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A screening method for polyester films-degrading microorganisms and enzymes

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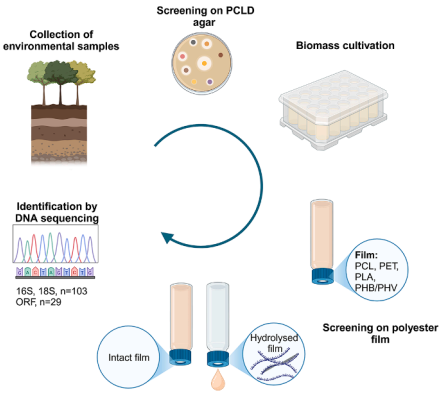
HIGHLIGHTS

- A method for screening of polyester film-degrading enzymes was proposed.
- In total, 102 bacteria were tested on PCL and PET high crystalline biaxial films.
- The 29 PCL-, PET-, PLA- or PHB/PHV-degrading polyesterases were identified.
- Robust enzymes can be screened by the proposed method.

GRAPHICAL ABSTRACT



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ABSTRACT

Enzymatic degradation of plastic pollution offers a promising environmentally friendly waste management strategy, however, suitable biocatalysts must be screened and developed. Traditional screening methods using soluble or solubilised polymers do not necessarily identify enzymes that are effective against solid or crystalline polymers. This study presents a simple, time-saving and cost-effective method for identifying microorganisms and enzymes capable of degrading polymeric films. The method was tested on polycaprolactone (PCL), polyethylene terephthalate (PET), polylactate (PLA) and polyhydroxybutyrate/polyhydroxyvalerate (PHB/PHV) films. It involves two steps: first, screening for PCL diol (PCLD)-degrading microorganisms on agar plates, and second, testing these microorganisms on polyester films. Using this screening method, over 100 PCLD-degrading microorganisms and 27 *E. coli* clones carrying genomic or metagenomic DNA fragments have been isolated. In addition, recombinant cutinases from *Streptomyces scabiei* and *Thermobifida fusca* have been tested. Approximately 66 % of the microorganisms forming halos on PCLD agar plates hydrolysed PCL and 6 % – the biaxially

Abbreviations: BHET, *bis*2-hydroxyethyl terephthalate; DMT, dimethyl-terephthalate; MMT, monomethyl-terephthalate; PCL, polycaprolactone; PCLD, polycaprolactone diol; PET, polyethylene terephthalate; PLA, polylactic acid; PHB/PHV, polyhydroxybutyrate/polyhydroxyvalerate, PS, polystyrene; TPA, terephthalic acid.

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oriented PET film. In addition, five PLA- and four PHB/PHV-degrading esterases have been identified. The proposed method is effective for detecting both wild-type and recombinant microorganisms, as well as recombinant enzymes from *in vitro* transcription-translation reactions. Screening for thermostable and thermophilic enzymes, including those resistant to organic solvents or environmental inhibitors, is also easily implemented.

1. Introduction

The cumulative global mass (~8 Gt) of synthetic plastics has become a major threat to the biosphere [1]. The detrimental effects of human-made polymers are observed both on natural ecosystems [2] and even on crop productivity, particularly in dryland agriculture [3–5]. The plastic residues especially in the form of microplastic, emissions of which to the environment are estimated to be between 10 and 40 million tonnes per year, rise numerous ecological impacts and risks as well as putative adverse effects on human health [6].

Enzymatic degradation has the potential to significantly accelerate the natural biodegradation of plastics, thereby reducing the time it takes for plastics to break down in the environment. Interestingly, microorganisms that have evolved to degrade natural polymers can also degrade synthetic plastic polymers. In this context, hydrolytic enzymes such as phosphatases, esterases, proteases/peptidases, oxidases and dehydrogenases are crucial for the degradation of these polymers [7–9]. However, most current knowledge on the microbial degradation of synthetic plastics is based on a limited number of bacteria, representing less than 0.1 % of the estimated bacterial population in the biosphere. Recent studies have identified more than 30.000 non-redundant enzyme homologues in databases with the potential to degrade 10 different types of plastics [10,11]. To date, most of the identified plastic-degrading microorganisms have been isolated from soils (27.8 %), plastic waste landfills (9.6 %) and composts (5.3 %), while a significant proportion have been obtained from culture collections of microorganisms (15.9 %) [12,13]. This suggests that a large number of different microorganisms and enzymes capable of degrading different polymers already exist in nature, but remain unexplored.

The biodegradation process of plastics is typically detected by observing changes in the surface structure of the plastic, such as colour changes, the formation of cracks and holes, roughening of the surface and the formation of microbial colonies. More than half of the studies on plastic degrading enzymes are based on functional screening or selection of new enzymes by culturing microorganisms in nutrient media enriched with appropriate substrates that form halo zones [12]. Screening or selection in vivo requires soluble and non-toxic substrates, which is challenging because some plastics contain additives that inhibit microbial growth. As a result, the number of microbes capable of degrading pure plastic polymers may be underestimated. Synthetic plastics, including bioplastics, are generally insoluble in water, making the preparation of plastic-containing media labour-intensive and time-consuming. Two common methods for incorporating plastics into the medium are emulsification and the incorporation of semi-water-soluble plastics. Emulsification involves dissolving plastics in an organic solvent such as dichloromethane, mixing with surfactants and growth medium, and evaporating the solvent to form small droplets of plastic in the medium. This method is difficult to reproduce, to maintain a stable dispersion and to exclude the use of potentially cytotoxic solvents or surfactants. The bulk film method is simpler, but offers limited surface area for microorganism/plastic interaction, reducing biodegradation efficiency and increasing test duration. In addition, in some cases microbial degradation of only plastic additives or low molecular weight oligomers is observed [12,14,15]. The most commonly used additives in plastic are plasticizers, flame retardants, antioxidants, acid scavengers, UV and thermal stabilizers, lubricants, pigments, antistatic agents, and slip agents [16]. Thus, such approach can sometimes give false positives or false negatives throughout the screening of plastic degrading microorganisms.

Weight loss measurement is another popular technique used to assess the biodegradation of plastics, but accurate cleaning of samples is crucial as the release of soluble and volatile contaminants can cause weight loss unrelated to microbial activity. Gel permeation chromatography (GPC) is commonly used to analyse the molecular weight (Mw) and number average molecular weight (Mn) of plastics after treatment [17]. This method is often combined with other techniques such as clear zone formation, scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), X-ray powder diffraction (XRD), differential scanning calorimetry (DSC), transmission electron microscopy (TEM) and scanning force microscopy (SFM) [18–20].

Techniques such as targeted metagenomics, stable-isotope probing as well as exoproteome analysis are used for screening plastic-degrading enzymes [21]. Detection of the degradation products by a colorimetric screening system [22], a high-throughput liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis method [23] or open-port sampling interface-mass spectrometry [24] have been applied for identification of the polymer depolymerizing enzymes, too. Selection of polymer-degrading enzymes based on homology to known enzymes is also common [25–27].

