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Brief Report

Agar plate-based screening methods for the identification of polyester hydrolysis by *Pseudomonas* species

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Summary

Hydrolases acting on polyesters like cutin, polycaprolactone or polyethylene terephthalate (PET) are of interest for several biotechnological applications like waste treatment, biocatalysis and sustainable polymer modifications. Recent studies suggest that a large variety of such enzymes are still to be identified and explored in a variety of microorganisms, including bacteria of the genus *Pseudomonas.* For activity-based screening, methods have been established using agar plates which contain nanoparticles of polycaprolactone or PET prepared by solvent precipitation and evaporation. In this protocol article, we describe a straightforward agar plate-based method using emulsifiable artificial polyesters as substrates, namely Impranil® DLN and liquid polycaprolactone diol (PLD). Thereby, the currently quite narrow set of screening substrates is expanded. We also suggest optional pre-screening with short-chain and middle-chain-length triglycerides as substrates to identify enzymes with lipolytic activity to be further tested for polyesterase activity. We applied these assays to experimentally demonstrate polyesterase activity in bacteria from the *P. pertucinogena* lineage originating from contaminated soils and diverse marine habitats.

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Introduction

Recent attention of both the scientific community and the public was drawn to microorganisms with enzymatic capabilities to degrade the plastic polymer polyethylene terephthalate (PET) (Wei *et al.*, 2016; Wierckx *et al.*, 2018) that was assumed to be biologically inert for a long time (Moharir and Kumar, 2019). Probably the most prominent example is the β-proteobacterium *Ideonella sakaiensis* isolated from a plastic-polluted site (Yoshida *et al.*, 2016) which produces an enzyme named *Is*PETase (Austin *et al.*, 2018; Gong *et al.*, 2018; Joo *et al.*, 2018) that was shown to be responsible for the biodegradation of PET. Crystallographic studies revealed that this enzyme shows a cutinase-like structure (Joo *et al.*, 2018) which is in line with other studies on enzymatic degradation of PET by enzymes that were initially described as cutinases (Nikolaivits *et al.*, 2018).

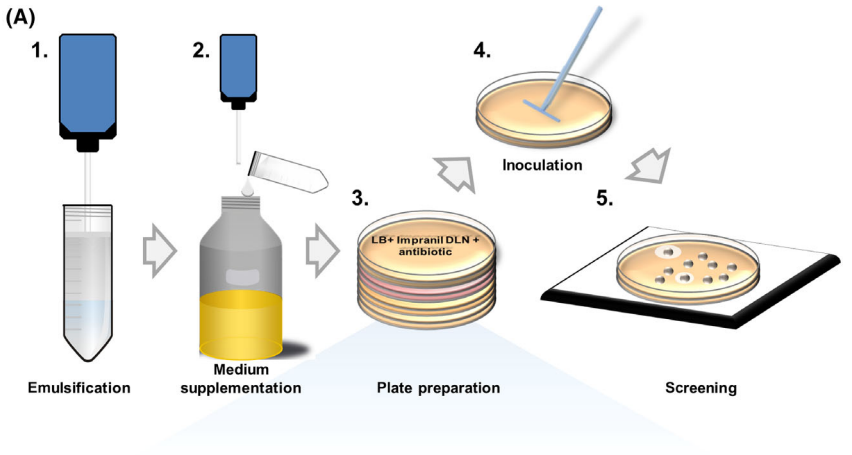
Cutinases are lipolytic enzymes and thus primarily active on carboxylic ester bonds (EC 3.1.1) but defined by activity on polyesters like the plant surface material cutin (Nikolaivits *et al.*, 2018) and, as a consequence, were assigned to a distinct enzyme subclass (EC 3.1.1.74). Cutinases are now spotlighted in the development of new strategies to deal with man-made plastic pollution: Most studies attempting to hydrolyze artificial polyesters are conducted applying such cutinase-like enzymes (Korpecka *et al.*, 2010; Austin *et al.*, 2018; Nikolaivits *et al.*, 2018). However, lipolytic enzymes clustering within other families (Arpigny and Jaeger, 1999), e.g. family VIII (β-lactamase like), were likewise associated with polyesterase activity very recently (Biundo *et al.*, 2017; Müller *et al.*, 2017; Hajighasemi *et al.*, 2018). Besides biodegradation of artificial polyesters like PET, cutinases/

polyesterases are discussed for different biotechnological applications, e.g. sustainable polymerization and polymer modification processes or biocatalytic transesterification and ester synthesis reactions (Nikolaivits *et al*., 2018). Most polyesterases known today are secreted into the extracellular medium, potentially facilitating industrial production with either wild-type or suitable recombinant host strains.

