shown), which ultimately contributed to prolonging this time to 1 month. Screening of the pre-treatment methods was performed using supernatant collected from 96 h of AJD 2 pAD CUT_FS and AJD 2 pAD PET_IS yeast cultures. As a control, supernatant derived from the AJD 2 strain was used. The mentioned factors were tested for their effects on the degradation of various forms of PET plastic, such as commercially available low-crystallinity film, PET film generated through recycling, shredded post-recycled PET bottles, and a mixture of PET plastic containing recycled film and melts (Fig. 1).

First, the supernatants derived from the culture (AJD2 pAD-CUT_Fs and AJD2 PET-IS) were tested for enzymatic activity (Table S1). It was noted that while PETase has a higher affinity for the substrate, cutinase is the enzyme with higher enzymatic activity. Since the *Y. lipolytica* strain produces a wide range of native extracellular esterases, the control strain AJD 2 was also tested for enzyme activity. Interestingly, it was observed that for this strain the enzymatic activity at 37 °C was 5.5 UmL^{-1} (Fig. S1A). In the supernatant after 96 h for strains AJD2 pAD-CUT_Fs and AJD2 PET-IS the activity was 119.23 UmL^{-1} and 52.79 UmL^{-1} at 37 °C, respectively. Both of the strains showed a comparable rate of glucose uptake. Due to the long incubation time (14 days) at 37 °C, the stability of the enzymes present in the supernatant was also assessed. Interestingly, prolonged incubation at 37 °C does not impair enzyme activity, either for cutinase from *F. solani* or for PETase from *I. sakaiensis* (Fig. S2A). The stability of the enzymes during direct PET degradation at 28 °C was also evaluated (Fig. S2B), and it was found that the enzymes present in the supernatant were not significantly affected by a fall in activity during cultivation. Temperatures applied in this experiment correspond with the optimal one for tested enzymes (37 °C) and used during direct PET degradation in yeast cultures (28 °C). The pH used in our study, both in the kinetic studies and in the determination of enzymatic activity, was 8.5 and corresponded to the previously reported range of optimal pH for the action of the overexpressed enzymes (Urbanek et al., 2021). The obtained results of the kinetic parameters of cutinase and PETase are in agreement with previously published reports (Xi et al., 2021).

The next step was measurement of PET film degradation level by monitoring the amount of released hydrolysis products (mono-(2-hydroxyethyl)terephthalic acid (MHET) and terephthalic acid (TPA), which might vary in dependence of the used type of material and strains, by using UPLC analysis. Verification of the PET plastic pre-treatment methods using the supernatant collected from the AJD 2 pAD CUT_FS culture is presented in Fig. 2. For commercially available amorphous PET film (Fig. 2A), only the milling process of the film into 1 mm sized fragments and the artificial aging process significantly increased the amount of TPA and MHET formed during the experiments. It should be noted that the sample with untreated film (control) resulted in the release of 247.5 mgL^{-1} TPA and 22.8 mgL^{-1} MHET and this was the highest among all untreated films. The amounts of TPA measured after incubation of commercial film pre-treated with grinding and artificial ageing in seawater were 1.5- and 1.1-fold higher relative to the control (untreated film), respectively. From the untreated recycled PET film 24.7 mgL^{-1} of TPA was measured. Significantly, MHET was not observed in any case of the use of post-recycled PET material while no pre-treatment method was applied. As seen in Fig. 2B, almost all of the pre-treatment methods resulted in an elevated amount of released TPA; however, a statistically significant increase occurred for samples incubated in Triton X-100, NaOH and in the case of 1 mm fragments. For these materials, the amounts of TPA obtained were 6.7-, 2.9- and 3.9-fold higher than the control, respectively. MHET was only detected after incubation of the film with surfactant and after the grinding process. During this experiment recycled PET bottles were found to be the most resistant to enzymatic hydrolysis (Fig. 2C). Only 3.88 mgL^{-1} of TPA was detected in the untreated material sample, and each of the treatment methods used contributed to higher levels of PET plastic degradation. No significant effect on the amount of TPA and MHET released among the treatments used was observed only for the incubation of the bottles with Triton X-100. The largest amount of TPA was measured for the PET plastic ground into 1 mm sized fragments, and this represented as much as 25.8 times the amount of TPA relative to the amount of this compound for the untreated PET plastic. A significant increase in the amount of TPA released was observed for the plastic substrate mixture containing recycled PET film with pre-treated melts in each case (Fig. 2D). For the untreated control sample, the amount of measured TPA was 43 mgL^{-1}, and the best result was obtained when using material ground into 1 mm fragments, where the amount of released terephthalic acid is 12 times higher; at the same time it was the sample with the largest amount of TPA measured during all screening experiments (515 mgL^{-1}). An interesting finding is the absence of MHET in the recycled PET plastic samples. As can be seen, incubation with surfactant stimulates the formation of this compound in the case of recycled film and recycled bottles, whereas the process of grinding into the smallest fragments and artificial ageing stimulates the release of MHET in each case. The ratio of TPA released during incubation of pre-treated PET plastic material to untreated plastic (control) with supernatant derived from a cutinase-producing strain is shown in Fig. S3A.