To intensify the biological processes of valorisation of polymers, the stable and robust biocatalysts are required. For instance, the most efficient PET-degrading biocatalysts reported to date are thermostable enzymes that exhibit optimal hydrolysis activity near the PET glass transition temperature (Tg ~65–80 °C) [25]. However, none effective high-throughput methods for screening of thermophilic or robust to inhibitors plastic degrading enzymes have been published to date.

This study proposes a simple, rapid and user-friendly screening system to identify plastic-degrading microbial cultures, recombinant enzymes from (meta)genomic libraries, cell lysates or purified enzyme libraries. Our study confirms that not all microorganisms that degrade polymeric compounds with low degree of polymerization (DP) can hydrolyse polyester films: approximately 30 % of the hits screened on polycaprolactone diol, DP= 530, (PCLD) were unable to hydrolyse polycaprolactone (PCL) films (DP=80,000). However, six microorganisms isolated on PCLD-agar hydrolysed PET films. In addition, a functional screening presented here allowed isolation of 27 polymeric films degrading esterases, which hydrolysed various biodegradable polyester films (PCL, PLA films or PHB/PHB copolymer film), moreover three of screened esterases have a potential to be novel PETases and one esterase hydrolysed all four tested polyester films. We have also implemented the screening method for identification of thermostable or organic solvent resistant enzymes within the stability limit of the film.

2. Materials and methods

2.1. Materials

Polycaprolactone (DP 80,000) and polycaprolactone diol (DP 530) were purchased from Sigma (Germany). PET biaxially oriented films 0.05 mm and 0.013 mm thickness, polylactic film biaxially oriented (0.05 mm) and polyhydroxybutyrate/polyhydroxyvalerate 8 % (0.01 mm) were purchased from Goodfellow (Sigma, Germany). Restriction endonucleases, Phusion DNA polymerase, aLICator™ LIC Cloning and Expression System Kit 3/1, PageRuler™ Prestained Protein Ladder were purchased from Thermo Fisher Scientific, Vilnius, Lithuania. Pierce™ Coomassie Plus (Bradford) Assay Reagent and His-Pur™ Ni-NTA spin columns were purchased from Thermo Fisher Scientific, Rockford, USA. Nutrient medium was purchased from Roth,

Germany. "ZR Soil Microbe DNA MidiPrep™", was purchased from Zymo Research, Freiburg, Germany. Oligonucleotides (Table S1) were purchased from Azenta, Germany. The plasmids pEE065 (Addgene plasmid # 176822; http://n2t.net/addgene:176822; RRID: Addgene_176822) and pEE075 (Addgene plasmid # 176832; http://n2t.net/addgene:176832; RRID: Addgene_176832) were a gift from Gregg Beckham [25].

2.2. DNA extraction and construction of the metagenomics and genomic libraries

Metagenomic libraries were constructed from soil and decayed wood samples DNA (Vilnius region, Lithuania) and pUC19 vector. The total DNA was extracted by using" ZR Soil Microbe DNA MidiPrep™" (Zymo Research, Freiburg, Germany). Purified DNA was partially digested with restriction endonuclease *Bam*HI after that DNA fragments were inserted into the pUC19 vector and used to transform *E.coli* DH5α competent cells by electroporation [28]. Genomic libraries were constructed analogically as metagenomic by using genomic DNA of individual microorganisms.

2.3. Screening of microorganisms on PCLD agar

PCLD is a thick liquid that mixes easily with LB agar to produce turbid screening plates. Various environmental soil samples were collected, samples were suspended in 0.9 % NaCl and incubated with shaking for 1 h, then 50 µL aliquots were plated on the LB agar containing 1 % polycaprolactone diol (PCLD). The plates were incubated at 30 °C for 1–2 days and were visually inspected for colonies that were able to produce clear hydrolysis zones, positive hits were isolated by repeated streaking.

2.4. DNA sequencing and gene annotation

Nucleotide sequences of genes were determined at Azenta (Germany) and using the following sequencing primers: M13F-pUC (5′-GTTTTCCCAGTCACGAC-3′), M13R-pUC (5′-CAGGAAACAGCTATGAC-3′), T7 Promoter (5´- TAATACGACTCACTATAGGG-3´) and LIC Reverse Sequencing primer, 24-mer (5′-GAGCGGATAACAATTTCACACAGG-3′). The 16S rRNA gene sequences were determined as described previously (Godon,et.al., 1997) [29] using the primers Woo1 (5′-AGAGTTT GATCMTGGCTC-3′) and Woo2 (5′-GNTACCTTGTTACGACTT-3′). The 18S rRNA gene sequences were determined using EukA (5′-AACCTG GTTGATCCTGCCAGT-3′) and EukB (5′-GATCCWTCTGCAGGTTCA CCTAC-3′) primers [30].

Plasmids with long DNA inserts (>3 kb) were sequenced using Oxford Nanopore platform on R10.4.1 flow cell using Kit 14 chemistry by Seqvision (Lithuania). ORFs were analysed by using Proksee [31] and Benchling tools, and homology search was conducted using the Blast server [32]. Phylogenetic analysis was conducted using the Maximum Likelihood Tree routine of MEGA 11 software [33]. The sequence alignment was performed using ClustalW in MEGA 11.

2.5. PCL film formation from PCL pellets

PCL (DP 80,000) pellets were dissolved in chloroform to obtain 5 % w/v solution. The solution was mixed at room temperature until PCL pellets completely dissolved (2–3 h at 22–23 °C) and a homogenous solution was obtained. Then, 4 mL of such a mixture was transferred to a glass 9 cm diameter Petri dish (~0.06 mL/cm^2) and left to dry overnight [27]. Intact films (~0.03–0.035 mm thick) were used for further experiments.

2.6. Hydrolysis of the PCL (DP 80,000), PLA, PHB/PHV and PET films

Microorganisms were cultivated in LB liquid medium up to exponential grow phase and then transferred to the ND9 vials (clear glass, 1.5 mL, screw neck, ROTH) or to the 4 mL glass vials. Screw caps with bore holes were used for sealing polyester films. Vials were placed upside down in the multipurpose tubes (PS, transparent, with conical base). The multiple wells device was prepared by using PCR plates and the plastic films were anchored by PCR caps with holes. The plates with samples were stacked with other PCR plates upside down. For holding the plates, a plastic tubes holder was used (Fig. S1). The samples were incubated in the tubes with plastic film membrane for an appropriate time. The liquid entry from the top well to the bottom well after short centrifugation indicated an enzymatic activity towards the polymer film. The 0.5 mM phenol red indicator (from 10 mM stock in water) was added to the liquid to facilitate visual assessment and to monitor the change in pH [28].

