Science of the Total Environment 846 (2022) 157358

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Production of PETase by engineered *Yarrowia lipolytica* for efficient poly (ethylene terephthalate) biodegradation

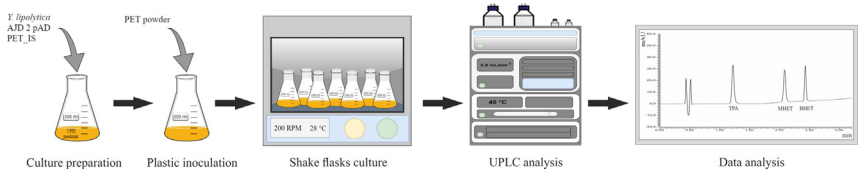
Katarzyna E. Kosiorowska [a], Antonio D. Moreno [b], Raquel Iglesias [b], Karol Leluk [c], Aleksandra M. Mirończuk [a,*]

[a] Wrocław University of Environmental and Life Sciences, Department of Biotechnology and Food Microbiology, Chełmońskiego 37, 51-630 Wrocław, Poland
[b] Advanced Biofuels and Bioproducts Unit, Department of Energy, Research Centre for Energy, Environment and Technology (CIEMAT), Avda. Complutense 40, 28040 Madrid, Spain
[c] Wroclaw University of Science and Technology, Faculty of Environmental Engineering, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland

HIGHLIGHTS

- Overexpression of PETase in *Y. lipolytica* results in PET films degradation.
- Yeast strains used in this study are able to assimilate ethylene glycol.
- Significant surface defects of PET film were observed.
- Supplementation with 1 % olive oil improves PET hydrolysis.
- No PET pre-treatment required

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damia Barcelo

Keywords:
PETase
Yarrowia lipolytica
Poly(ethylene terephthalate)
PET degradation
Plastic waste
Genetic engineering

ABSTRACT

There has been a growing interest in poly(ethylene terephthalate) PET degradation studies in the last few years due to its widespread use and large-scale plastic waste accumulation in the environment. One of the most promising enzymatic methods in the context of PET degradation is the use of PETase from *Ideonella sakaiensis*, which has been reported to be an efficient enzyme for hydrolysing ester bonds in PET. In our study, we expressed a codon-optimized PETase gene in the yeast *Yarrowia lipolytica*. The obtained strain was tested for its ability to degrade PET directly in culture, and a screening of different supplements that might raise the level of PET hydrolysis was performed. We also carried out long-term cultures with PET film, the surface of which was examined by scanning electron microscopy. The efficiency of PET degradation was tested based on the concentration of degradation products released, and the results showed that supplementation of the culture with olive oil resulted in 66 % higher release of terephthalic acid into the medium compared to the mutant culture without supplementation. The results indicate the possibility of ethylene glycol uptake by both strains, and, additionally, the PETase produced by the newly engineered strain hydrolyses MHET. The structure of the PET film after culture with the modified strain, meanwhile, had numerous surface defects, cracks, and deformations.

1. Introduction

Plastics are now one of the most widely produced materials for commercial and industrial use. Those synthetic compounds are mainly used in the packaging, construction, automobile, and electrical industries. Globally, 367 million tonnes of primary plastics were produced in 2020, and poly(ethylene terephthalate) (PET) contributed to 8.4 % of this amount and was mainly used in the production of plastic bottles (Plastics Europe – Association of Plastics Manufacturers, 2020; PlasticsEurope Market Research Group (PEMRG)/Conversio Market &amp and Strategy GmbH, 2021). The high production level of plastics directly impacts the quantity of plastic waste generated nowadays. In 2020, 29 million tonnes of post-consumer plastic trash were collected in Europe, of which 34.6 % have been recycled. Encouragingly, the amount of plastic dumped in landfills fell from 12.9 to 6.9 million tonnes from 2006 to 2018, while the amount of plastic recycled doubled (PlasticsEurope Market Research Group (PEMRG)/Conversio Market &amp and Strategy GmbH, 2021).

* 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), Karol.leluk@pwr.edu.pl (K. Leluk), aleksandra.mironczuk@upwr.edu.pl (A.M. Mirończuk).

http://dx.doi.org/10.1016/j.scitotenv.2022.157358
Received 30 March 2022; Received in revised form 7 July 2022; Accepted 10 July 2022
Available online 16 July 2022

PET is a thermoplastic polymer categorized as non-biodegradable. Despite this, investigations into the feasibility of enzymatic degradation of PET have identified several enzymes capable of hydrolysing the ester bonds present in PET. The possibility of PET degradation has been confirmed by CALB lipase from *Candida antarctica*, cutinases from *Fusarium solani*, *Humicola insolens,* and *Thermobifida fusca*, and PETase from *Ideonella sakaiensis* (Carniel et al., 2017; Tanasupawat et al., 2016; Then et al., 2015; Tournier et al., 2020). The action of PET degrading enzymes is to break the internal bonds present in the polymer, resulting in the release of bis (2-hydroxyethyl) terephthalate (BHET), mono-(2-hydroxyethyl)terephthalic acid (MHET), terephthalic acid (TPA) and ethylene glycol (EG) (Mohanan et al., 2020). To improve the efficiency of PET degradation by enzymes, studies conducted so far have mostly focused on the use of genetic engineering methods to obtain higher enzyme secretion enabling further purification of the protein (Kim et al., 2020; Shi et al., 2021; Wang et al., 2020). Following reports that the most efficient enzymatic degradation of PET occurs at temperatures close to the glass transition temperature of 75 °C due to the greater mobility of the polymer chain, studies related to changing the structure of proteins to increase their thermostability have also been conducted (Ronkvist et al., 2009; Son et al., 2019). Considering the fact that the use of high temperatures for PET degradation is economically unfavourable, despite the ability of the produced proteins to act at high temperatures, it is necessary to look for further, superior solutions that are efficient and can be used on a large scale.

In this study, we overexpressed the PETase from *I. sakaiensis* in the yeast *Yarrowia lipolytica* and we optimized the PET degradation process by medium supplementation. In this study we performed PET degradation directly in the culture of the engineered strain of *Y. lipolytica*. Due to the capability for assimilation of atypical carbon sources which would allow for the assimilation of degradation products released in the process and good expression of the heterologous protein, the engineered yeast *Y. lipolytica* is a suitable organism for PET degradation.

2. Materials and methods

2.1. Microorganisms, media, and culture conditions

The yeast strains used in this study were AJD 2 pAD PET_IS (PETase overexpressing strain) and AJD2 (the control strain). LB medium was used for the growth of *Escherichia coli* (DH5α) strains. YPD (1 % yeast extract, 2 % peptone, 2 % glucose) medium was used for *Yarrowia lipolytica* inoculum preparation. For RNA isolation, a YNB (yeast nitrogen base) medium with 2 % glucose was used. The assimilation capacity of PET degradation products by *Y. lipolytica* was checked using a YPD medium containing 50 gL^{-1} glucose and the test compound. The final concentration of TPA and MHET in culture was 100 mgL^{-1}, while that of glycol was 10 gL^{-1}. Solutions of TPA and MHET were prepared by dissolving them in DMSO and methanol, respectively, and sterilized using 0.22 μM syringe filters (Merck Millipore). PET degradation experiments were performed in deep-well plates (DWP) and 0.3 L Erlenmeyer flasks. The growth curve analysis was conducted using a YPD medium with a dextrose content of 50 gL^{-1}. Semi-crystalline PET powder (particular size of 300 μm) and amorphous PET film (0.25 mm thickness) used as a plastic substrate in the media was purchased from GoodFellow Cambridge Limited (England) (ES306031/1; ES301445/7). PET material was previously sterilized by 10 min of UV irradiation. Supplements added to the medium to test their effect on degradation efficiency were sterilized using 0.22 μm syringe filters (Merck Millipore). Salt additives included: manganese (II) sulfate monohydrate ($MnSO_4$ x H_2O), zinc sulfate heptahydrate ($ZnSO_4$ x $7H_2O$), copper (II) sulfate pentahydrate ($CuSO_4$ x $5H_2O$), calcium chloride ($CaCl_2$), and magnesium sulfate heptahydrate ($MgSO_4$ x $7H_2O$). Salt stocks' molarity (50 mM) was calculated with consideration of their hydration level. Each supplement was added directly to the medium immediately before inoculation of the microorganisms and addition of plastic material. Tests were provided in 4 mL of YPD medium with glucose content of 50 gL^{-1} and the addition of 0.26 g of PET powder previously sterilized by 10 min of UV radiation. The addition of salt to the final concentration (1 mM and 2.5 mM) and olive oil (1 % and 2 %) was done before the inoculation of the culture. Control strain (AJD 2) and AJD 2 pAD PET_IS cultures were standardized to OD_{600} 0.1 and the experiment was conducted for 168 h (7 days); samples in triplicate were taken daily and the first sampling was after 72 h of culture. PET powder was added to the culture before the beginning of incubation. Based on the experiments performed in DWP we evaluated the most promising results and used them to scale up the process. For this purpose, we applied the conditions which gave a significant increase in the number of released degradation products on the larger laboratory scale. Experiments were performed in 0.3 L Erlenmeyer shake flasks in 30 mL of medium with addition of 2 g of PET powder previously sterilized for 10 min with UV light. Cultures were standardized to OD_{600} 0.5. Supplements and PET powder were added to the culture before incubation. Shake flask culture was performed at 28 °C at 200 RPM for 10 days (240 h) and the first samples were taken after 72 h of culture. All cultures conducted in this study were performed in three biological repetitions.