To determine whether the pre-treatment methods of PET material depend on the applied enzyme, we repeated the same experiments for supernatant derived from AJD 2 pAD PET_IS. Surprisingly, we noted a lower biodegradation rate than for cutinase, but we observed improvement for the pre-treatment sample in comparison to untreated PET plastic.

The results obtained for PETase including a commercial PET film and a recycled mixture of PET film and melts are shown in Fig. 3. In agreement with our previous study (Kosiorowska et al., 2022b) for the

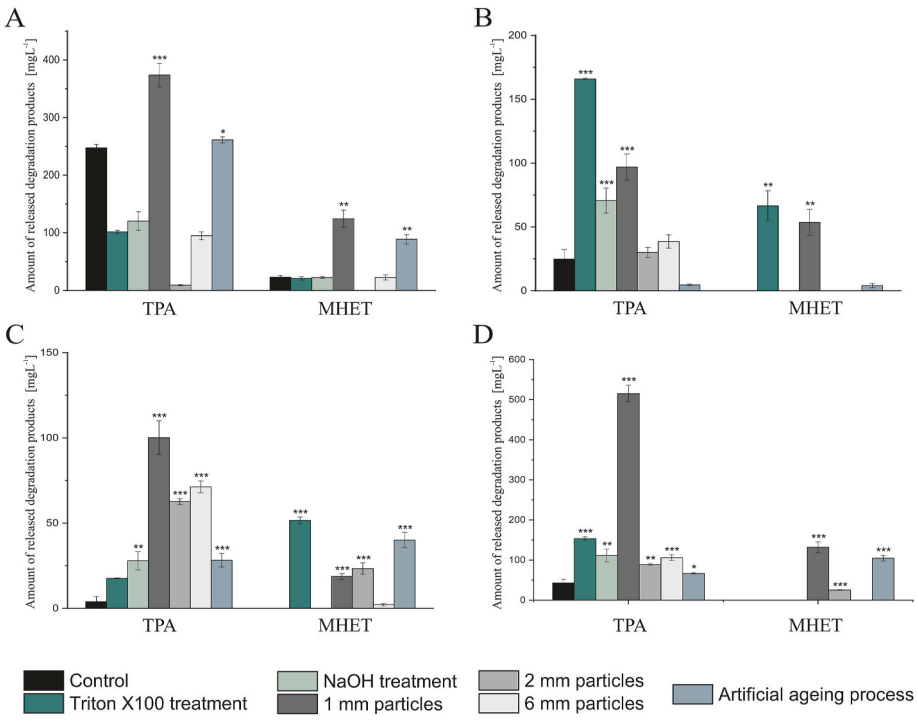


Fig. 2. Screening of PET film pre-treatment methods based on the amount of released degradation product. Plastic materials were incubated for 2 weeks with supernatant of AJD 2 pAD CUT_FS. A- Commercial, amorphous PET film, B- Recycled PET films, C- Recycled PET bottles, D- Recycled PET films with melts.

AJD 2 pAD PET_IS strain, no MHET was observed. The presence of BHET was not observed in the AJD 2 pAD CUT_FS or the AJD 2 pAD PET_IS supernatant. Because very low levels of PET hydrolysis were obtained when recycled film and bottles were used as a substrates, these materials were excluded from further experiments (Fig. S4). The effect of pre-treatment methods on the level of TPA released from commercial PET film is shown in Fig. 3A. The untreated film degrades slightly, and the measured amount of TPA was only 1 mgL^{-1}. A significant increase in hydrolysis efficiency was found for the film exposed to alkaline incubation with NaOH and artificial aging, where 92.2 mgL^{-1} and 10 mgL^{-1} TPA were detected, respectively. Similar improvement was observed when a mixture of recycled PET film with melts was used (Fig. 3B). The relative correlation of the TPA yields of films treated during incubation with PETase relative to controls (untreated films) is shown in Fig. S3B.