An ethanol resistance test was performed in 60 µl of reaction mixtures containing 50 mM potassium phosphate buffer (pH 7.5), 45 µg cutinase from *S. scabiei* 87.22 and different concentrations of ethanol (0 %, 30 % or 50 %) were added to tubes sealed with PCL film (DP 80,000). All reactions were performed in triplicate. Samples were incubated at room temperature (23 °C) for up to 2 hours. The tubes were centrifuged at low speed after 10, 15, 20, 30, 60 and 120 minutes of incubation.

2.7. Cloning, heterologous expression and purification of esterases

Esterases encoding genes were amplified with Phusion DNA polymerase using primers listed in Table S1. The cloning and overexpression procedures and purification of enzymes were performed as described previously [28].

2.8. An in vitro transcription-translation

The transcription-translation reaction was performed by using PURExpress® In Vitro Protein Synthesis Kit (NEB, USA). The reaction mix contained solution A, 10 µL, Solution B, 7.5 µL, nuclease-free H_2O, 3.5 µL and template DNA (plasmid with *Thermobifida fusca* gene, Addgene plasmid # 176832) 4 µl (50 ng/µL). The reaction mixtures were incubated 2 h at 37°C.

2.9. Hydrolysis of terephthalate esters

The PET films hydrolysis was analysed by incubating the enzymes (~100 µg) with ~10 mg of PET films in 50 mM potassium phosphate buffer, pH 7.5 at 37 °C for 120 h. The activity of hydrolases towards PET monomers: dimethyl terephthalate (DMT), bis(2-hydroxyethyl) terephthalate (BHET) and monomethyl terephthalate (MMT) was analysed by incubating the cells with overexpressed enzyme in 50 mM potassium phosphate buffer, pH 7.5, containing 10 mM substrate (from 100 mM stock in DMSO) at 37 °C for 3–18 h. *E. coli* cells cultures carrying appropriate hydrolase genes were used for the analysis. In general, 5 µL of cultures were added into 20 µL of reaction volume. The hydrolytic activity was monitored by thin-layer chromatography (TLC) and high-performance liquid chromatography-mass spectrometry (HPLC-MS). TLC was conducted on the Merck silica gel 60 F254 plates, using the chloroform and methanol (5:1) mixture of solvents. The samples were exposing to UV light. HPLC-MS analysis were performed using a high-performance liquid chromatography system (Shimadzu, Japan) and a mass spectrometer (LCMS-2020, Shimadzu, Japan) equipped with an ESI source. The chromatographic separation was conducted using a YMC Pack Pro C18 column, 3 × 150 mm (YMC, Japan) at 40 °C and a mobile phase that consisted of 0.1 % formic acid water solution (solvent A), and acetonitrile (solvent B) delivered in gradient elution mode at a flow rate of 0.45 mL/min. The elution program was used for DMT and MMT analysis as follows: isocratic 5 % B for 1 min, from 5 % to 95 % B over 5 min, isocratic 95 % B for 2 min, from 95 % to 5 % B over 1 min, isocratic 5 % B for 4 min. For BHET compound and PET hydrolysis products

the elution program was: isocratic 15 % B for 0.5 min, from 15 % to 30 % B over 4.5 min, isocratic 30 % B for 2 min, from 30 % to 95 % B over 2 min, isocratic 95 % B for 3 min, from 95 % to 15 % B over 2 min, isocratic 15 % B for 4 min. Mass scans were measured from *m/z* 50 up to *m/z* 700, at 350 °C interface temperature, 250 °C DL temperature, ± 4500 V interface voltage, neutral DL/Qarray, using N_2 as nebulizing and drying gas. Mass spectrometry data was acquired in both the positive and negative ionization modes. The data was analyzed using the LabSolutions LCMS software.

3. Results and discussion

3.1. Proof of principal of the screening method

The proof of principal of the proposed method is presented in the Fig. 1. Initially, two sources of enzymes (environmental microorganisms, and recombinant *E. coli* cells transformed with metagenomic DNA libraries from various soil samples) were chosen. In short, PCLD (DP 530) was used as a substrate for the pre-screening of esterases-producing colonies on agar plates. The positive hits were successfully identified by the formation of clear zones. Then, individual halo-forming colonies were cultured in liquid LB medium, transferred into wells and sealed with a polymer film, which has to be watertight. PCL (DP 80,000), PLA, PHB/PHV and biaxially oriented high crystallinity (~30 %) PET films [16] were tested as substrates. The sealed samples were incubated for an appropriate period of time, and the movement of liquid from the upper to the lower well due to a damage of the plastic film indicated an enzymatic degradation of polymer. Three known enzymes with the ability to degrade plastics were used as a positive control. Those included the recombinant cutinase from *Streptomyces scabiei* 87.22 [34], and two variants of cutinases from *Thermobifida fusca* (Addgene plasmids # 176832 and # 176822) [25]. Cutinase from *S. scabiei* 87.22 exhibit 100 % similarity to cutinase/suberinase Sub1 from *S. scabiei* EF-35 which is able to act towards PET [35,36].

3.2. Screening of polycaprolactone diol-, PCL- and PET film-degrading microorganisms

Various environmental samples (soil, decaying wood) were collected and plated on LB agar containing 1 % PCLD. Microorganisms with activity towards PCLD were detected by the formation of clear halos. These were streaked repeatedly on selective LB agar containing 1 % PCLD until homogeneous cultures were obtained. In total, 103 different positive hits were isolated. Notably, the PCLD12 culture formed an opaque zone instead of a clear halo. Then all 103 different positive hits were identified by 16S or 18S rRNA gene sequencing (Fig. 2, Table S2). Among isolates, Gram-positive bacteria such as *Bacillus* spp. (33 hits, 32 % from all isolated samples) and *Paenibacillus* spp. (12 hits, 12 %), and Gram-negative bacteria such as *Pseudomonas* sp. (30 hits, 29 %) were predominant (Fig. 2). Other positive hits (26 %) included *Seratia*, *Moraxella*, *Gibbsiella*, *Micrococcus*, *Staphylococcus*, *Acinetobacter*, *Xenophilus*, *Vibrio*, *Pseudorhodoferax*, *Aeromonas*, *Cellulomonas*, *Lysinibacillus*, *Burkholderia*, *Priestia* and *Janibacter* species (Fig. 2). In addition, one of the hits, PCLD155, was identified as fungus belonging to *Cryptococcus* genus. This step effectively screened a wide range of microorganisms capable of degrading PCLD, opening up the potential for further isolation of efficient biocatalysts for the degradation of plastic films.

In the second step, microorganisms that exhibited hydrolytic activity towards PCLD were tested on PCL (DP 80,000), PLA, PHB/PHV and PET films. Hence, the selected positive hits from the PCLD agar screening were cultivated in liquid LB medium for 5–24 hours until the cultures reached an optical density (OD_{600}) of 0.5–1.5. Then 200–500 µL of this liquid culture was added to tubes or wells and sealed with polymeric film (Fig. S1). Samples were incubated at 30°C for up to 30 days, with daily checks for hydrolytic activity, indicated by liquid entering the bottom well after low speed and short time (2–5 seconds) centrifugation.