2.2. Strain construction

A codon-optimized PETase gene (GAP38373.1) from *I. sakaiensis* containing the XPR2 signal sequence from *Y. lipolytica* (915 bp) was cloned into the previously described pAD vector (Mirończuk et al., 2017) using SgsI and *Nhe*I FastDigest restriction enzymes and T4 DNA Ligase (Thermo Fisher Scientific). Digestion and ligation were conducted according to the manufacturer's protocols. *E. coli* transformation was executed using the standard protocol. Isolation of the obtained plasmid was done using the Plasmid Mini Kit (A&A Biotechnology) and the correctness of the overexpression cassette was verified by sequencing (Genomed, Poland). The constructed vector was linearized with the MssI FastDigest enzyme and the expression cassette was introduced to the *Y. lipolytica* genome based on rDNA integration using the lithium acetate-based transformation method described previously (Mirończuk et al., 2019), resulting in strain AJD 2 pAD PET_IS. Yeast genomic DNA isolation was performed with the Genomic Mini AX Yeast Spit Kit (A&A Biotechnology) and the verification of obtained clones was checked by standard PCR reaction.

2.3. Relative gene expression level and growth profile analysis

Y. lipolytica RNA isolation procedure and gene expression quantification were performed as described before (Kosiorowska et al., 2021). Primers used in qRT-PCR analysis to determine PETase expression level were qPET_IS_F (5′-GCCATGAAGCTCGCTACCGC-3′) and qPET_IS_R (5′-AGACGGCCATCAGACCACCC-3′) and the resultant product size was 109 bp. The relative expression level was normalized to the actin gene. Growth profiles were estimated using Spark Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). *Y. lipolytica* inoculum cultures grew for 24 h at 28 °C at 200 RPM. Culture cells were centrifuged and double rinsed in Milli-Q water. The experiment was conducted in 200 μL of YP medium with 50 gL^{-1} of glucose content. The effects of degradation products and solvents on yeast growth were evaluated on a YP medium with 50 gL^{-1} addition of glucose and relevant compounds: 100 mgL^{-1} TPA, 100 mgL^{-1}, MHET, 10 gL^{-1} EG, 10 gL^{-1} DMSO, 10 gL^{-1} MeOH. Experiments were carried out for 72 h with continuous orbital agitation at 28 °C. The initial OD was standardized to 0.05 and subsequent measurements of culture density were made at OD600 every 30 min of culture growth.

2.4. Analytical methods and statistical analysis

Samples obtained from cultures conducted in deep well plates (200 μL) and 0.3 L Erlenmeyer flasks (500 μL) supplemented with PET powder were centrifuged for 10 min at 10000 G and subsequently diluted in 99.9 % methanol (Chempur) followed by further centrifugation for 10 min at 10000 G at 10 °C. Terephthalic acid standard (TPA, Sigma-Aldrich) was prepared by dissolving in DMSO (Sigma-Aldrich). 2-Hydroxyethyl-terephthalic acid (MHET, AChemBlock) and bis(2-hydroxyethyl) terephthalate (BHET, Sigma) were

dissolved in 99.9 % methanol (P. P. H. STANLAB). Standards of compounds were prepared in stock with a final concentration of 10 gL^{-1} and were stored at −20 °C. Analysis of compounds released during PET degradation was performed by Ultra Performance Liquid Chromatography (UPLC) (Dionex-Thermo Fisher Scientific, UK) using a C18 type column (Hypersil Gold 100 × 2.1 mm, 3 μm) with a universal guard column (Hypersil Gold 10 × 2.1 mm, 3 μm). A gradient of two mobile phases was used during the analysis: acetonitrile with 0.1 % trifluoroacetic acid (*v*/v) and Milli-Q water with 0.1 % trifluoroacetic acid (v/v). The column temperature was 45 °C, the flow rate was 0.8 $mLmin^{-1}$ and the volume of the sample injected was 1 μL. Identification of analytes was performed with the UV detector at 243 ± 2 nm. The concentration of glucose and ethylene glycol was studied using a HyperREZ XP Carbohydrate H + 8 μm column (Thermo Scientific, Waltham, MA) and a refractive index (RI) detector (Shodex, Ogimachi, Japan). As the eluent 0.25 mM trifluoroacetic acid was used, the flow rate was 0.6 $mLmin^{-1}$ and the column temperature was 65 °C. The UPLC program used in this study was described in detail previously (Kosiorowska et al., 2022).

Statistical analysis was performed using Student's *t*-test to determine the significance of the influence of the supplements used in our study. The means of the control culture (without additives) were compared with the supplemented cultures (two-tailed, unequal variance). In addition, the results presented in the graphs include error bars that indicate the standard deviation of the samples calculated from the three biological replicates.

2.5. Scanning electron microscope analysis

Samples for examining plastic surfaces were derived from cultures conducted for 4 weeks at 28 °C at 200 RPM in 50 mL of YP medium containing 50 gL^{-1} of glucose, 2 g of PET film (GoodFellow ES301445/7, England), and from cultures performed in the same media with olive oil supplementation to a final concentration of 1 %. The analysis of the plastic surface was performed using a VEGA Tescan 3 scanning electron microscope and dedicated software. To prevent excessive accumulation of electric load plastic samples were coated with a thin layer of gold using a Cressington 108 Auto Sputter Coater with the following conditions: current 40 mA, sputtering time 60 s.

3. Results and discussion

3.1. Overexpression of I. sakaiensis PETase (PET_IS) and growth of the engineered Y. lipolytica strain

In recent years, the need to remove PET plastic wastes from the environment has been the subject of numerous studies. The enhancement of the natural plastic degradation processes can be improved by the use of genetic engineering methods. In our work, we used an unconventional yeast, *Y. lipolytica*, as the host organism; it possesses a GRAS (Generally Recognised as Safe) status, is able to assimilate atypical carbon sources such as alkanes and can grow in a wide pH range (Rzechonek et al., 2020). The codon optimized PETase gene (GAP38373.1) with the *Y. lipolytica* XPR2 signal sequence was expressed in the AJD 2 strain devoid of acidic (AXP) and alkaline (XPR2) extracellular proteases to eliminate the risk of heterologous protein degradation (Janek et al., 2020). The choice of this microorganism to secrete a PETase (6EQD_A) protein capable of poly (ethylene terephthalate) (PET) degradation (Yoshida et al., 2016) may carry the additional benefit of the ability of this type of yeast to assimilate atypical carbon sources. Over the past few years, this enzyme has been the target of numerous studies involving attempts to increase its heterologous expression in various host microorganisms (Kim et al., 2020; Shi et al., 2021) as well as modifying its structure to enhance protein stability (Chen et al., 2021; Han et al., 2017). PETase is an enzyme with an optimum temperature of 40 °C and exhibits activity at a wide pH range of 6–10 (Chen et al., 2021). It belongs to the class of hydrolases and its catalytic triad consists of the amino acids conventionally present in the active site of esterases: Asp-His-Ser (Fecker et al., 2018). The mechanism of hydrolysis of the ester bonds present in PET is based on the nucleophilic attack of serine on the carbonyl carbon present in this polymer (Feng et al., 2021). The products of PET hydrolysis are BHET (bis(2-hydroxyethyl) terephthalate), MHET (2-hydroxyethyl-terephthalic), TPA (terephthalic acid), and EG (ethylene glycol) (Mohanan et al., 2020).

First, to verify the functional expression of the *Y. lipolytica* strain expressing PETase from *I. sakaiensis*, the expression level of the cloned gene was analysed using the qRT-PCR method. Due to the use of a hybrid $UAS1B_{16}$-TEF promoter, whose highest activity was recorded after 24 h of growth (Blazeck et al., 2013), RNA isolation from the tested strain and from the control strain (AJD2) that was modified was performed on 24 h cultures. The relative expression level of the PET_IS gene is shown in Fig. 1A. This result indicates that the AJD 2 pAD PET_IS strain functionally expresses the target gene.

Considering the fact that genetic modifications of microorganisms can adversely affect their growth (Ko et al., 2020), we compared the growth profile of the AJD 2 pAD PET_IS strain with the control strain (AJD2). The experiment was conducted for 72 h in a YPD medium containing 50 gL^{-1} glucose, which was used at all further stages of this research. As shown in Fig. 1B there was no significant difference in the growth of the tested strain in comparison with the control strain. Both strains presented an extended lag phase occurring up to 15 h of culture and an exponential phase persisted up to 40 h of culture. In agreement with previous studies, no significant delay in the growth of the modified strains was observed (Dobrowolski and Mirończuk, 2020; Kosiorowska et al., 2021).

3.2. Capability of the engineered Y. lipolytica strain to assimilate PET degradation products

Consistent with reports on the capability of *Y. lipolytica* yeast to assimilate atypical carbon sources (Mirończuk et al., 2018), we tested the capability of the yeast used to grow on PET degradation products. These studies were crucial to verify that the possible uptake of hydrolysis products would not disturb the actual concentration of degradation products measured in the culture supernatant. Experiments were carried out in YPD medium supplemented with 50 gL^{-1} glucose supplemented with TPA, MHET, EG, DMSO or MeOH as required. Regarding the fact that BHET is released during PET degradation in negligible amounts (Vertommen et al., 2005), and was not detected in our preliminary studies, it was not included in the tests. The results shown in Fig. 2A demonstrate that neither the control strain nor the modified strain takes up TPA. A slight increase in the concentration of this compound in the medium was observed; however, considering the duration of the culture (168 h) this increase is associated with the evaporation process. This increase is not statistically significant. The glucose was consumed up until 48 h of culture and its uptake was identical for both strains. These results are in contrast with previous studies performed by Costa et al. in 2020, where TPA consumption by *Y. lipolytica* IMUFRJ 50682 in YPD media was observed. However, the authors used a YPD medium with lower glucose content. In addition, they observed a decrease in TPA assimilation in the glucose-supplemented medium compared to the medium without the additional carbon source (da Costa et al., 2020).