Currently, high-throughput activity-based screening assays are a frequently applied method to identify novel biocatalysts within environmental isolates or metagenomic libraries (Popovic *et al*., 2015; Martin *et al*., 2016; Peña-García *et al*., 2016; Thies *et al*., 2016). These assays are of key importance for reducing the experimental workload to allow assigning of an activity of interest to an individual clone which can then be further characterized. To this end, agar plate-based activity assays are typically applied. Here, clear or coloured zones which are formed around the bacterial colonies indicate the production of a catalytically active enzyme. Suchlike approaches to identify organisms or clones with polyesterase activity currently mostly rely on clearance of media containing polycaprolactone (PCL) or PET nanoparticles prepared by solvent precipitation and evaporation techniques (Jarrett *et al*., 1984; Nishida and Tokiwa, 1993; Wei *et al*., 2014). Notably, these assays imply safety hazards and the production of organic solvent waste. In this protocol article, we describe water-emulsifiable polyesters (Fig. S1) as substrates for rapid and straightforward agar plate-based screening assays as an alternative or at least complementary strategy to identify polyesterase activity in bacterial clones, here exemplified by the identification of such enzymatic activities exhibited by yet unexplored *Pseudomonas* species. These assays generally allow for high-throughput identification of relevant clones, e.g. in metagenomic or genomic libraries (Fig. 1).

Step-by-step protocols for agar plate preparation and polyesterase activity screening

Polyesterases are lipolytic enzymes and are thus detected by non-specific esterase assays like an agar plate-based screening with the substrate tributyrin. The use of this universal substrate with a short-chain fatty acid triglyceride will also detect activities of esterases, true lipases,



(B)

Plate substrate	Composition	Target enzymes
Pre-screening		
Tributyrin	Triacylglycerides (C4)	Polyesterases, esterases, lipases, Acyl transferases, other hydrolases
Coconut oil	Triacylglycerides (~60% C12-C14)	Polyesterases, lipases
Polyesterase screening		
Polycaprolactone	Aliphatic polyester	Polyesterases
Polycaprolactone diol (M_n530)	Aliphatic polyester	Polyesterases
Impranil® DLN	Aliphatic polyester polyurethane	Polyesterases, polyurethanase

Fig. 1. Workflow for agar plate-based screening for polyesterase active clones.
A. Steps of plate preparation and screening: 1. Prepare an emulsion/suspension with the respective substrate (if necessary). 2. Combine substrate emulsion/suspension and molten agar-containing nutrient medium. 3. Pour the warm medium into suitable Petri dishes and let the agar solidify. Suitable supplements for induction of gene expression or selection may be included as well. 4. Plate bacteria either by transfer of single colonies using autoclaved toothpicks, 96 pin replicators or a robotic colony picker, or spread appropriate cell suspensions with glass beads or a Drigalski spatula. Incubate for at least 16 h at a temperature optimal for the applied organism. 5. Document the appearance of halos and/or fluorescence if applicable.
B. Overview on the described substrates (including the chain lengths of the dominant fatty acid for the triacylglycerides) and the enzymatic activities that can be identified with the respective screening plates.

phospholipases or even peptidases and acyl transferases. The use of triglycerides with long-chain fatty acids (FA) like olive oil instead is more selective for lipases because activity towards substrates with fatty acid chains > C10 is a characteristic of these enzymes (Kouker and Jaeger, 1987). However, cutinases have been categorized between esterases and true lipases because they are reported to have higher affinities for short-chain to middle-chain FA ester substrates with chain lengths up to C8 or C12 (Nikolaivits *et al*., 2018); as a result of this substrate specificity, established lipase-specific screenings with long-chain plant oils like olive oil (Kouker and Jaeger, 1987) may miss lipolytic enzymes with additional polyesterase activity. The application of coconut oil that contains, in contrast, a large portion of C6-C14 FA esters (Sankararaman and Sferra, 2018), may bridge the gap between too universal and too lipase-specific substrates used for screening (tributyrin and olive oil respectively). Here, we suggest using the substrates tributyrin and coconut oil for an optional pre-screening to identify lipolytic activity because both substrates are inexpensive and easily available. As a second step, we describe the utilization of easy-to-emulsify polyesters which can serve as appropriate substrates, i.e. Impranil® DLN, an anionic aliphatic polyester polyurethane, and polycaprolactone diol PCD_{Mn530} as a polycaprolactone derivative of lower molecular weight. Impranil® DLN emulsion was already described as a substrate in agar plates for polyurethanase screening (Howard *et al*., 2001). PCD_{Mn530} constitutes a viscous liquid which can be emulsified in liquid media in contrast to amorphous or crystalline solids like the commonly applied polycaprolactone.