The results showed that 68 out of 103 microorganisms selected on PCLD agar (66 % of the samples tested) exhibited hydrolytic activity towards polymeric PCL films. The distribution of microorganism species with hydrolytic activity towards PCL films was as follows: 35 % of *Pseudomonas* spp., 88 % of *Bacillus* spp., 92 % of *Paenibacillus* sp. and

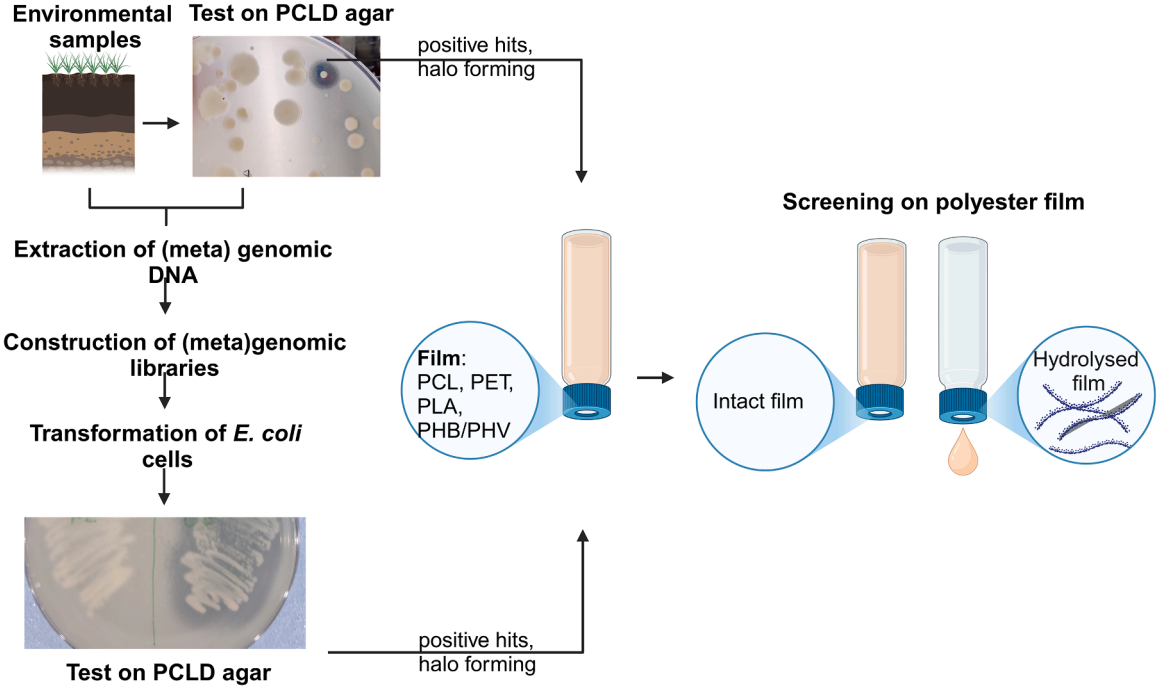

Fig. 1. Proof of principle of the screening method for plastics degrading microorganisms and enzymes. First, the microorganisms/clones were tested on LB agar containing 1 % PCLD and the positive hits were selected by the halo zone formation. In a second step, the ability of positive hits to hydrolyse the PCL (DP 80, 000), PLA, PHB/PHV and PET films was evaluated. Created in https://BioRender.com.

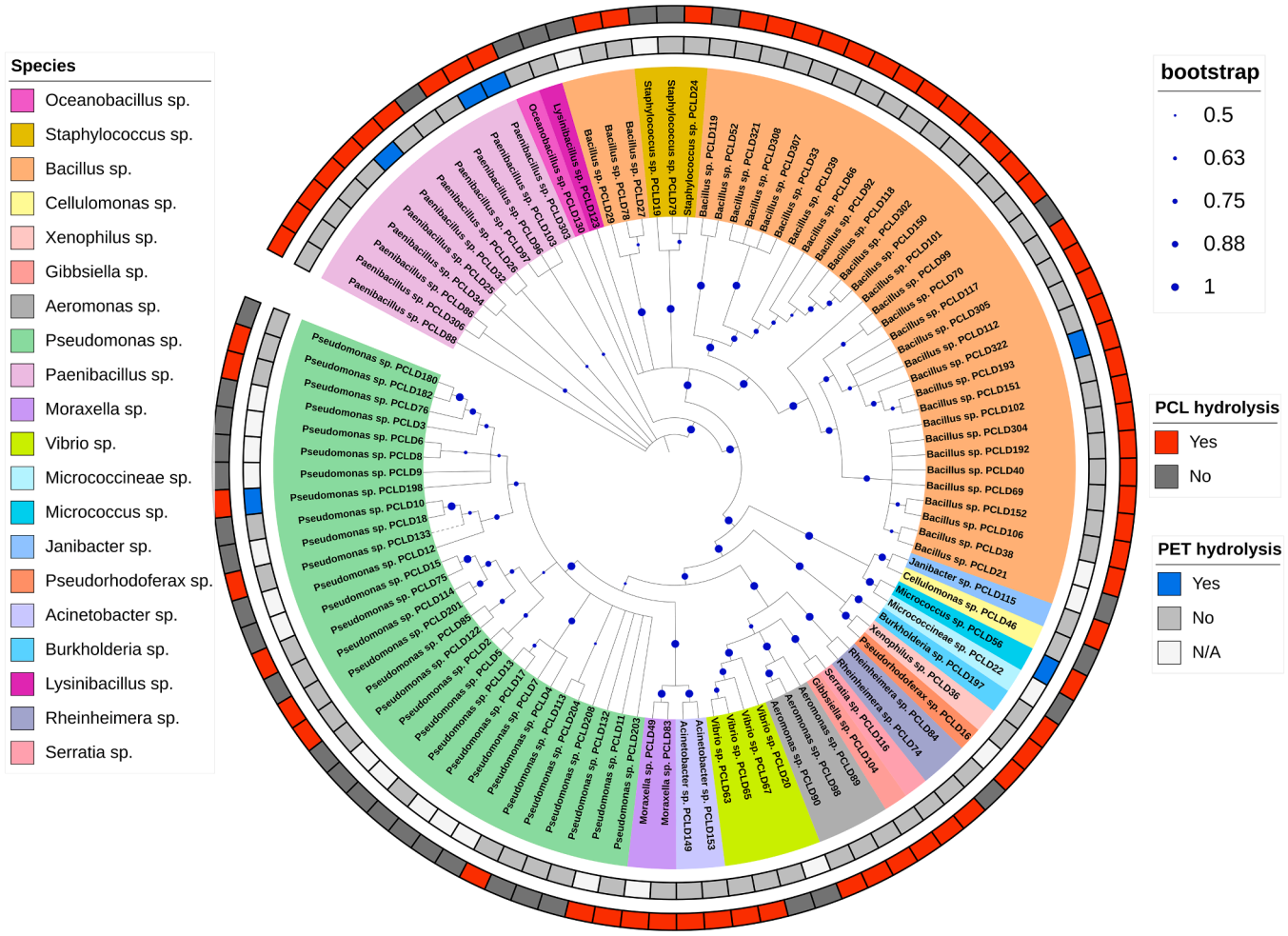

Fig. 2. The distribution of the isolated microorganisms that exhibited hydrolytic activity towards PCLD (coloured according to genus). The red and blue bars represent the species with detected PCL or PET film-degrading activity, respectively. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The bootstrap consensus tree inferred from 50 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. Evolutionary analyses were conducted in MEGA11. The tree was created by using the Interactive Tree of Life tool (iTOL) [37]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