To investigate the ability of MHET hydrolysis by AJD 2 and AJD 2 pAD PET_IS strains, an experiment using media supplemented by this compound was carried out. As shown in Fig. 2B, the AJD 2 pAD PET_IS strain completely hydrolysed MHET to TPA after only 96 h of culture. In the control culture, no decrease in the MHET concentration was observed and the very low concentration of TPA detected is due to the spontaneous hydrolysis of MHET. The results indicate that a PETase from *I. sakaiensis* produced by the modified *Y. lipolytica* strain is capable of efficient hydrolysis of MHET without the support of the other overexpressed hydrolases. This finding concurs with other studies which have shown that the PETase enzyme requires additional MHETase enzyme responsible for MHET hydrolysis during the PET degradation process (Joo et al., 2018; Yoshida et al., 2016).

It can be seen from Fig. 2C that both the AJD 2 and AJD 2 pAD PET_IS strains have the capability to assimilate ethylene glycol (EG) under the

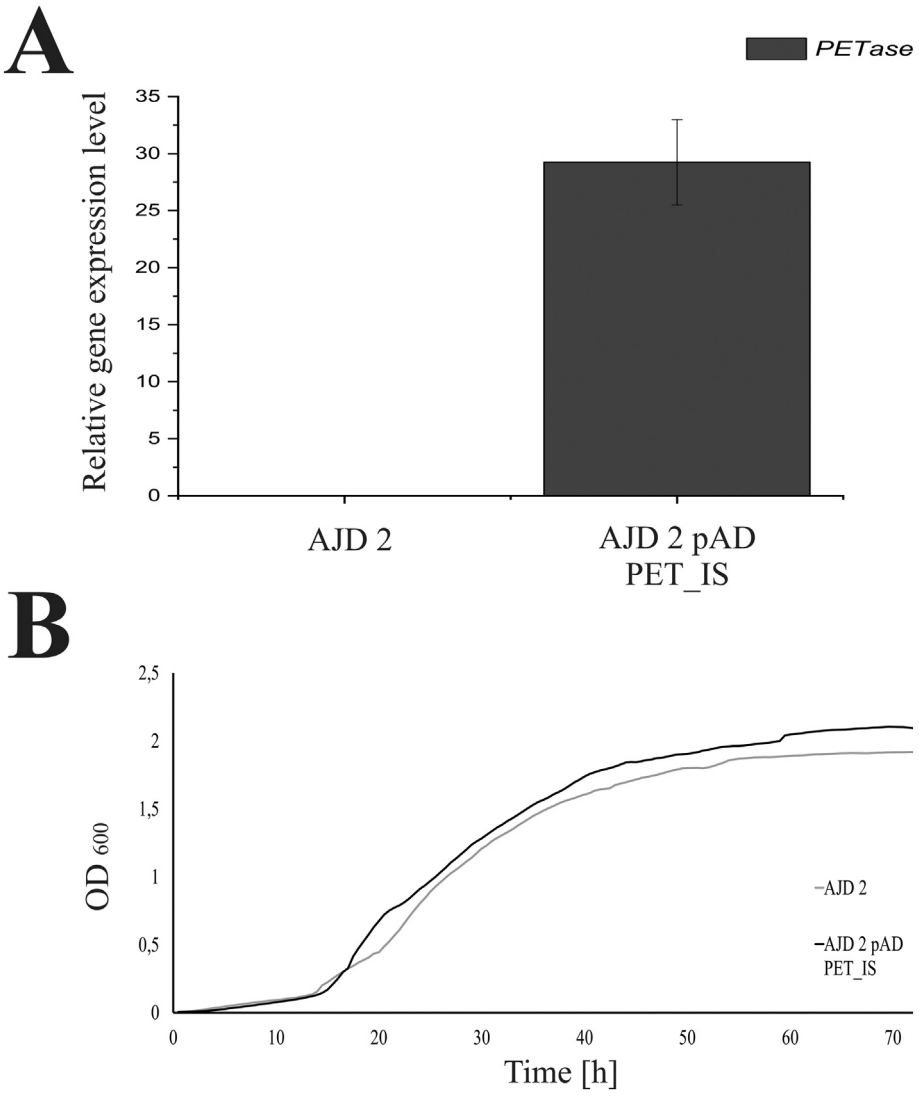


Fig. 1. Relative PETase gene expression level (A) in the obtained *Y. lipolytica* AJD 2 pAD PET_IS strain and control strain (AJD 2). Growth curve analysis (B) between AJD 2 and newly derived strain in YPD medium containing 50 gL^{-1} of glucose.

given conditions. It was observed that EG is taken up concomitantly with glucose, which is consumed with lower rates in contrast to the medium supplemented with TPA or MHET. To date, there are no studies regarding the capacity of yeast to assimilate EG, and metabolic pathways for natively existing pathways have been found only in bacterial species (Mückschel et al., 2012; Pandit et al., 2021).

Due to the use of compounds that have not been previously tested for their effect on the growth of *Y. lipolytica* yeast and the fact that TPA and MHET components were dissolved in DMSO and MHET respectively, whose toxicity toward microorganisms has been confirmed (Sadowska-Bartosz et al., 2013; Vartiainen et al., 2019), growth curves were constructed using the medium supplemented with an appropriate concentration of each of the tested supplements. As can be seen from S. Fig. 1, the applied concentrations of compounds and solvents do not have a negative effect on the growth of either the control or the tested strain.

3.3. Process optimization by the engineered Y. lipolytica strain

A key limitation of PET degradation by PETase is the low secretion level of this hydrolase by the *I. sakaiensis* bacterium, which directly affects the efficiency of the process. The literature concerning the use of genetic engineering methods demonstrates a variety of approaches in the host-organism selection that may alter the secretion level of this protein. To date, successful expression of PETase protein from *I. sakaiensis* has been performed in green algae (Kim et al., 2020), *Escherichia coli* (Shi et al., 2021), and *Bacillus subtilis* (Xi et al., 2021). The purpose of our study was to use *Y. lipolytica* as a host organism for extracellular PETase production and also to test the effect of supplementation on the PET degradation rate in the yeast culture. To determine the modified *Y. lipolytica* strain's ability to degrade PET, we first provided a small-scale experiment in deep-well plates (DWP). Small scale experiments were also used for estimation of the influence of supplementation of various salt and olive oil concentrations on degradation efficiency.

Following the exclusion of TPA assimilation in YPD medium with 50 gL^{-1} glucose, we performed degradation studies on PET powder added to cultures with the modified *Y. lipolytica* strain. In parallel, we investigated the effect of supplements such as inorganic salts and olive oil at different concentrations on the PET degradation level measured in the form of released degradation products. In agreement with a previous experiment, where we observed hydrolysis of MHET to TPA by the enzyme PETase and assimilation of ethylene glycol with the applied substrate composition, MHET and EG were not detected, and we determined the effect of supplementation from the measured concentration of TPA in the supernatants. The BHET compound was also not observed in any of the tested samples. The results are shown in Fig. 3.