In parallel with the tests conducted using supernatants from modified strains, incubation of each type of film was carried out using the supernatant obtained from the control strain AJD 2. In neither case was the presence of PET degradation products observed, which confirms the lack of ability of *Yarrowia lipolytica* yeast to hydrolyse this polymer in applied conditions and is in agreement with our previous studies (Kosiorowska et al., 2022a, 2022b). More importantly, this fact also excludes the possibility of PET autohydrolysis resulting from the pre-treatment methods used. MHET as a product of PET hydrolysis for untreated materials was observed in the screening study only when the strain producing cutinase from *F. solani* was applied on commercial PET film. Intriguingly, it was not detected when recycled films were used as a substrate.

3.2. Degradation of PET plastic films in the yeast culture

Following preconditioning experiments with post-culture supernatant containing cutinase and PETase, long-term cultures were carried out. Based on statistical analysis of the results obtained in the screening process, the selected PET pre-treatment methods were used to prepare the substrates, which were subsequently used in yeast cultures. Direct PET degradation studies with pre-treated PET material were conducted for 4 weeks. Samples were collected at weekly intervals and analysed by UPLC in terms of PET hydrolysis product release (TPA and MHET). Furthermore, we analysed the enzymatic activity of the esterase present in the post-culture supernatant in order to verify whether the applied conditions and prolonged incubation time affect the reduction of this parameter. Regarding a previous report (Kosiorowska et al., 2022b) related to the inability of the AJD 2 pAD PET_IS strain to assimilate EG and, simultaneously, the ability of this strain to completely hydrolyse MHET to TPA, we performed analogous experiments involving the AJD 2 pAD CUT_ FS strain, due to the lack of experimentally validated data on this aspect. As expected, TPA was not assimilated by the *Y. lipolytica* AJD 2 pAD CUT_FS strain during cultivation (Fig. S5A), whereas MHET hydrolysis occurred, albeit at a much slower rate than was previously observed with AJD 2 pAD PET_IS (Fig. S5B). In addition, we carried out an additional assay for ethylene glycol (EG) uptake by AJD 2 pAD CUT_FS (Fig. S5C), which indicated that this compound could be completely assimilated within 96 h of culture, and this result is consistent with the data developed for AJD 2 and AJD 2 pAD PET_IS strains in prior studies (Kosiorowska et al., 2022b). The obtained results pointing to the maintenance of enzyme activity during the culture of the used strains are in agreement with previous reports regarding long-term incubation of PETase at 30 °C and 40 °C, where the enzyme retained stability during a 10-day experiment (Son et al., 2019) and for 14-day incubation of cutinase at 30 °C (Macedo and Pio, 2005).

To verify the stability of the produced enzymes and their effects in the long term, we performed 28-day incubation with pre-treated materials. As seen in Fig. 4, for strain AJD 2 pAD CUT_FS all PET plastic pre-treated samples, except recycled bottles treated with NaOH and artificially aged, showed a statistically significant increase in the amount of released degradation products, in comparison to the control untreated material. For the commercial PET film (Fig. 4A), the amount of released TPA for the untreated material is smaller than in the screening study (Fig. 2A), while for the treated samples the values achieved are at a comparable level. The samples incubated with NaOH and grinding yielded a much higher result of 0.26 gL^{-1} and 0.2 gL^{-1} of released TPA, respectively. For the pre-treatments performed with recycled bottles, we