63 % of other microorganisms (Fig. 2). Most of the hydrolytic activity was detected within one week of incubation at 30 °C. *Paenibacillus* sp. showed activity within 1–2 days, *Bacillus* sp. within 1–8 days and *Pseudomonas* sp. within 1–2 weeks. An incubation over two weeks did not give positive results. However, a significantly extended incubation up to 3–8 weeks was required for detection of the PET film hydrolysis. Six cultures from the 103 samples tested showed activity against 0.013 mm PET film, representing less than 6 % of the positive hits screened on PCLD agar. The active cultures were PCLD56 (*Micrococcus* sp.), PCLD198 (*Pseudomonas* sp.), PCLD32, PCLD103, PCLD303 (*Paenibacillus* sp.) and PCLD193 (*Bacillus* sp.).

Published studies have frequently identified various micromycetes and bacterial microorganisms involved in plastic degradation. Commonly detected species include *Fusarium*, *Aspergillus*, *Trichoderma*, *Bacillus*, *Clostridium*, *Amycolatopsis*, *Pseudomonas*, *Micrococcus*, *Lysinibacillus*, *Rhodococcus*, *Serratia* and *Prestia* species [38,39]. In addition, Pang et al. [13] identified 184 fungal and 55 bacterial cultures in plastic waste samples that were capable of degrading PCL. The predominant bacteria in their study were *Streptomyces*, *Jonesia* cf. *quinghaiensis* and *Pseudomonas* sp. Their selection was based on clear zone formation on agar plates with plastic powders incubated at 28 °C for 10–14 days. Also, other authors Danso et al. [11] clustered 504 enzyme candidates based on amino acid similarity and found that PET hydrolases are mainly found in the phyla Actinobacteria, Proteobacteria and Bacteroidetes. The microbial species detected in our study are consistent with those reported in the literature. These results indicate that the developed screening method is effective in identifying diverse microorganisms capable of hydrolysing polymeric films, with different species showing different levels of activity and incubation times required for both PCL and PET degradation.

3.3. Screening of the recombinant enzymes active towards films

To identify the enzymes responsible for polymeric film hydrolysis, genomic libraries prepared from several microorganisms that tested positive on PCLD agar and metagenomic libraries created from environmental samples (Table S3). In total, 14 genomic and 7 metagenomic libraries were used to transform *E. coli* DH5α strain cells (Table S3) and screened using our method. Clones with PCLD hydrolytic activity were first detected on LB agar containing 1 % PCLD. Fourteen clones with PCLD hydrolytic activity were identified from the genomic libraries and twelve from the metagenomic libraries. Notably, some clones such as PCLD8, PCLD9, PCLD420CE1 and PCLD514D formed opaque zones instead of clear halos. Plasmid DNA from these positive clones was isolated and sequenced. Sequence analysis of the open reading frames (ORFs) revealed that clones PCLDk103, PCLDk198, and PCLD7 each

contained two different hydrolase genes, while other clones contained one hydrolase gene each. Comparison of these sequences with proteins in the GenBank database showed high sequence identity (93–100 %) for the genomic library clones (Table 1), but lower identity (48–92 %) for the metagenomic library clones (Table 2). Both groups had significantly lower sequence similarity to proteins with solved 3D structures in the Protein Data Bank (Table S4).

The majority of positive hits belonged to the AB hydrolase superfamily (SSF53474), with 12 genomic hydrolases (Table 1) and 7 metagenomic hits (Table 2). In addition, PCLDk17A and both hydrolases of PCLDk198 contained the C-terminal domain of serralysins (SSF51120), known as the "parallel β-roll", which is required for secretion. This domain is organised as a 21-strand β-sandwich with tandem repeats of the GGXGXDX(L/I/FV)X motif for binding calcium ions. Each of the identified hydrolases contained two such repeats: PCLD17A contains GGKGSDYLE (382–390 a.a.) and GGAGNDTFVG (520–529 a.a.), PCLDk198–1 — GGAGNDFLE (329–337 a.a.) and GGAGNDVMT (474–482 a.a), and PCLDk198–2 — GGSGNDYLE (411–419 a.a.) and GGSGNDVFVG (549–557 a.a.). The hydrolases from clones PCLD16C and PCLD7–2 were homologous to members of the SGNH/GDSL superfamily (SSF52266), while PCLDk83/514D/6/600 were similar to the *β*-lactamase/transpeptidase-like superfamily (SSF56601) proteins. One metagenomic hydrolase (PCLD514B) was identified as a patatin-like phospholipase (SSF52151). The putative hydrolases from clones PCLDk103_PE and PCLD3E2 could not be associated with any superfamily, but were assigned to the Notum carboxylesterase family. Sequence alignment of all identified hydrolases showed that our screening method yielded a large diversity of PCLD hydrolases with no common conserved region (Fig. S2).

To confirm hydrolytic activity, 22 intact hydrolase genes (excluding PCLDk39, k102, k106, k193, k198 and PCLD600) were PCR amplified with specific primers (Table S1) and cloned into expression vectors. *E. coli* HMS174 (DE3) or KRX strains were transformed with these recombinant plasmids and reseeded on PCLD agar plates to determine hydrolytic activity. All but one of the cloned hydrolases formed clear halos on PCLD plates, with the PCLDk103_CE hydrolase identified as responsible for the hydrolysis from the original PCLDk103 clone, which contained two hydrolase genes in its DNA fragment.

Activity towards PCL film was detected with genomic clones prepared from microorganisms with PCL film hydrolytic activity (Table 1). However, clone PCLDk29–3 and PCLDk103 did not hydrolyse PCL film, unlike the microorganism from which the genomic library was prepared. An exception was observed for clone PCLDk52 and PCLDk83: hydrolysis of the PCL film was detected in samples with the expression plasmids but not with the initial clone in pUC19 vector, suggesting that expression intensity may influence the detection of hydrolysis. In total, 12 genomic and 9 metagenomic esterases showed activity towards PCL film (Table 2). However, it remains unknown which esterase in the PCLD198 clone hydrolysed PCL as they were not separately cloned into the inducible vector.