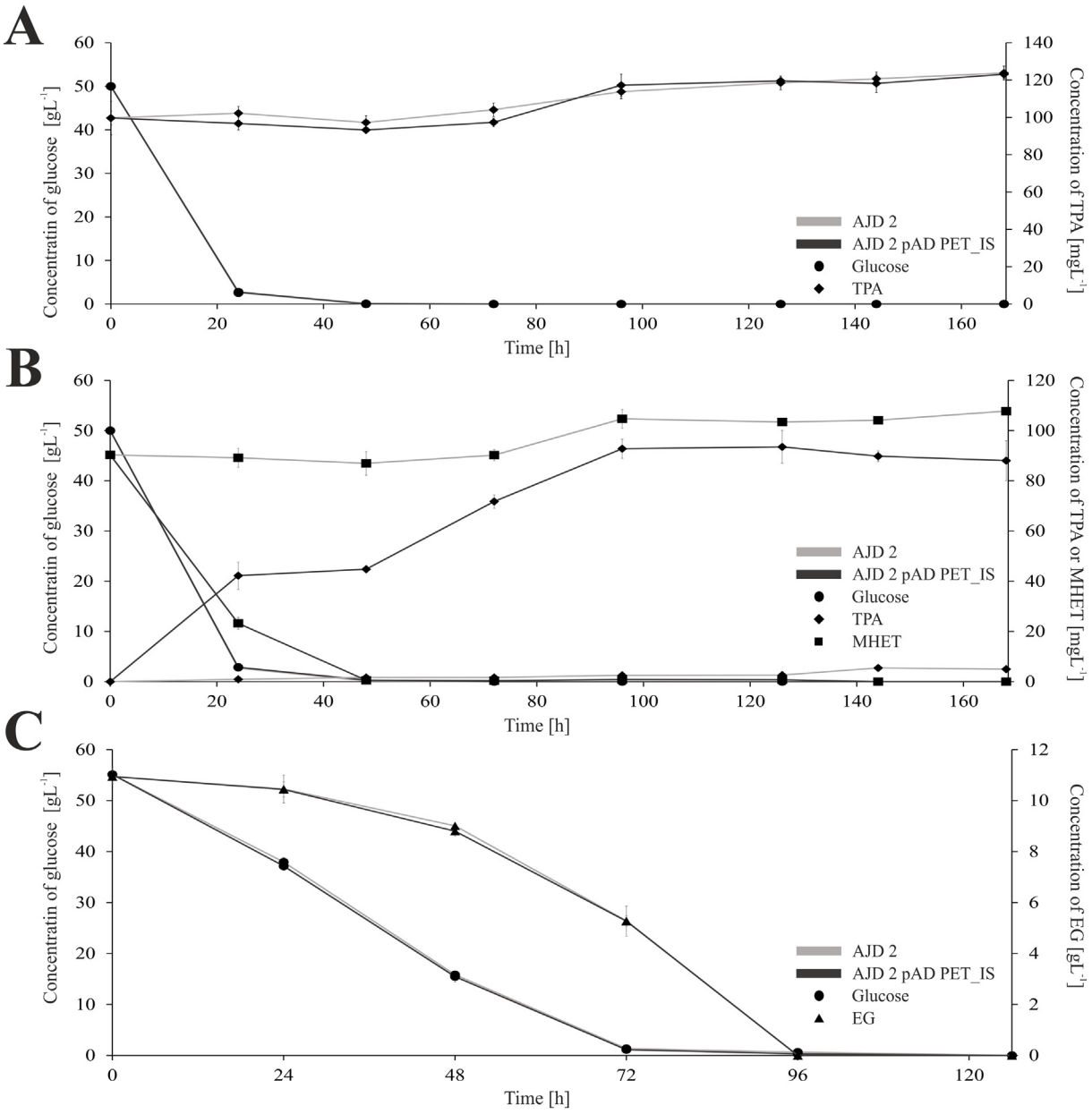


Fig. 2. Growth analysis of AJD 2 control strain and AJD 2 pAD PET_IS mutant with respect to PET hydrolysis products: panel A presents results derived from culture in the medium containing TPA, panel B contains results from cultures with the addition of MHET compound, panel C shows data from the culture with EG additive. Each graph also presents the profile of glucose depletion in the medium.

The supplements used in this study were selected based on previous scientific reports regarding the positive effects of salt ions on esterase enzymatic activity (Liu et al., 2019). Studies conducted on esterases also indicate that salt ions such as Mg^{2+}, Zn^{2+}, and Mn^{2+} used in concentrations of 2.5 mM and 5 mM enhance their enzymatic activity (Kawai et al., 2014; Metin et al., 2006; Park et al., 2021). Scientific literature also points to the increase in the stability of hydrolytic enzymes by Ca^{2+} and Mg^{2+} ions (Gricajeva et al., 2021; Urbanek et al., 2021). In our research, the use of *Y. lipolytica* as a host organism offers additional opportunities for supplementation in terms of induction of native lipase production using olive oil (Papanikolaou et al., 2007), for which the capacity for PET hydrolysis was previously described (Carniel et al., 2017). Moreover, lipases can increase the hydrophilicity of synthetic polyesters, thus facilitating access of the PETase enzyme to the hydrophobic substrate (Kim and Song, 2010). A further advantage of this yeast is its ability to assimilate atypical carbon sources, which allows the assimilation of one of the degradation products released into the medium (Fig. 2C).

In the first phase of our study on PET degradation by the AJD 2 pAD PET_IS strain, we focused on the screening of the supplements that could enhance the hydrolysis of this polymer during cultivation. First, we performed small-scale experiments for 168 h, in which we measured the concentration of TPA released to the supernatant since 72 h of cultivation. The daily increase of terephthalic acid in the collected samples is shown in S. Fig. 2. The first measurable concentrations of TPA in the supernatants occur after 120 h of culture, which is associated with an increase in the pH of the culture to the pH optimal for PETase activity (S. Fig. 4A). In the experiments performed on the AJD2 strain, which was tested for PET hydrolysis under identical conditions as the AJD2 pAD PET_IS strain, the presence of TPA in the supernatants was not recorded in any case. Additionally, we did not note the BHET compound in any of the tested samples. This result is contrary to reports (da Costa et al., 2020) where the tested *Y. lipolytica* strain hydrolysed PET, resulting in the release of approximately 23 mgL^{-1} MHET after 96 h of culture in YPD with amorphous PET.

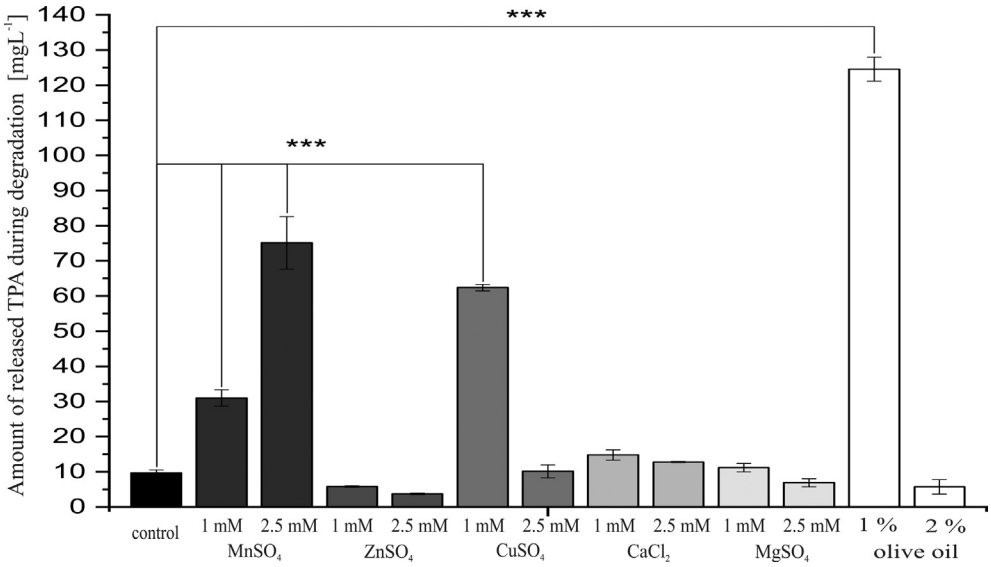


Fig. 3. Screening of the influence of the various supplementary additives on the PET degradation level in the cultures carried out in deep well plates for 168 h. Statistical analysis was performed using Student's *t*-test; error bars represent standard deviation. All experiments were performed in triplicate.

The results shown in Fig. 3 present the measured TPA concentration in the AJD 2 pAD PET_IS culture. The control labelled in the graph refers to the amount of terephthalic acid released to the media during cultivation with the engineered *Y. lipolytica* (AJD 2 pAD PET_IS) strain without supplementation and was 10 mgL^{-1}. The statistical analysis of the results was performed using Student's *t*-test, where the results from the supplemented cultures were compared to the control culture. PET degradation was significantly enhanced by the addition of $MnSO_4$ salt at both concentrations used (1 mM and 2.5 mM), where the measured amount of TPA was 30 mgL^{-1} and 75 mgL^{-1}, respectively. The hydrolysis of PET was also significantly influenced by the addition of 1 mM $CuSO_4$, where the concentration of TPA was 62 mgL^{-1}. In the experiment conducted with a higher concentration of this salt (2.5 mM), no positive effect on degradation efficiency was observed. The other additions of salts to the culture medium did not significantly affect the level of PET decomposition. Moreover, the addition of $ZnSO_4$ reduced it in both concentrations used. The data obtained are not surprising in the context of previous scientific reports on the effect of salt on esterase activity. The established effect of culture supplementation with $MnSO_4$ salt concurs with the experiments performed by Senga et al. in 2020. The relative activity against PBSA by examined esterase showed that in the presence of 2.5 mM Mn^{2+} ions it reached 65 % of the activity measured in 2.5 mM Ca^{2+} (Senga et al., 2020). A statistically insignificant increase in TPA concentration with the addition of $CaCl_2$ and $MgSO_4$ was observed. The highest observed amount of TPA released occurred with the addition of olive oil to a final concentration of 1 % in the culture and was 124 mgL^{-1}. This could be related to the induction of lipase production, which could act synergistically with secreted PETase to increase the level of PET degradation. In a previous study, it was noted that the cooperative action of cutinase and lipase effectively degraded PET (Liu et al., 2018). Interestingly, the addition of this supplement at a final concentration of 2 % had

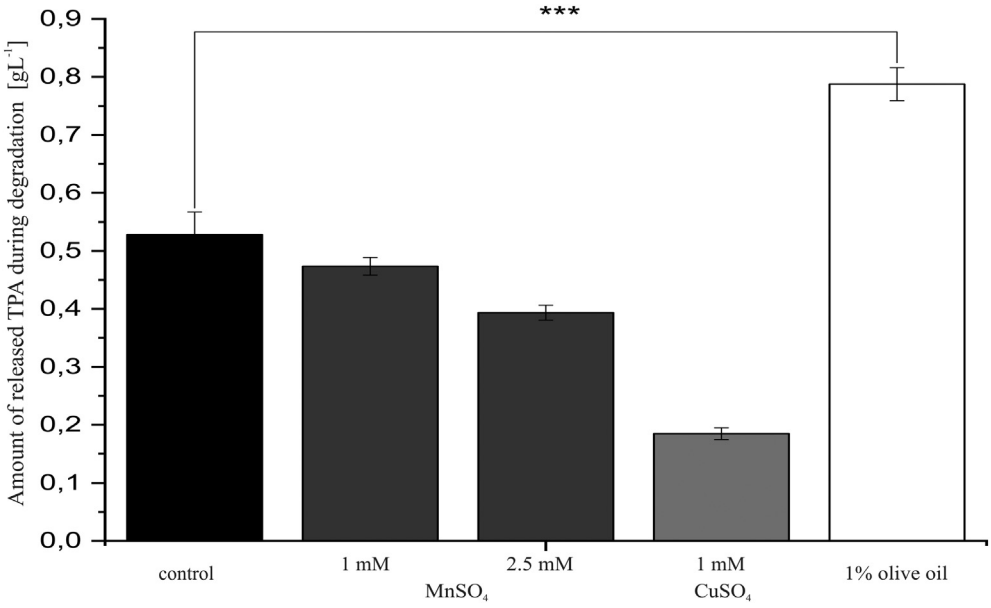


Fig. 4. Amount of TPA released during PET degradation process carried out in 0.3 L Erlenmeyer shake cultures with or without supplementation. Cultivation was carried out for 240 h in YPD medium containing 50 gL^{-1} of glucose and 2 g of PET powder at 28 °C at 200 RPM. Student's t-test was used for the statistical analysis; error bars represent standard deviation. All cultures were performed in triplicate.

no effect on the degradation efficiency. Supplementation of the culture with olive oil to a final concentration of 1 % resulted in a statistically significant increase in the amount of TPA released. Therefore, the results obtained may also indicate the influence of biosurfactants produced by *Y. lipolytica*, where at a lower olive oil concentration their production of them is induced, and at a higher concentration the phenomenon of their affiliation with the cell wall occurs (Fontes et al., 2010). The production of biosurfactants is closely related to the assimilation of hydrophobic products. The study by Fontes et al., 2010 also confirmed that high glucose concentration in the medium promotes the production of natural surfactants. Other researchers have used carbohydrate media supplemented by vegetable oils for surfactant production in the *Candida lipolytica* yeast strain (Sarubbo et al., 2007). Previous research has shown that surfactant addition improves PET degradation (Samak et al., 2020).