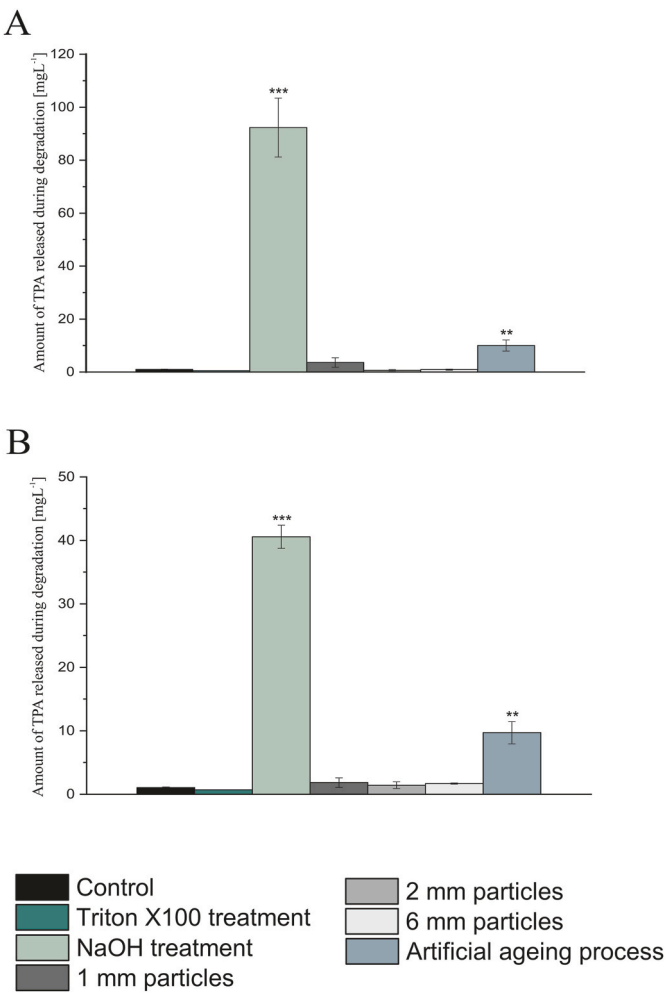


Fig. 3. Screening of PET film pre-treatment methods based on the amount of released degradation product. Plastic materials were incubated for 2 weeks with supernatant of AJD 2 pAD PET_IS. A- Commercial, amorphous PET film, B- Recycled PET films with melts.

noted that incubation of the PET in NaOH and artificial aging of the material did not provide similar results as we reported in the previous experiment. Clearly, a very large amount of TPA was retrieved for samples milled into 1 mm fragments, in which 0.59 gL^{-1} of this compound was measured, and this value was 16.1-fold higher than in the control culture with untreated PET. Milling the recycled bottles into 2 mm and 6 mm fragments gave very similar results, which may be related to the fact that abrasion of the material during the milling treatment affects the degradation process more than its fragmentation. The recycled mixture of recycled films and PET melts in cultures with modified yeast underwent less degradation compared to the incubation of this material with supernatant at 37 °C; however, each time the treatment used increased the level of PET plastic hydrolysis relative to the untreated material.

In contrast to the results obtained for cutinase and in parallel with a previous experiment conducted using a supernatant, in the culture of the AJD 2 pAD PET_IS strain, the degradation of commercial PET film proceeds with much higher efficiency, compared to both the former screening assay and the use of the AJD 2 pAD CUT_FS strain. The results obtained for *Y. lipolytica* AJD 2 pAD PET_IS are shown in Fig. 5. In the control culture with PET film the TPA level reached 0.5 gL^{-1}, and both pre-treatment methods used significantly affected the PET hydrolysis efficiency. In the culture with the artificially aged PET plastic, 2.18 gL^{-1} TPA was measured, and this was the highest content of TPA obtained during all the assays included in this research. In the mixture of recycled film and PET melt used, there was also a noticeably higher amount of TPA released than in the small-scale experiment performed using the supernatant (Fig. 5B). In the culture with the untreated material 0.039 gL^{-1} TPA was released. The highest level of degradation was observed when PET was exposed to artificial aging, in which the amount of TPA was 0.17 gL^{-1}. Interestingly, for commercial PET film, this pre-treatment method also had the greatest impact on the level of degradation. Interestingly, in the process performed directly in culture, a greater effect on the level of PET degradation was observed in the AJD 2 pAD PET_IS strain for the artificially aged samples than that seen in the screening study for the NaOH-treated material (Fig. 3). Each time, the AJD2 strain was also cultured with the appropriate type of PET material form tested. As in previous studies, no hydrolysis products such as TPA and MHET were observed in the post-culture fluid. BHET was also not observed, which is explained by the fact of enzymatic hydrolysis of this monomer by extracellularly produced enzymes to TPA and MHET. Ethylene glycol was absent from the post-culture fluid, and this phenomenon was also observed previously for the AJD 2 pAD PET_IS strain. The EG uptake ability, with the applied culture medium, of the AJD 2 pAD CUT_FS strain is shown in Sup. Fig. 5C.