No hydrolases active on PET film were detected from the genomic library clones. However, clones PCLDk52 and PCLDk149 hydrolysed PET monomers, specifically the terephthalate esters *bis*(2-hydroxyethyl) terephthalate (BHET), monomethyl terephthalate (MMT) and dimethyl terephthalate (DMT) (Table 1, Fig. S3-S5). In contrast, the metagenomic hydrolases PCLD8, PCLD514B and PCLD514D exhibited slow activity towards PET film, with hydrolysis observed after 3–4 weeks of incubation (Table 2). The HPLC analysis of PET film degradation reaction mixtures reveal the presence of various compounds including terephthalic acid released during incubation with the enzymes PCLD8, PCLD514B, and PCLD514D (Fig. S6–8). These hydrolases also hydrolysed the PET monomers BHET, DMT and MMT. Clones PCLD1E1, PCLD5BE1, PCLD514B and PCLD420CE1 showed activity towards BHET and DMT but did not hydrolyse MMT (Table 2, Fig. S4–S5). The product of BHET hydrolysis catalysed by PCLD8 and PCLDk52 was terephthalic acid (TPA), whereas PCLD1E1, PCLD5BE1, PCLD149, PCLD514B, PCLD514D and PCLD420CE1 hydrolysed BHET to mono(2-hydroxyethyl)terephthalate (MHET). TPA was also detected in the

Table 1
The list of isolated genomic hydrolases and their properties. The number and colour indicate the incubation time, after which a hydrolysis was detected.

0 no activity; 1 after 1-2 month; 2 after 14 day; 3 after 5-8 day; 4 after 72h; 5 after 48 h; 6 up to 24 h; 7 within 3-18h; N/A- not analysed

Clone	Genbank Acc.No.	Closest homologue [genus, species], GenBank accession No.	Identity, %	*In vivo* hydrolytic activity							*In vitro* hydrolytic activity	
				Initial clone		Over-expressed		terephthalate esters				
				PCL	PET	PCL	PET	BHET	MMT	DMT	PLA	PHB/PHV
PCLDk16C	XBM56763	SGNH/GDSL hydrolase [*Variovorax boronicumulans*], WP_145551826.1	93	N/A	N/A	5	0	N/A	N/A	N/A	N/A	N/A
PCLDk17A	XBM56782	Polyurethanase [*Pseudomonas viridiflava*], WP_122566918.1	100	N/A	N/A	4	0	0	0	0	N/A	N/A
PCLDk29-3	XAM23852	AB hydrolase [*Priestia megaterium*], WP_074895752.1	99	N/A	N/A	0	0	N/A	N/A	N/A	N/A	N/A
PCLDk32	XBM56782	AB fold hydrolase [*Paenibacillus* sp. FSL H7-689], WP_036608131.1	98	N/A	N/A	6	0	0	0	0	N/A	N/A
PCLDk33-10	XBM56787	Carboxylesterase/lipase [*Bacillus amyloliquefaciens*], WP_253600429.1	99	3	0	2	0	0	0	0	N/A	N/A
PCLDk39	XBM56792	MULTISPECIES: lipase LipA [*Bacillales*], WP_003246250.1	100	6	0	N/A	N/A	0	0	0	N/A	N/A
PCLDk52	XBM56795	Carboxylesterase/lipase family protein [*Bacillus*], WP_003179394.1	99	0	0	5	0	7	7	7	0	2
PCLDk83	XBM56800	Serine hydrolase [*Moraxella osloensis*], WP_282849630.1	100	0	N/A	2	0	0	0	0	0	2
PCLDk102	XBM56805	AB hydrolase [*Bacillus altitudinis*], WP_096881477.1	100	3	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
PCLDk103_PE	XBM56810	Pectinacetylesterase [*Paenibacillus amylolyticus*], WP_249912589.1	100	0	0	0	0	0	0	0	0	N/A
PCLDk103_CE	XBM56808	Carboxylesterase family protein [*Paenibacillus amylolyticus*], MCL6662027.1	99			0	0	0	0	0	0	N/A
PCLDk106	XBM56812	AB hydrolase [*Bacillus altitudinis*], WP_249855746.1	100	6	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
PCLDk149	XBM56815	AB hydrolase [*Acinetobacter johnsonii*], WP_184118327.1	98	3	0	2	0	7	7	7	2	N/A
PCLDk193	XBM56817	MULTISPECIES: triacylglycerol lipase [*Bacillus*], WP_024424844.1	100	2	0	N/A	N/A	0	0	0	2	N/A
PCLDk198-1	XBM56822	Polyurethanase [*Pseudomonas* sp. PB103], WP_158829089.1	99	3	0	N/A	N/A	0	0	0	N/A	N/A
PCLDk198-2	XBM56823	Polyurethanase [*Pseudomonas* sp. PB103], WP_158829091.1	99									

Table 2
The list of isolated metagenomic hydrolases and their properties. The number and colour indicate the incubation time, after which a hydrolysis was detected.

hydrolysis was detected:

0	no activity	2	after 14 day	4	after 72h	6	up to 24 h	N/A- not analysed
1	after 1-2 month	3	after 5-8 day	5	after 48 h	7	within 3-18h	

Clone	Genbank Acc.No.	Closest homologue [genus, species], GenBank accession No.	Identity, %	*In vivo* hydrolytic activity							*In vitro* hydrolytic activity	
				initial clone		Over-expressed		terephthalate esters				
				PCL	PET	PCL	PET	BHET	MMT	DMT	PLA	PHB/PHV
PCLD1E1	XAM23849	AB hydrolase [*Gammaproteobacteria* bacterium] MDD9964011.1	61	N/A	N/A	5	0	7	0	7	0	3
PCLD3E2	XAM23850	Pectin acetylesterase [*Comamonadaceae* G21597-S1] MCZ4315433.1	79	N/A	N/A	0	0	N/A	N/A	N/A	N/A	N/A
PCLD5BE1	XBM56719	AB hydrolase [*Mycobacterium* sp. KBS0706]	67	N/A	N/A	2	0	7	0	7	N/A	N/A
PCLD6	XBM56723	AB hydrolase [*Deltaproteobacteria* bacterium] MBV8136698.1	66	N/A	N/A	2	0	N/A	N/A	N/A	N/A	N/A
PCLD7-1	XBM56726	AB hydrolase [Candidatus *Binataceae* bacterium], ID: MBF6560469.1	80	N/A	N/A	2	0	N/A	N/A	N/A	3	N/A
PCLD7-2	XBM56727	SGNH/GDSL hydrolase [*Deltaproteobacteria* bacterium], MBI3758430.1	70	N/A	N/A	5	0	N/A	N/A	N/A	0	N/A
PCLD8	XBM56730	AB hydrolase [*Planctomycetia* bacterium], MBI2827008.1	55	N/A	N/A	3	1	7	7	7	4	3
PCLD9	XBM56737	Serine hydrolase [*Sphingomonas hylomeconis*] WP_261292862.1	92	N/A	N/A	6	0	N/A	N/A	N/A	N/A	N/A
PCLD514A	XBM56740	EST1 [uncultured microorganism], ADR31550.1	60	N/A	N/A	0	0	N/A	N/A	N/A	N/A	N/A
PCLD514B	XBM56750	Patatin-like phospholipase [*Myxococcales bacterium*], MDD9932651.1	48	N/A	N/A	0	1	7	0	7	0	0
PCLD514D	XBM56759	*β* -lactamase family protein [Candidatus *Hydrogenedentes*], MCH7910645.1	59	N/A	N/A	3	1	7	7	7	0	3
PCLD_420CE1	XAM23851	AB hydrolase [*Vicinamibacterales bacterium*], HZB25512.1	61	N/A	N/A	0	0	7	0	7	N/A	N/A
PCLD600	XBM56761	*β*-lactamase family protein [*Pseudomonadota*], MDQ2642018.1	81	5	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A