Subsequent to screening for potential supplements that could enhance PET degradation efficiency in the yeast culture, we made an attempt to scale up the degradation process. For this purpose, we used 0.3 L Erlenmeyer flasks and culture conditions where a statistically significant effect on PET hydrolysis was observed. The culture was conducted for 240 h and the results of TPA concentration at the final checkpoint are shown in Fig. 4. The samples were collected each day starting at 72 h and the daily increase in released product amount is shown in S. Fig. 3. Considering the pH as an important factor affecting the enzyme activity, it was monitored for each checkpoint. From S. Fig. 4B, it can be seen that the pH was stable at 8 throughout the sampling period for both AJD 2 and AJD 2 pAD PET_IS strain. Consistent with the cultivation carried out in deep well plates, we did not observe PET degradation by the AJD 2 strain, and TPA was observed as the sole hydrolysis product in the strain AJD2 pAD-PET_IS. The supplements used were selected based on a previous screening experiment where a statistically significant effect on PET degradation was observed in cultures containing 1 mM and 2.5 mM $MnSO_4$, 1 mM $CuSO_4$, and 1 % olive oil. The control culture labelled in the graph refers to AJD 2 pAD PET_IS culture in a medium without supplementation. The obtained results of salt supplementation in flask cultures did not confirm their positive effect on the degradation process. In the 2 pAD PET_IS culture without supplementation, the measured amount of released TPA was 0.53 gL^{-1}, while the highest amount of TPA released in the case of salt supplementation was obtained in the medium containing 1 mM $MnSO_4$ and was 0.47 gL^{-1}. When cultures containing 2.5 mM $MnSO_4$ and 1 mM $CuSO_4$ were grown, the TPA concentration was 0.39 gL^{-1} and 0.18 gL^{-1}, respectively, which is 1.35- and 2.9-fold lower than in the control culture. Contrary to the lack of a positive effect of salt on PET degradation when scaling up the process, the addition of olive oil to a final concentration of 1 % in the culture did have a statistically significant effect on the degradation level of this polymer. The concentration of TPA was 0.88 gL^{-1}, which is 66 % higher than in the culture without olive oil added. The non-positive effect of the addition of salts to the growth medium for cultures conducted in 0.3-L Erlenmeyer flasks may be related to the aeration conditions and cell growth rate. In comparison to the small scale, the yeast cells had a better environment for growth and the salts under these conditions had the opposite effect to the previously observed results. Particularly important for the growth of *Y. lipolytica* is aeration, the crucial effect of which has been described before (Mirończuk et al., 2019). Analysis of the daily increment of a degradation product is shown in S. Fig. 3. Cultures without supplementation to salt-supplemented cultures show no remarkable differences in the rate of growth of the amount of the TPA. The rise of the compound concentration is gradual, and the highest daily increase is observed on the last day of culture. Regarding the olive oil-supplemented culture, fundamentally different dynamics of TPA accumulation in the supernatant can be observed. In this case, three points of spiking increase of terephthalic acid concentration can be noted: between 96 h and 120 h, 120 h and 144 h, and on the last day of culture.

The degradation of PET plastic material during the cultivation of a microorganism capable of secreting PETase enzyme has not been the subject of much research. Studies published so far have been mostly focused on the production, isolation, and purification of PETase for further incubation with the plastic under optimized conditions. In this research, the novel degradation of PET in *Y. lipolytica* yeast culture can provide an interesting alternative in studies on the hydrolysis of this polymer. In the method proposed, purification of the enzyme is not required, the steady pH of the culture corresponds with the optimum for the enzyme action, and the low temperature of cultivation ensures the stability of the protein. Moreover, as illustrated previously, *Y. lipolytica* can assimilate ethylene glycol as a carbon source, enabling yeast to grow despite the depletion of the main carbohydrate source. In previous studies on PET degradation by PETase from *I. sakaiensis*, the number of degradation products released varied depending on the applied incubation conditions and the concentration of enzyme employed. In the first study reporting the promising capabilities of PETase, approximately 0.09 mM TPA and 0.2 mM MHET were obtained (Yoshida et al., 2016). These results were noted after 18 h of incubation at 30 °C and a pH of 7.0. An interesting study was conducted by Chen et al. in 2021, where PETase was expressed in *E. coli*. Incubation on PET film with purified enzyme caused the release of approximately 0.12 mM of total TPA and MHET after 6 days of incubation at 30 °C with PET. Interestingly, when a temperature of 40 °C was used, the total amount of released degradation products was slightly higher and was about 0.13 mM (Chen et al., 2021). Conversely, in the study of Shi et al., 2021, PETase containing the signal peptide pelB after 48 h of incubation with PET powder resulted in the release of a total of 531 μM of TPA and MHET. From the measured amount, approximately 400 μM was TPA and 130 μM was MHET, which is 64 mgL^{-1} and 30 mgL^{-1}, respectively. Intriguingly, no significant increase in the amount of degradation products was observed between the established control points in this study (18 h, 24 h, and 48 h) (Shi et al., 2021). A further study with PETase from *I. sakaiensis* performed by Son et al. in 2019 showed that during incubation of the enzyme with PET at 30 °C after 72 h, 11.5 μM of total TPA and MHET was released, whereas, at 40 °C, the total amount of degradation products was 8.7 μM. Enzyme stability during PET incubation for 10 days at both temperatures used demonstrated that PETase maintains its activity throughout the experiment (Son et al., 2019). A different study on PET degradation showed that, using 5 μg of purified PETase enzyme, approximately 2.5 mM TPA (400 mgL^{-1}) after 48 h of PET film incubation at 30 °C was released (Ma et al., 2018). Among the studies devoted to PET degradation by PETase, Kim et al., 2020 reported a very interesting application, whereby green microalgae were used as a host organism for production of this hydrolase. 30 mg of PET powder was incubated in cell lysate for 4 weeks at 30 °C, resulting in a quantity of TPA of 9.12 mg (Kim et al., 2020).

Notably, in addition to PETase, another promising enzyme for PET biocatalytic recycling research is cutinase produced by such microorganisms as: *Fusarium solani*, *Fusarium oxysporum*, *Thermobifida fusca*, *Saccharomonospora viridis* (Furukawa et al., 2019; Groß et al., 2017; Nimchua et al., 2007). Similar to PETase investigations described above, studies conducted using different cutinases relied on employing purified protein and executing the reaction under the most optimal conditions for enzyme action. Interesting results were obtained in a study comparing the action of two cutinases from *Fusarium solani* and *Fusarium oxysporum*, where enzymes with activity of 80 U and PET yarn as substrate, were used. Regarding the first enzyme, after 168 h of reaction at pH 7.0 and temperature 30 °C, approximately 9 $ugmL^{-1}$ TPA was released, while hydrolysis of PET by *F. oxysporum* resulted in the release of approximately 16 $ugmL^{-1}$ TPA. Intriguing findings were reported by Eugenio et al. in 2021 during a study on cutinase from *Humicola insolens*, where post-consumer PET was used as a substrate. PET flakes have been incubated for 96 h with 1.0 $mg_{protein}mL^{-1}$ at 70 °C in pH 7.0, and the total amount of hydrolysis products released (TPA, MHET and BHET) was 129 mM (Eugenio et al., 2021). Another noteworthy study is the leaf-branch compost cutinase, described by Sulaiman et al., 2014, which inherently exhibits high thermostability and the ability to hydrolyze PET, and within 24 h caused a loss of about 5 mg of PET film weight (initial PET weight 20–25 mg) at 70 °C (Sulaiman et al., 2014). However, other researchers, in their work on LCC, proved that the protein is susceptible to aggregation in its native state, but this effect can be

inhibited by protein glycosylation (Shirke et al., 2018) . In this work, the LCC cutinase was expressed in *Pichia pastoris* (*Komagataella phaffii*), and the results of PET film degradation indicated that after 48 h the polymer weight loss was more than 95 %. The synergistic effect of hydrolytic enzymes produced by *Microbacterium oleivorans* and cutinase from *Thermobifida fusca* was recently observed by Yan et al., 2021. Here, it was proven that the combination of *M. oleivorans* culture and 120 μgmg^{-1} of cutinase from *T. fusca* can contribute to more efficient degradation of PET films as low as 35 °C, with 47 nM TPA and 330 nM MHET released during 15 h of incubation (Yan et al., 2021).