To confirm the degradation performance and illustrate the differences between the PET plastic form, applied yeast strain and pretreatment method used, the weight loss of the plastic was determined (Fig. 6). The results obtained are in agreement with the chromatographic analysis of the post culture supernatant, where the amount of monomers (TPA and MHET) released into the medium was quantified. Using the AJD2 pAD CUT_FS strain, the highest weight loss was recorded for the mix of recycled film and artificially aged PET melts (42.17%) (Fig. 6A). It is noteworthy that for the untreated sample (control), the weight loss amounted to only 1.4%, and this material was characterized by high resistance to the degradation process in previous studies (data not shown). Interestingly, the weight loss of the PET plastic was lower when commercial material was used, with a maximum of 24.23% for 1 mm particles. Similarly, when recycled bottles was used as tested material, the highest decrease in the mass was when 1 mm particles was used (16.93%), where for the untreated control sample, the weight loss observed amounted only for 2.6%. For recycled films, the maximal weight loss was observed for NaOH treatment resulted in 16.93%, however it is comparable with 1 mm particles achieved during grinding process (15.07%). Applying the AJD 2 pAD PET_IS strain in degradation process confirmed the highest impact of artificial ageing process, which led to 72.43% mass loss of commercial PET plastic. NaOH treatment of commercial PET caused 30.43% decrease in weight and differed slightly from the untreated sample (control) when PETase was used (Fig. 6B). When recycled PET film and melt mix was used, maximal observed weigh loss was 17.37% and occurred also for material subjected to artificial ageing.

To verify that the PET material used in this study varied in crystallinity, a TGA-DSC analysis was performed (Supplementary Fig. 8). Crystallinity of commercially available amorphous PET film was 4.00%, for recycled film it was 15.76%, while for recycled bottles was 19.22%. These findings agree with literature reports, which indicate that the crystallinity of recycled PET (films and bottles) is in the range of 12–23% (Liu et al., 2004; Wang and Salmon, 2022). According to the lack of cold crystallization peak for recycled melts, which were selected for analysis, it was impossible to calculate the crystallinity level of this material. The samples also differed in glass transition temperature, where the lowest was recorded for commercially available PET film (78.84 °C) and the highest for recycled bottles (102.26 °C).

4. Discussion

In the present study, the effect of PET pre-treatment methods on its degradation directly in the culture of cutinase and PETase enzyme-secreting microorganisms was examined. We have focused on