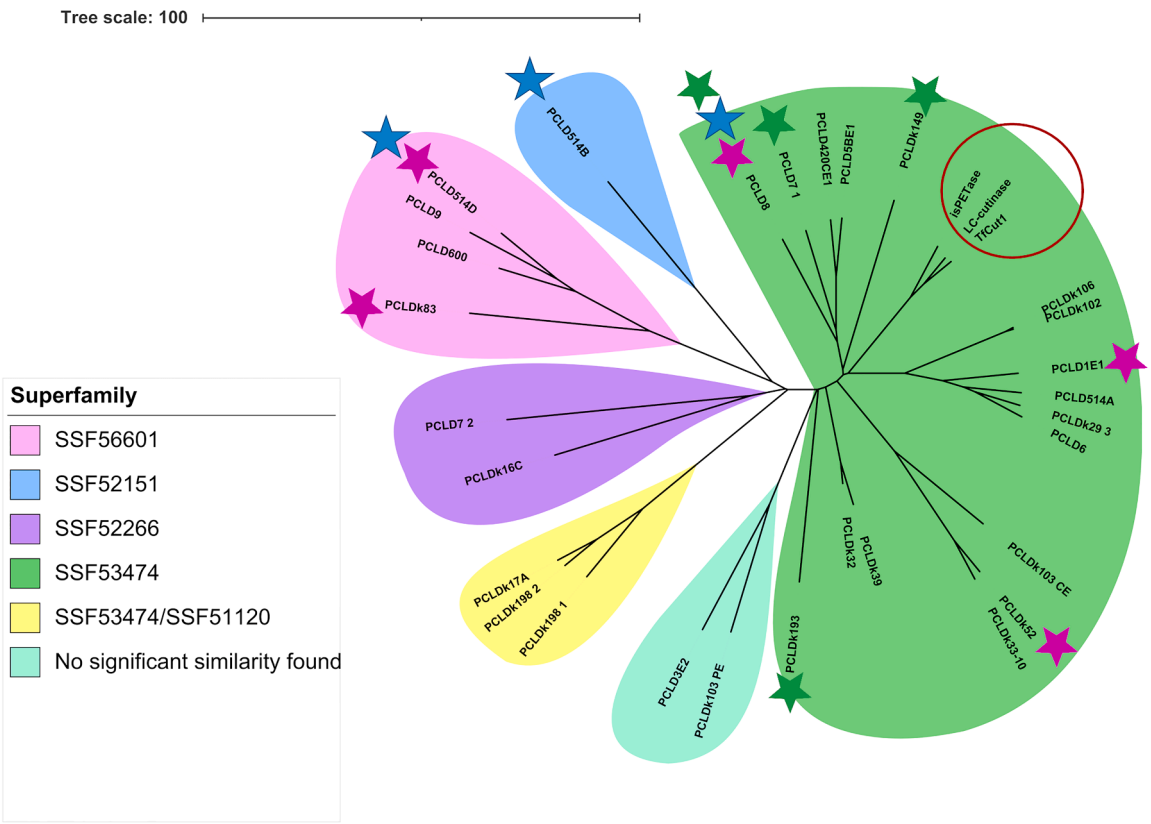

Fig. 3. Phylogenetic tree of the selected esterases by 3D structures. The structure models of PCLD esterases were performed by using ColabFold. IsPETase (PDB:5YFE), LC-cutinase (PDB:4EB0) TfCut1(PDB:5ZOA) crystal structures were used for comparison. The phylogenetic analysis was conducted using the Dali server for comparing protein structures in 3D. The active esterases towards PET films are marked by blue, PLA – green and PHB/PHV – pink stars. The published PET-hydrolases are labelled by the red circle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

DMT hydrolysis reaction with PCLDk52, PCLD8, PCLD514D and PCLD149 esterases. DMT is a component widely used in the manufacture of industrial plastics. An environmental fate of DMT is of concern due to its global use and the severe toxicity [40]. Hence, novel variants of DMT-hydrolysing enzymes is a welcomed addition to this enlarging of a collection of industrially-relevant but still understudied esterases [40–42].

Studies with other bio-plastics PLA and PHB/PHV have shown that PLA is degraded by far fewer enzymes than PCL. Hence, hydrolytic activity of 14 purified polyesterases were tested towards PLA films and activity of six enzymes towards PHB/PHV film. In the case four enzymes (PCLD8, PCLD7_1, PCLD149 and PCLD193) hydrolysed PLA polymer film and five degraded PHB/PHV film (PCLD1E1, PCLD8, PCLDk52, PCLDk83 and PCLD514D) (Tables 1 and 2). The hydrolysis was detected within 5 days of incubation at 30 °C. One polyesterase (PCLD8) was active towards all four tested polyester film (Fig. 3, Fig. S9, Table 2).

The identified hydrolases are highly diverse (Fig. S2) with different secretion signal peptides and extra domains. To compare these enzymes, a phylogenetic tree was constructed using only catalytic domains, identified using the InterPro database [43] (Fig. S9). However, the phylogenetic analysis of the catalytic domains of the esterases did not show any clustering based on their hydrolytic activity towards different films. The structural models of the esterases were then compared. The 3D structural models were generated using ColabFold [44] software, and phylogenetic analysis 3D models was performed using the Dali server [45]. Well-studied PET-hydrolysing enzymes such as the leaf-branch compost metagenome LC-cutinase (LCC) [46], hydrolases from *T. fusca* (TfCut) [47] and *I. sakaiensis* (isPETase) [48] were included into phylogenetic 3D analysis. The isolated putative PET hydrolases PCLD8, PCLD514B and PCLD514D clustered on different branches of the phylogenetic tree and showed low identity with the known ones (Fig. 3).

Current research often focuses on searching for homologues of isPETase, TfCut cutinase or LCC cutinase and evolving these homologues [11,21,25,49]. This strategy may reduce enzyme diversity and potentially overlook enzymes of different origin. In contrast, the method we developed identified potential targets from other families, highlighting the need for broader screening approaches to uncover a wider variety of active hydrolases.