In another study, various hydrophobic or hydrophilic monomers (TBMA, HEMA, DMAEMA, MA) were used to improve the catalytic

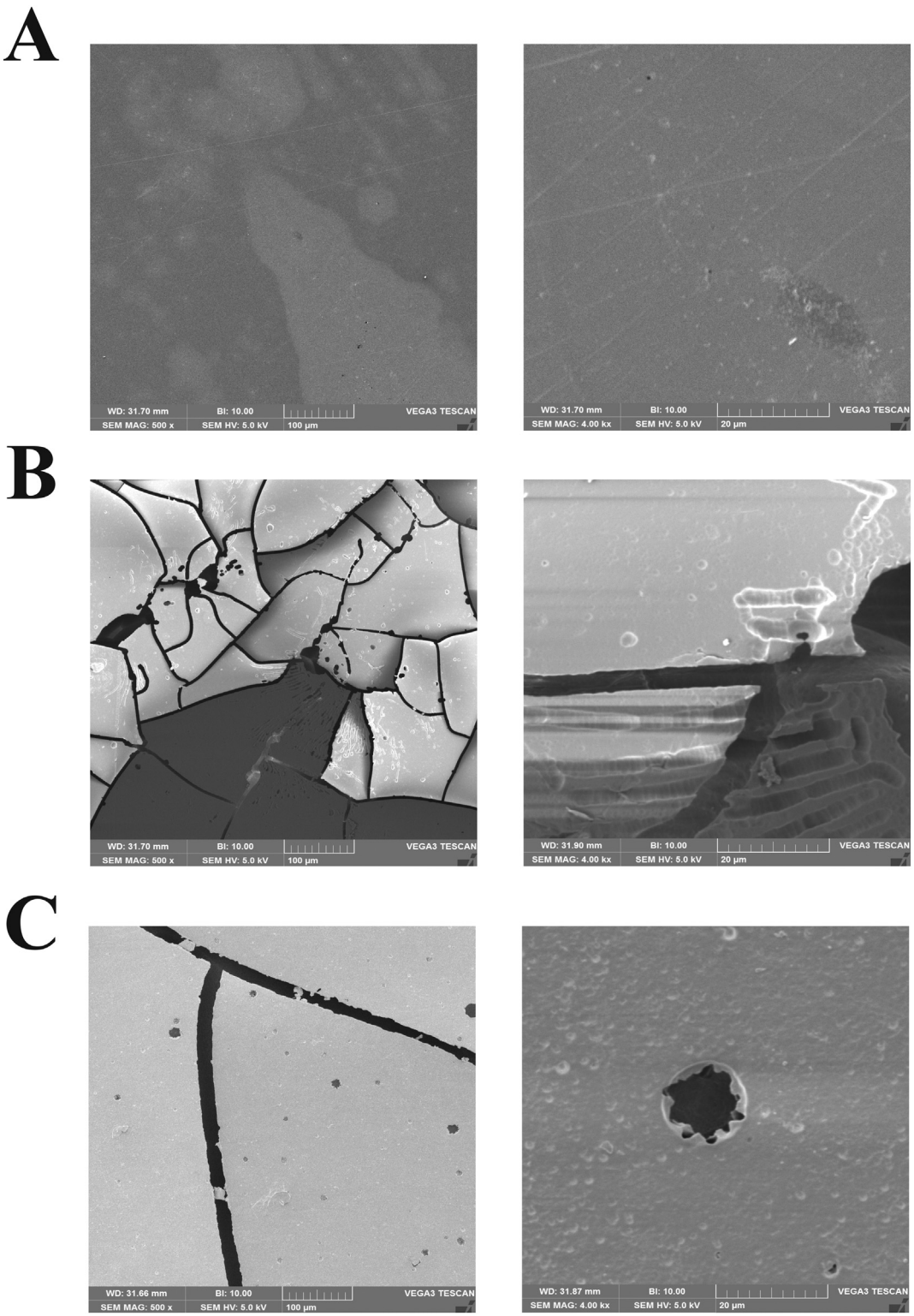


Fig. 5. Images of PET film surface derived from scanning electron microscope after 4 weeks of PET film incubation of control strain (AJD 2) (A) and AJD 2 pAD PET_IS (B) in YP medium containing 50 gL^{-1} of glucose. Fig. C presents PET film obtained in the mutant culture in a medium supplemented with olive oil. All cultures were incubated at 28 °C at 200 RPM. Detailed description (magnification, scale) is shown under every presented sample.

performance of PETase and the protein thus conjugated was tested with PET film at 40 °C. The best PET hydrolysis results were obtained using TBMA-PETase and DMAEMA-PETase, where the total amount of TPA and MHET released was 82.5 μM and 77.5 μM, respectively. Artificial intelligence was also used in the development of the GRAPE (*greedy accumulated strategy for protein engineering*) algorithm, with the help of which a DuraPETase mutant was obtained, possessing as many as 10 mutations in the amino acid chain. The generated PETase mutant, after 10 days of incubation with PET film, resulted in the release of 3.1 mM total PET degradation products (TPA, MHET, BHET) (Cui et al., 2021). In the work of Liu et al., 2022, this variant (DuraPETase) underwent further modifications in the amino acid chain to increase the thermostability of the enzyme (N233C/S282C/H214S/S245R). When degrading the amorphous PET powder at 60 °C, the total amount of degradation products released after 96 h with the purified DuraPETase-4 M enzyme was 15.8 mM, which was 2.77 fold higher than the initial mutant tested under the same conditions (Liu et al., 2022).

Protein engineering has also targeted cutinases, and among the most promising cutinase modifications, was the one made by Tournier et al., 2020. In this work, the LCC cutinase was subjected to a series of mutations, two of which, named WCCG (F243I/D238C/S283C/Y127G) and ICCG (F243W/D238C/S283C/Y127G), caused depolymerization of more than 90 % of the PET substrate in 10.5 h and 9.3 h at 72 °C, respectively (Tournier et al., 2020). The comparison of PET degradability by enzymes described above, with respect to reaction conditions, degradation time and amount of hydrolysis products released can be found in Supplementary Table 1.

3.4. PET film surface morphology analysis

After cultivation of *Y. lipolytica* strains in medium with the addition of PET powder, we used a more difficult to degrade type of substrate, i.e. PET film. For that purpose, the medium supplemented with olive oil to a final concentration of 1 % in the medium was used. To compare the effect of supplementation, a culture without supplements was conducted in parallel. The addition of PET film to the medium was 2 g of equally divided film fragments with dimensions of 1 cm × 1 cm. The collected film samples were analysed using a scanning electron microscope (SEM). Images showing the performance of the AJD 2 pAD PET_IS strain are shown in Fig. 5. The visual changes in the appearance of the films were also noticeable in the samples without SEM analysis and are shown in S. Fig. 5. No significant damage to the plastic surface was observed in the culture of strain AJD 2 at either of the magnifications used, 500 x or 4000 x (Fig. 5A). The surface of the film after culture with the AJD 2 pAD PET_IS strain in a medium with no olive oil added is shown in Fig. 5B. Both magnifications used demonstrate extensive damage to the surface of the material. The film is significantly cracked and numerous gouges can be seen on the surface. The depicted fragments also exhibit cavities in the plastic material. Fig. 5C presents the PET film after culture with a modified strain of *Y. lipolytica* in the medium supplemented with olive oil. The structure of the film obtained from this culture exhibits visible craters of irregular shape in parallel with the cracks. It can also be noted that the frequency of the breaks on this PET material is lower than in the films obtained from the experiment using the same strain but without supplementation (Fig. 5B). The films collected after long-term culture with yeast strains were also subjected to an estimated mass loss analysis (Supplementary Fig. 6). According to the data obtained, the addition of olive oil to the culture medium caused not only more corrosion on the film surface but also a greater polymer mass loss of 53.05 % of the initial mass. In the case of the substrate without supplementation, there was a 44.74 % decrease in film weight. For the culture of the control strain (AJD 2), the mass loss was imperceptible.

The analysis of changes in the polymer surface after PETase treatment plays a crucial role in determining the PET degradation level. The results obtained in our study correspond with previously reported data on plastic surface corrosion arising using the PETase enzyme (Shi et al., 2021; Yoshida et al., 2016). However, due to the extended culture of the *Y. lipolytica* modified with PET film, degradation level in our study is more apparent. Interestingly, in the 6-day incubation of PETase enzyme at 40 °C at pH 9.0 with PET film in the study by Chen et al. in 2021, changes in the plastic structure were barely noticeable (Chen et al., 2021). Considering the culture time, similar to our research, Kim et al. in 2020 incubated PET film for 4 weeks in the cell lysate of PET-producing green microalgae. In their study, the surface changes are slightly different due to the absence of visible cracks in the material, but numerous depressions and cavities could also be seen (Kim et al., 2020).

In summary, here we present the possibility to use an unconventional yeast strain as a host organism for the extracellular production of PETase. The methodology used in this work allowed us to determine the ability of the obtained strain to degrade PET, and its universal applicability in research on the degradation of this polymer allows us to compare the results with other reports on the discussed topic.

4. Conclusion

The present study showed that the modified *Y. lipolytica* yeast is capable of PET degradation directly in the culture. The obtained data showed that olive oil addition significantly improves the PET hydrolysis efficiency. However, we also observed that the addition of salt can reduce the degradation rate of the polymer, and the optimisation of the substrate composition has a key role in the degradation of PET by the method used. Furthermore, our study showed that *Y. lipolytica* cannot assimilate TPA; nevertheless, with the medium composition used, it can take up ethylene glycol. The applied novel approach has a great potential for further PET degradation during yeast culture growth.

CRediT authorship contribution statement

Katarzyna E. Kosiorowska: Investigation, Validation, Formal analysis, Visualization, Writing - original draft. **Antonio D. Moreno:** Supervision, Writing - review & editing. **Raquel Iglesias:** Supervision, Writing - review & editing. **Karol Leluk:** Investigation, Visualization. **Aleksandra M. Mirończuk:** Conceptualization, Supervision, Writing - review & editing, Project administration, Funding acquisition.