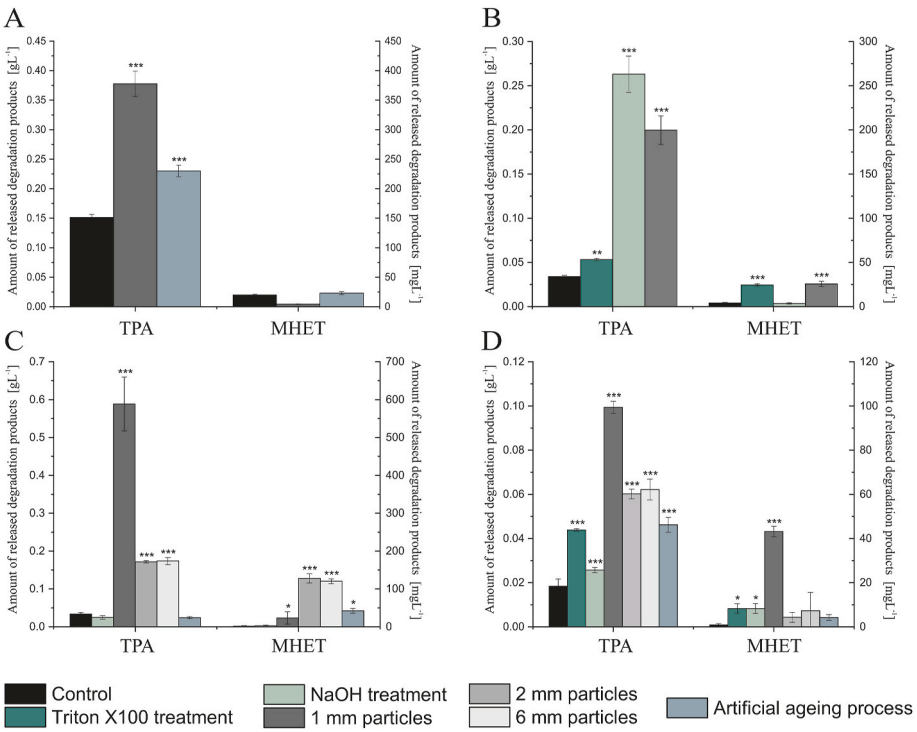
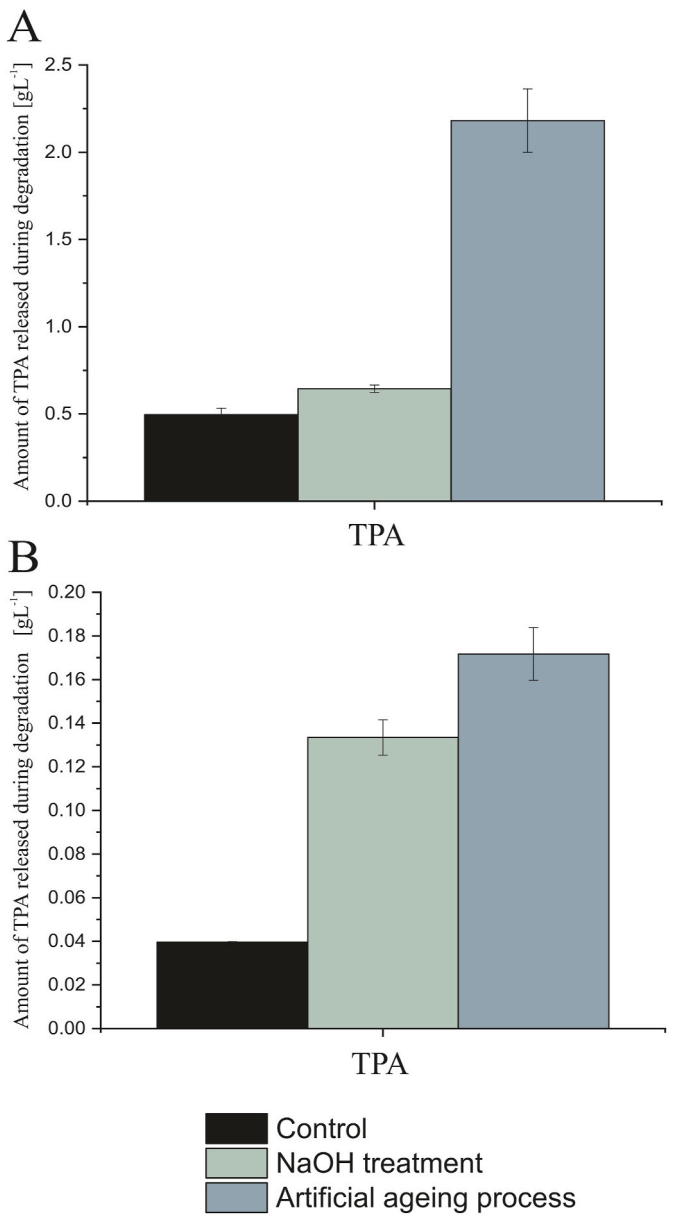


Fig. 4. Amount of PET degradation products released during 28-days culture with pre-treated plastic material with AJD 2 pAD CUT_FS. A- Commercial, amorphous PET film, B- Recycled PET films, C- Recycled PET bottles, D- Recycled PET films with melts.

exploring new directions in the pretreatment of substrates, with a special attention given to the current possibilities of obtaining already pre-treated material (collected from the seas and oceans) or used in recycling processes (surfactants in the washing, shredding process). The results obtained on the degradation of various types of PET films directly in culture with engineered *Y. lipolytica* yeast indicated that the PETase enzyme hydrolyses the ester bonds present in PET with higher efficiency than cutinase. The data obtained for the two esterases used show that although the mechanism of action of both is similar, the influence of the pre-treatment method on the level of degradation differs. In the case of the strain producing cutinase, a markedly higher level of degradation was obtained for PET plastic subjected to grinding into 1 mm fragments, where this process in the case of use of PETase was eliminated already at the screening stage. The process of grinding the PET enlarged the surface area available for enzymes, which resulted in an increase of its degradation level. It is also worth noting that in the case of recycled PET plastic, more applied factors can increase the level of its degradation using cutinase, which proves that accessibility to this type of material can be enhanced in many ways.