3.4. Evaluation of polymer degradation by using the selected purified recombinant enzymes

The efficiency of the proposed method was also evaluated using known polymeric film hydrolysing enzymes: the recombinant cutinase from *S. scabiei* 87.22 [34–36] and *T. fusca* cutinases variants (Addgene plasmid # 176822 and # 176832) [25]. First, the activity towards PCLD, PCL and PET films was tested using *E. coli* cultures containing cutinases genes. All three clones hydrolysed PCLD in agar plates. The hydrolytic activity towards PCL film of cell cultures with *S. scabiei* cutinase 87.22 and *T. fusca* cutinases (Addgene plasmid # 176822 and # 176832) was detected after 2 days of incubation at 23°C and 55 °C, respectively.

Also used to evaluate the efficacy of our plastic film degradation assay using purified enzymes. Different amounts of the enzyme (0.9 μg, 4.5 μg, 9 μg or 45 μg) were added to tubes and sealed with PCL film, and all reactions were tested in triplicate. Samples were incubated at room temperature (23°C) for up to 18 hours. Tubes were centrifuged at low speed after various incubation times (10, 15, 20, 25, 30, 40, 50, 60 minutes and 18 hours). Activity was detected after 15 minutes in the sample containing 45 μg of cutinase, after 25 minutes in the sample containing 9 μg of cutinase, after 50 minutes in the sample containing 4.5 μg of cutinase and after 18 hours in the sample containing 0.9 μg of cutinase.

Subsequent testing of the purified *S. scabiei* 87.22, *T. fusca* cutinases (Addgene plasmid # 176822 and176832) on polymeric PET film showed no positive results, except for pH changes indicating slow hydrolytic activity in reactions with *T. fusca* cutinase (Addgene plasmid #76832). These reactions were incubated in glass tubes with sealed PET films for up to 1 month at 30°C (*S. scabiei* cutinase) or 60°C (Addgene plasmid # 176822 and176832) in 0.3 mL reaction mixtures containing 10 mM potassium phosphate buffer (pH 7.5), ~0.2 mg enzymes and 1 mM phenol red. Previously published data demonstrated the hydrolytic activity of *T. fusca* cutinase (Addgene plasmid #76832) by detecting aromatic compounds after 48–72 hours of incubation with amorphous PET film and powder [25].

In vitro transcription-translation is becoming an increasingly popular method for various high-throughput screening approaches to design functional proteins [50,51]. Hence, we assessed the applicability of our screening method for polyester film-degrading enzymes *in vitro T. fusca* gene (Addgene plasmid # 176832) in a plasmid. For this, 25 μl of transcription-translation reaction mixtures were added to tubes sealed with PCL film. All reactions were performed in triplicate. Samples were incubated at room temperature (23°C) for up to 48 hours. After the incubation period, the tubes were centrifuged at low speed. The degradation activity of the PCL film was detected after 48 hours of incubation, demonstrating the effectiveness of the method for assaying active enzymes obtained by in vitro transcription-translation.

Another advantage of the proposed method is its applicability to studies of enzyme stability in the presence of additives such as organic solvents. For this purpose, an ethanol resistance test was performed in reaction mixtures containing 50 mM potassium phosphate buffer (pH 7.5), 45 μg cutinase from *S. scabiei* 87.22 and different concentrations of ethanol (0 %, 30 % or 50 %). The hydrolytic activity towards PCL film (DP 80,000) was detected after 15 minutes of incubation in the sample without ethanol, after 20 minutes in the sample with 30 % ethanol and after 120 minutes in the sample with 50 % ethanol. This shows that the method can be effectively used to evaluate enzyme stability in the presence of various additives.

In summary, the proposed method can be successfully applied for screening hydro-lytic enzymes active towards various polyester films. As previous studies have shown, PCL polymer is one of the easily biodegradable plastics and is hydrolysed by a variety of esterases, cutinases, PETases and other plastic degrading hy-drolases [9,52]. Therefore, pre-screening on soluble PCLD is a convenient step to improve the identification of enzymes active on different polymers and to reduce the number of clones to be further tested on the appropriate polymeric films. To further improve the yield of the desired en-zymes, different substrates can be used for the pre-enrichment step. An initial screening on oligomers or low DP polymers, PLA emulsions, BHET or, for example, commercially available polylactide-*block*-poly(ethylene glycol)-*block*-polylactides as well as PET-*block*-polymers of varying crystallinity hence containing polyethylene chains to improve solubility/emulsification and prepared as described previously [53,54] is worth of considering. Moreover, a pre-incubation in the presence of the target polymer containing different es-ters, for example, a copolymer of polyethylene terephthalate and polycaprolactone [55] could be considered for future studies. Moreover, a pre-incubation in the presence of the target polymer containing different esters, for example, a copolymer of polyethylene terephthalate and polycaprolactone might be consider for the future studies.

4. Conclusion

The method developed in this study allows the screening of polymeric film-degrading enzymes produced by native and recombinant microorganisms. The microorganism lysates and purified proteins can *in vitro* transcription-translation can be carried out. In principle, the proposed technique can be further extended to any film-forming polymer, a wide range of temperature modes, a wide range of media, buffers and the presence of various additives including organic solvents and other inhibitory compounds, facilitating the discovery or engineering of novel robust polymer-degrading enzymes.

Environmental implication

Synthetic polymers are hazardous as they accumulate in the environment, causing harm to various ecosystems as well as to human body. Enzymatic degradation has been shown to be a proper alternative to conventional waste recycling strategies. By efficiently identifying of enzymes capable of degrading polymer films, without the need for sophisticated equipment and techniques, this work proposes a more effective alternative for screening the desired biocatalyst applicable to polymer-waste reduction.

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CRediT authorship contribution statement

Rita Meškienė: Investigation. **Justas Vaitekūnas:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Conceptualization. **Renata Gasparavičiūtė:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization. **Rolandas Meškys:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Renata Gudiukaitė:** Writing – review & editing, Resources. **Povilas Kruopis:** Investigation. **Urtė Valantinaitė:** Investigation. **Nina Urbelienė:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Chat GPT/4 in order to language editing. After using this tool, the authors reviewed and edited the content as needed and takes full responsibility for the content of the published article.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nina Urbeliene, Renata Gasparaviciute, Renata Gudiukaite and Rolandas Meskys has patent #EP23187384.5 pending to Vilnius University. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2025.137177.

Data availability

All data needed to evaluate the conclusions in the paper a represent in the paper and/or the Supplementary Materials. The list of microorganisms screened on PCLD and their assigned GenBank accession numbers by 16S or 18S sequences are listed in the table S2 and selected metagenomic esterase GenBank accession numbers — in the Table 1 and 2. The materials can be provided by the Department of Molecular Microbiology and Biotechnology, Institute of Biochemistry, Life Sciences Center, Vilnius University pending scientific review and a completed material transfer agreement. Requests for the materials should be submitted to N.U. (nina.urbeliene@bchi.vu.lt)..

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