Funding

This study was financed by the National Science Centre, Poland, project UMO-2017/27/B/NZ9/02218, and the International Academic Partnership Programme by the Polish National Agency for Academic Exchange (NAWA), project INCREaSE, PPI/APM/2018/1/00013/U/001.

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.

Acknowledgments

The authors would like to thank José Luis Fernández Rojo for the HPLC analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.157358.

References

Blazeck, J., Reed, B., Garg, R., Gerstner, R., Pan, A., Agarwala, V., Alper, H.S., 2013. Generalizing a hybrid synthetic promoter approach in yarrowia lipolytica. Appl. Microbiol. Biotechnol. 97, 3037–3052. https://doi.org/10.1007/s00253-012-4421-5.

Carniel, A., Valoni, É., Nicomedes, J., Castro, A.M.de, Gomes, A.da C., 2017a. Lipase from Candida antarctica (CALB) and cutinase from Humicola insolens act synergistically for PET hydrolysis to terephthalic acid. Process Biochem. 59, 84–90. https://doi.org/10.1016/J.PROCBIO.2016.07.023.

Chen, K., Quan, M., Dong, X., Shi, Q., Sun, Y., 2021. Low modification of PETase enhances its activity toward degrading PET: effect of conjugate monomer property. Biochem. Eng. J. 175, 108151. https://doi.org/10.1016/j.bej.2021.108151.

Cui, Y., Chen, Y., Liu, X., Dong, S., Tian, Y., Qiao, Y., Mitra, R., Han, J., Li, C., Han, X., Liu, W., Chen, Q., Wei, W., Wang, X., Du, W., Tang, S., Xiang, H., Liu, H., Liang, Y., Houk, K.N., Wu, B., 2021. Computational redesign of a PETase for plastic biodegradation under ambient condition by the GRAPE strategy. ACS Catal. 11, 1340–1350. https://doi.org/10.1021/acscatal.0c05126.

da Costa, A.M., de Oliveira Lopes, V.R., Vidal, L., Nicaud, J.M., de Castro, A.M., Coelho, M.A.Z., 2020. Poly(ethylene terephthalate) (PET) degradation by yarrowia lipolytica: investigations on cell growth, enzyme production and monomers consumption. Process Biochem. 95, 81–90. https://doi.org/10.1016/j.procbio.2020.04.001.

Dobrowolski, A., Mirończuk, A.M., 2020. The influence of transketolase on lipid biosynthesis in the yeast yarrowia lipolytica. Microb. Cell Factories 19, 1–9. https://doi.org/10.1186/s12934-020-01398-x.

Eugenio, E.de Q., Campisano, I.S.P., de Castro, A.M., Coelho, M.A.Z., Langone, M.A.P., 2021. Kinetic modeling of the post-consumer poly(ethylene terephthalate) hydrolysis catalyzed by cutinase from Humicola insolens. J. Polym. Environ. https://doi.org/10.1007/s10924-021-02301-4.

Fecker, T., Galaz-Davison, P., Engelberger, F., Narui, Y., Sotomayor, M., Parra, L.P., Ramírez-Sarmiento, C.A., 2018. Active site flexibility as a Hallmark for efficient PET degradation by I. Sakaiensis PETase. Biophys. J. 114, 1302–1312. https://doi.org/10.1016/j.bpj.2018.02.005.

Feng, S., Yue, Y., Zheng, M., Li, Y., Zhang, Q., Wang, W., 2021. IsPETase- andIsMHETase-catalyzed Cascade degradation mechanism toward polyethylene terephthalate. ACS Sustain. Chem. Eng. 9, 9823–9832. https://doi.org/10.1021/acssuschemeng.1c02420.

Fontes, G.C., Fonseca Amaral, P.F., Nele, M., Zarur Coelho, M.A., 2010. Factorial design to optimize biosurfactant production by yarrowia lipolytica. J. Biomed. Biotechnol. 2010, 821306. https://doi.org/10.1155/2010/821306.

Furukawa, M., Kawakami, N., Tomizawa, A., Miyamoto, K., 2019. Efficient degradation of Poly(ethylene terephthalate) with thermobifida fusca cutinase exhibiting improved catalytic activity generated using mutagenesis and additive-based approaches. Sci. Rep. 9, 1–9. https://doi.org/10.1038/s41598-019-52379-z.

Gricajeva, A., Nadda, A.K., Gudiukaite, R., 2021. Insights into polyester plastic biodegradation by carboxyl ester hydrolases. J. Chem. Technol. Biotechnol. 97, 359–380. https://doi.org/10.1002/jctb.6745.

Groß, C., Hamacher, K., Schmitz, K., Jager, S., 2017. Cleavage product accumulation decreases the activity of cutinase during PET hydrolysis. J. Chem. Inf. Model. 57, 243–255. https://doi.org/10.1021/acs.jcim.6b00556.

Han, X., Liu, W., Huang, J., Ma, J., Zheng, Y., Ko, T., Xu, L., Cheng, Y., Chen, C., Guo, R., 2017. Structural insight into catalytic mechanism of PET. Nat. Commun. 8. https://doi.org/10.1038/s41467-017-02255-z.

Janek, T., Mirończuk, A.M., Rymowicz, W., Dobrowolski, A., 2020. High-yield expression of extracellular lipase from yarrowia lipolytica and its interactions with lipopeptide biosurfactants: a biophysical approach. Arch. Biochem. Biophys. 689, 108475. https://doi.org/10.1016/j.abb.2020.108475.

Joo, S., Seo, H., Cho, I.J., Lee, S.Y., Choi, S.Y., Son, H.F., Shin, T.J., Sagong, H.-Y., Kim, K.-J., 2018. Structural insight into molecular mechanism of poly(ethylene terephthalate) degradation. Nat. Commun. https://doi.org/10.1038/s41467-018-02881-1.

Kawai, F., Oda, M., Tamashiro, T., Waku, T., Tanaka, N., Yamamoto, M., Mizushima, H., Miyakawa, T., Tanokura, M., 2014. A novel Ca2 + -activated, thermostabilized polyesterase capable of hydrolyzing polyethylene terephthalate from saccharomonospora viridis AHK190. Appl. Microbiol. Biotechnol. 98, 10053–10064. https://doi.org/10.1007/s00253-014-5860-y.

Kim, H.R., Song, W.S., 2010. Lipase treatment to improve hydrophilicity of polyester fabrics. Int. J. Cloth. Sci. Technol. 22, 25–34. https://doi.org/10.1108/09556221011008785.

Kim, J.W., Park, S.Bin, Tran, Q.G., Cho, D.H., Choi, D.Y., Lee, Y.J., Kim, H.S., 2020. Functional expression of polyethylene terephthalate-degrading enzyme (PETase) in green microalgae. Microb. Cell Factories 19, 1–9. https://doi.org/10.1186/s12934-020-01355-8.

Ko, Y.S., Kim, J.W., Lee, J.A., Han, T., Kim, G.B., Park, J.E., Lee, S.Y., 2020. Tools and strategies of systems metabolic engineering for the development of microbial cell factories for chemical production. Chem. Soc. Rev. 49, 4615–4636. https://doi.org/10.1039/d0cs00155d.

Kosiorowska, K.E., Połomska, X., Wang, G., Borodina, I., Mirończuk, A.M., 2021. Efficient biodegradation of aliphatic polyester by genetically engineered strains of the yeast yarrowia lipolytica. Int. Biodeterior. Biodegrad. 161, 105232. https://doi.org/10.1016/j.ibiod.2021.105232.

Kosiorowska, K.E., Biniarz, P., Dobrowolski, A., Leluk, K., Mirończuk, A.M., 2022. Metabolic engineering of yarrowia lipolytica for poly(ethylene terephthalate) degradation. Sci. Total Environ. 831, 154841. https://doi.org/10.1016/j.scitotenv.2022.154841.

Liu, B., He, L., Wang, L., Li, T., Li, C., Liu, H., Luo, Y., Bao, R., 2018. Protein crystallography and site-direct mutagenesis analysis of the poly(Ethylene terephthalate) hydrolase petase from ideonella sakaiensis. ChemBioChem 19, 1471–1475. https://doi.org/10.1002/cbic.201800097.

Liu, C., Shi, C., Zhu, S., Wei, R., Yin, C.C., 2019. Structural and functional characterization of polyethylene terephthalate hydrolase from ideonella sakaiensis. Biochem. Biophys. Res. Commun. 508, 289–294. https://doi.org/10.1016/j.bbrc.2018.11.148.

Liu, Y., Liu, Z., Guo, Z., Yan, T., Jin, C., Wu, J., 2022. Enhancement of the degradation capacity of IsPETase for PET plastic degradation by protein engineering. Sci. Total Environ. 834, 154947. https://doi.org/10.1016/j.scitotenv.2022.154947.

Ma, Y., Yao, M., Li, B., Ding, M., He, B., Chen, S., Zhou, X., Yuan, Y., 2018. Enhanced Poly(ethylene terephthalate) hydrolase activity by protein engineering. Engineering 4, 888–893. https://doi.org/10.1016/j.eng.2018.09.007.

Metin, K., Ateslier, Z.B.B., Basbulbul, G., Biyik, H.H., 2006. Characterization of esterase activity in Geobacillus sp. HBB-4. J. Basic Microbiol. 46, 400–409. https://doi.org/10.1002/jobm.200510121.

Mirończuk, A.M., Biegalska, A., Dobrowolski, A., 2017. Functional overexpression of genes involved in erythritol synthesis in the yeast yarrowia lipolytica. Biotechnol. Biofuels 10, 1–12. https://doi.org/10.1186/s13068-017-0772-6.