A significant impact of surfactants on PET degradation efficiency has been reported previously (Samak et al., 2020). Among a number of surfactants, a positive impact was observed with non-ionic and anionic agents (Caparanga et al., 2009; Furukawa et al., 2018). The first hypothesis, presented by Furukawa et al. (2018), that the use of surfactants with long alkyl chains and negatively charged head groups enhances PETase activity, indicating that these surfactants increase the enzyme's access to the PET surface. The authors suggested, that enzymatic degradation of PET can be improved by pre-incubating a low-crystallinity PET film with anionic surfactants, leading to a 120-fold increase in PETase activity. The binding of surfactants to the PET film creates a negatively charged surface, attracting the positively charged PETase enzyme and aligning its active site for efficient hydrolysis. The key element responsible for increasing the efficiency of PET degradation by the anionic surfactant was a cationic surface region composed of the amino acids R53, R90 and K95 (Furukawa et al., 2018). Conversely, the work of Ribitsch et al., (2015) indicated that surfactants can act like amphiphilic proteins, in which the negatively charged main groups are oriented toward the aqueous phase by making the PET surface charged (Ribitsch et al., 2015). The pre-treatment of PET via surfactant was investigated by Ahmaditabatabaei et al., (2022). An anionic surfactant was examined as a factor to increase the ability to degrade PET by bacteria (*Idoenella sakaiensis* and *Pseudomonas mendocina*) with pro-longed incubation time (overnight). The authors noticed the positive impact of applied pre-treatment of PET during enzymatic hydrolysis by both tested bacteria (Ahmaditabatabaei et al., 2022).

The use of grinding as a pre-treatment method of PET is related to the current use of this process in mechanical recycling of plastics (Grigore, 2017; Farzi et al., 2019; Maurya et al., 2020). In this study, PET plastic material was ground into 1 mm, 2 mm and 6 mm fragments to increase the availability of the enzyme to the substrate. Moreover, the micro-damage to the material's surface caused by friction also expands the surface area being targeted by the applied enzymes (Austin et al., 2018). The results obtained when ground PET was used as a substrate are in agreement with previous reports, indicating shredding as a promising factor of PET plastic degradation during the action of cutinases. The lack of significant increase in degradation of all forms of PET material subjected to shredding and exposed to PETase may have resulted from the different types of hydrolysis. Previous studies have indicated the possibility of endo-type action, where internal bonds of PET are hydrolysed, as well as exo-type hydrolysis, observed when recombinant cutinase TfCut2 from *T. fusca* was used (Kawai et al., 2019; Wei et al., 2019; Maurya et al., 2020). In the present study, we observed that the grinding of the PET increases the level of degradation mainly for recycled plastics. These findings demonstrate the great potential of using such a pre-treatment method in the preparation of PET material for biological recycling using the AJD 2 pAD CUT_FS strain. The relatively low weight loss observed in graft degradation may be related to the form of PET plastic used. The best results in regard to this were obtained for material ground into 1 mm fragments, which is in agreement with the PET powder used in earlier studies (Kosiorowska et al., 2022a). When analysing mass loss, special attention should be paid to the risk of interfering with the results of the viable degradation rate, which is



Figre 5. Amount of PET degradation products released during 28-days culture with pre-treated plastic material with AJD 2 pAD PET_IS. A- Commercial, amorphous PET film, B- Recycled PET films with melts.