Mirończuk, A.M., Biegalska, A., Zugaj, K., Rzechonek, D.A., Dobrowolski, A., 2018. A role of a newly identified isomerase from yarrowia lipolytica in erythritol catabolism. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.01122.

Mirończuk, A.M., Kosiorowska, K.E., Biegalska, A., Rakicka-Pustułka, M., Szczepańczyk, M., Dobrowolski, A., 2019. Heterologous overexpression of bacterial hemoglobin VHb improves erythritol biosynthesis by yeast yarrowia lipolytica. Microb. Cell Factories 18, 1–8. https://doi.org/10.1186/s12934-019-1231-9.

Mohanan, N., Montazer, Z., Sharma, P.K., Levin, D.B., 2020. Microbial and enzymatic degradation of synthetic plastics. Front. Microbiol. 11. https://doi.org/10.3389/fmicb.2020.580709.

Mückschel, B., Simon, O., Klebensberger, J., Graf, N., Rosche, B., Altenbuchner, J., Pfannstiel, J., Huber, A., Hauer, B., 2012. Ethylene glycol metabolism by pseudomonas putida. Appl. Environ. Microbiol. 78, 8531–8539. https://doi.org/10.1128/AEM.02062-12.

Nimchua, T., Punnapayak, H., Zimmermann, W., 2007. Comparison of the hydrolysis of polyethylene terephthalate fibers by a hydrolase from fusarium oxysporum LCH I and fusarium solani f. Sp. Pisi. Biotechnol. J. 2, 361–364. https://doi.org/10.1002/biot.200600095.

Pandit, A.V., Harrison, E., Mahadevan, R., 2021. Engineering Escherichia coli for the utilization of ethylene glycol. Microb. Cell Factories 20, 1–17. https://doi.org/10.1186/s12934-021-01509-2.

Papanikolaou, S., Chevalot, I., Galiotou-Panayotou, M., Komaitis, M., Marc, I., Aggelis, G., 2007. Industrial derivative of tallow: a promising renewable substrate for microbial lipid, single-cell protein and lipase production by Yarrowia lipolytica. Electron. J. Biotechnol. 10, 425–435. https://doi.org/10.2225/vol10-issue3-fulltext-8.

Park, J.E., Jeong, G.S., Lee, H.W., Kim, S.K., Kim, J., Kim, H., 2021. Characterization of a novel family iv esterase containing a predicted czco domain and a family v esterase with broad substrate specificity from an oil-polluted mud flat metagenomic library. Appl. Sci. 11. https://doi.org/10.3390/app11135905.

Plasctic Europe - Association of Plastics Manufactures, 2020. Plastics – the facts 2020. Plast. Eur. 1–64.

PlasticsEurope Market Research Group (PEMRG)/Conversio Market &amp, Strategy GmbH, 2021. An analysis of European plastics production, demand and waste data. Plastics - The Facts 2021. PlasticsEurope Market Research Group (PEMRG) / Conversio Market & Strategy GmbH.

Ronkvist, Å.M., Xie, W., Lu, W., Gross, R.A., 2009. Cutinase-catalyzed hydrolysis of poly(ethylene terephthalate). Macromolecules 42, 5128–5138. https://doi.org/10.1021/ma9005318.

Rzechonek, D.A., Szczepańczyk, M., Wang, G., Borodina, I., Mirończuk, A.M., 2020. Hog-independent osmoprotection by erythritol in yeast yarrowia lipolytica. Genes (Basel) 11, 1–15. https://doi.org/10.3390/genes11121424.

Sadowska-Bartosz, I., Paczka, A., Moloń, M., Bartosz, G., 2013. Dimethyl sulfoxide induces oxidative stress in the yeast Saccharomyces cerevisiae. FEMS Yeast Res. 13, 820–830. https://doi.org/10.1111/1567-1364.12091.

Samak, N.A., Jia, Y., Sharshar, M.M., Mu, T., Yang, M., Peh, S., Xing, J., 2020. Recent advances in biocatalysts engineering for polyethylene terephthalate plastic waste green recycling. Environ. Int. 145, 106144. https://doi.org/10.1016/j.envint.2020.106144.

Sarubbo, L.A., Farias, C.B.B., Campos-Takaki, G.M., 2007. Co-utilization of canola oil and glucose on the production of a surfactant by Candida lipolytica. Curr. Microbiol. 54, 68–73. https://doi.org/10.1007/s00284-006-0412-z.

Senga, A., Hantani, Y., Bekker, G.J., Kamiya, N., Kimura, Y., Kawai, F., Oda, M., 2020. Metal binding to cutinase-like enzyme from saccharomonospora viridis AHK190 and its effects on enzyme activity and stability. J. Deaf. Stud. Deaf. Educ. 25, 149–156. https://doi.org/10.1093/jb/mvz020.

Shi, L., Liu, H., Gao, S., Weng, Y., Zhu, L., 2021. Enhanced extracellular production of IsPETase in Escherichia coli via engineering of the pelB signal peptide. J. Agric. Food Chem. 69, 2245–2252. https://doi.org/10.1021/acs.jafc.0c07469.

Shirke, A.N., White, C., Englaender, J.A., Zwarycz, A., Butterfoss, G.L., Linhardt, R.J., Gross, R.A., 2018. Stabilizing leaf and branch compost cutinase (LCC) with glycosylation: mechanism and effect on PET hydrolysis. Biochemistry 57, 1190–1200. https://doi.org/10.1021/acs.biochem.7b01189.

Son, H.F., Cho, I.J., Joo, S., Seo, H., Sagong, H.Y., Choi, S.Y., Lee, S.Y., Kim, K.J., 2019. Rational protein engineering of thermo-stable PETase from ideonella sakaiensis for highly efficient PET degradation. ACS Catal. 9, 3519–3526. https://doi.org/10.1021/acscatal.9b00568.

Sulaiman, S., You, D.J., Kanaya, E., Koga, Y., Kanaya, S., 2014. Crystal structure and thermodynamic and kinetic stability of metagenome-derived LC-cutinase. Biochemistry 53, 1858–1869. https://doi.org/10.1021/bi401561p.

Tanasupawat, S., Takehana, T., Yoshida, S., Hiraga, K., Oda, K., 2016. Ideonella sakaiensis sp. Nov., isolated from a microbial consortium that degrades poly(ethylene terephthalate). Int. J. Syst. Evol. Microbiol. 66, 2813–2818. https://doi.org/10.1099/ijsem.0.001058.

Then, J., Wei, R., Oeser, T., Barth, M., Belisário-Ferrari, M.R., Schmidt, J., Zimmermann, W., 2015. Ca2 + and Mg2 + binding site engineering increases the degradation of polyethylene terephthalate films by polyester hydrolases from thermobifida fusca. Biotechnol. J. 10, 592–598. https://doi.org/10.1002/biot.201400620.

Tournier, V., Topham, C.M., Gilles, A., David, B., Folgoas, C., Moya-Leclair, E., Kamionka, E., Desrousseaux, M.L., Texier, H., Gavalda, S., Cot, M., Guémard, E., Dalibey, M., Nomme, J., Cioci, G., Barbe, S., Chateau, M., André, I., Duquesne, S., Marty, A., 2020. An engineered PET depolymerase to break down and recycle plastic bottles. Nature 580, 216–219. https://doi.org/10.1038/s41586-020-2149-4.

Urbanek, A.K., Kosiorowska, K.E., Mirończuk, A.M., 2021. Current knowledge on polyethylene terephthalate degradation by genetically modified microorganisms. Front. Bioeng. Biotechnol. 9, 1–15. https://doi.org/10.3389/fbioe.2021.771133.

Vartiainen, E., Blomberg, P., Ilmén, M., Andberg, M., Toivari, M., Penttilä, M., 2019. Evaluation of synthetic formaldehyde and methanol assimilation pathways in yarrowia lipolytica. Fungal Biol. Biotechnol. 6, 1–13. https://doi.org/10.1186/s40694-019-0090-9.

Vertommen, M.A.M.E., Nierstrasz, V.A., Veer, M.Van Der, Warmoeskerken, M.M.C.G., 2005. Enzymatic Surface Modification of Poly (ethylene Terephthalate). 120, pp. 376–386. https://doi.org/10.1016/j.jbiotec.2005.06.015.

Wang, N., Guan, F., Lv, X., Han, D., Zhang, Y., Wu, N., Xia, X., Tian, J., 2020. Enhancing secretion of polyethylene terephthalate hydrolase PETase in Bacillus subtilis WB600 mediated by the SPamy signal peptide. Lett. Appl. Microbiol. 71, 235–241. https://doi.org/10.1111/lam.13312.

Xi, X., Ni, K., Hao, H., Shang, Y., Zhao, B., Qian, Z., 2021. Secretory expression in Bacillus subtilis and biochemical characterization of a highly thermostable polyethylene terephthalate hydrolase from bacterium HR29. Enzym. Microb. Technol. 143, 109715. https://doi.org/10.1016/j.enzmictec.2020.109715.

Yan, Z.F., Wang, L., Xia, W., Liu, Z.Z., Gu, L.T., Wu, J., 2021. Synergistic biodegradation of poly(ethylene terephthalate) using microbacterium oleivorans and thermobifida fusca cutinase. Appl. Microbiol. Biotechnol. 105, 4551–4560. https://doi.org/10.1007/s00253-020-11067-z.

Yoshida, S., Hiraga, K., Takehana, T., Taniguchi, I., Yamaji, H., Maeda, Y., Toyohara, K., Miyamoto, K., Kimura, Y., Oda, K., 2016. A bacterium that degrades and assimilates poly(ethylene terephthalate). Science (80-.) 351, 1196–1199. https://doi.org/10.1126/science.aad6359.