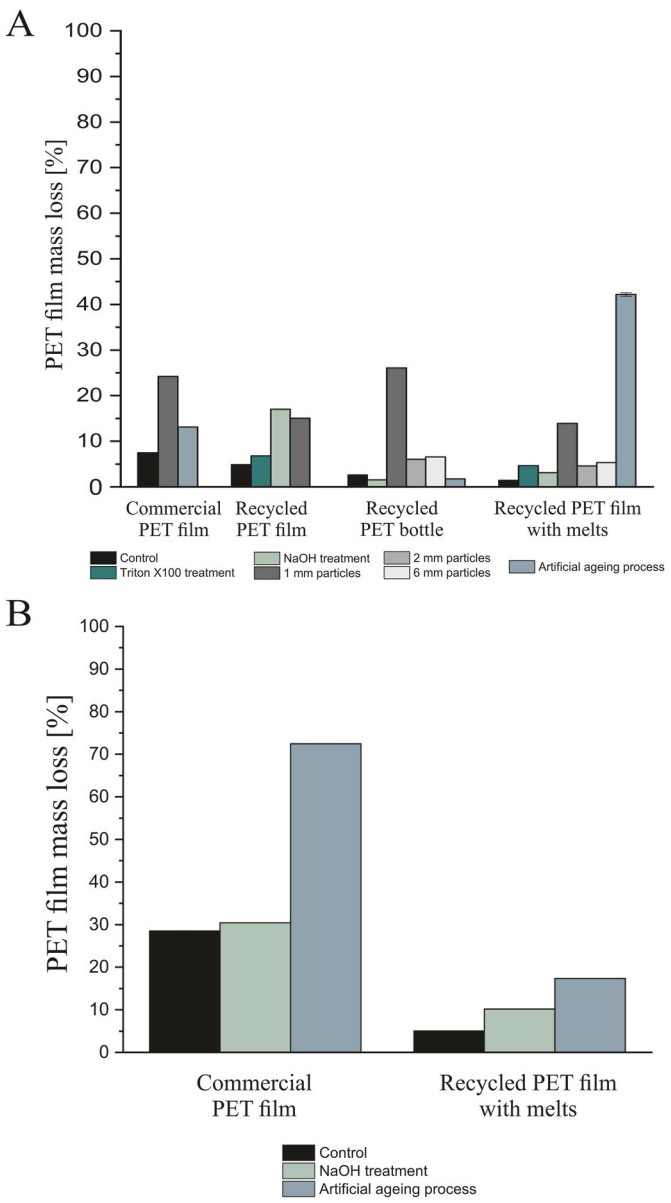


Fig. 6. Mass loss of PET films after prolonged (28-day) culture with A- AJD 2 pAD CUT_FS and B- AJD 2 pAD PET_IS.

associated with the nano and microplastic released during the process into the culture medium.

The application of the PET plastic artificial aging process in this study is related to reports on the effects of abiotic processes on synthetic materials during prolonged exposure to seawater (Manjunath et al., 2019). Furthermore, the employment of this method aimed to examine the possibility whether plastic materials collected from aquatic environments would be more susceptible to degradation by PET-hydrolysing enzymes. The results obtained in this study indicate a significant increase in the level of degradation of PET subjected to ageing and PETase treatment for both forms of plastic tested on a larger laboratory scale (both commercial film and recycled film with melts). Regarding PET exposed to cutinase action, significant enhancement of the amount of TPA released was only observed when the commercial film was used as a substrate. These findings suggest the necessity of applying an individual approach for each type of material in parallel with selection of the most promising pre-treatment taking into consideration the intended target enzyme to be applied in further studies.

Achieved outcomes, globally, have huge relevance, due to the ongoing efforts aimed at reducing the level of plastic pollution in the seas and oceans. We also confirmed that efficient PET degradation can occur at a low temperature, which significantly reduces the cost of the process, and that extracellularly produced enzymes retain activity at this temperature even after a month of incubation.

5. Conclusion

Summarising the results obtained in the present study, it is clear that untreated recycled plastic material is more difficult to degrade than virgin material. This study demonstrates, regarding the use of a cutinase-producing strain, the most promising plastic pre-treatment methods are the milling process and the artificial aging process. In contrast, when PETase is employed, the highest degradation rate was observed when alkaline pre-treatment was used, and, as for cutinase, the artificial aging process. Therefore, any enzyme used in biodegradation of commercial (bottle-grade) PET should have a tailored pre-treatment method for an

efficient process.

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CRediT authorship contribution statement

Katarzyna E. Kosiorowska: Writing – review & editing, Writing – original draft, Visualization, Investigation, Conceptualization. **Antonio D. Moreno:** Writing – review & editing, Supervision. **Raquel Iglesias:** Writing – review & editing, Supervision. **Piotr Biniarz:** Writing – review & editing, Supervision, Resources. **Aleksandra M. Mirończuk:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2024.105842.

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