General remarks

Media preparation. For the plate assays, we used autoclaved (121°C, 20 min) LB medium (Carl Roth, Karlsruhe, Germany), consisting of 10 g l^{-1} tryptone (peptone from casein), 5 g l^{-1} yeast extract and 10 g l^{-1} NaCl solubilized in deionized water supplemented with 15 g l^{-1} agar–agar (Carl Roth) as the growth medium because it proved suitable for growth of the selected *Pseudomonas* strains despite their partial marine origin and *Escherichia coli* that was included as negative control. However, other growth media or agar plates supplemented with antibiotics or expression inductors may also be tested, if required. As examples, polyesterase screening plates supplemented with the here introduced polyesterase substrates polycaprolactone diol and Impranil® DLN based on MME minimal medium (Vogel and Bonner, 1956) as well as artificial seawater medium (Passeri *et al*., 1992) with regard to the sea-born strains of the *P. pertucinogena* lineage (Fig. S2). The agar was melted just before plate preparation or, alternatively, applied immediately after autoclaving. An Ultra Turrax T25 basic (IKA Labortechnik, Staufen, Germany), previously rinsed with 70% (v/v) ethanol, was applied with 16 000 rpm for both the preparation of substrate emulsions in sterile deionized water (if applicable) and their homogeneous emulsification into molten LB agar (cooled to a temperature of about 60–70°C). Emulsification using an ultrasonic emulsifier according to manufacturer's instructions is also possible. Here, it was in particular applied for smaller volumes (≤ 1 ml). The emulsion of the substrates in hot agar is recommended to maintain sterility of the plates.

Bacterial clones. In the presented examples, single colonies of *Pseudomonas* sp. and *E. coli* BL21(DE3), respectively, are transferred from a master plate to the indicator plates using sterile toothpicks. In general, it should also be applicable to directly plate (meta-)genomic libraries prepared in *E. coli* (Katzke *et al*., 2017) using commercially available kits for TopoTA-cloning (Thermo Scientific, Waltham, MA, USA) or CopyControl™ Fosmid Library Production (epicentre, Madison, WI, USA), or mutagenesis libraries of specific genes in expression vectors prepared by standard molecular cloning methods. Agar plate-based screening assays are typically suitable for that application. As an example, tributyrin plates are an established tool for metagenomic library screenings (Peña-García *et al*., 2016). Plating of dilutions of environmental samples to isolate species of interest might also be tested. However, it has to be kept in mind that the applied growth medium and incubation conditions will in general select for a subpopulation of the plated microbial strains. Hence, a good part of the natural diversity may be lost.

In the here presented example, plates were incubated at the optimal growth temperature of the used bacterial strains to allow colony formation and afterwards incubated at 4°C. At low temperatures, halo formation proceeds, whereas bacterial growth is slowed down. Thereby, the perception of activity is often facilitated without the danger of overgrowing (see below, section 'example'). Hence, prolonged incubation of screening plates at a lower temperature is a common strategy to detect poor activities in activity-based metagenomic library screenings (Popovic *et al*., 2015, 2017; Thies *et al*., 2016). Incubation temperature, incubation time to establish growth and the necessity for prolonged incubation at 4°C depend of course on the investigated organisms and enzymes.

Universal screening for lipolytic enzymes

Tributyrin assay.

(i) Prepare a 50% (v/v) tributyrin (Applichem, Darmstadt, Germany) emulsion in sterile distilled water and add

50 g l^{-1} gum arabic (Carl Roth) (Jaeger and Kovacic, 2014). Gum arabic powder is used as an emulsifying agent for the triglyceride. Homogenize the mixture for at least 1 min to yield a stable emulsion, e.g. using an Ultra Turrax (see general remarks).

Note: Add the gum arabic powder to the respective volume of water. Filling water into a tube or a bottle with a layer of the powder at the bottom should be avoided because it will result in a hard-to-dissolve clot of gum.

(ii) Add 30 ml of tributyrin emulsion per 1 l of molten LB agar (see general remarks) and mix thoroughly, e.g. using an Ultra Turrax (see general remarks).
(iii) Pour 25 ml medium portions into appropriate Petri dishes and allow the agar to solidify for at least 15 min.
(iv) Plate bacterial clones (see general remarks) and incubate at optimal growth temperature for the specific organism for at least 16 h.
(v) Positive clones are identified by a clearing halo after overnight growth or after prolonged (2–7 days) incubation at 4°C for clones expressing low amounts or less active enzymes. Photodocument agar plates (e.g. with a digital camera) and further proceed with selected clones, which were identified as lipolytically active, as appropriate.

Coconut oil assay.

(i) Melt coconut oil (Biozentrale Naturprodukte, Wittibreut – Ulbering, Germany) by incubation at 30–37°C. Pre-heat sterile distilled water to 60°C. Heating the water in advance should avoid a drop of temperature below 30°C during the preparation of the emulsion in the next step and therefore prevent a partial hardening of the coconut oil which will hamper successful emulsification.
(ii) Prepare a 50% (v/v) coconut oil emulsion in the preheated water containing 50 g l^{-1} gum arabic (Carl Roth) and 0.35 g l^{-1} rhodamine B (Sigma-Aldrich/Merck, Darmstadt, Germany). Homogenize the mixture for at least 1 min to yield a stable emulsion.

Note: Add the gum arabic powder to the respective volume of water. Filling water into a tube or a bottle with a layer of the powder at the bottom should be avoided because it will result in a hard-to-dissolve clot of gum.

(iii) Add 20 ml of coconut oil emulsion per 1 l of molten LB agar (see general remarks) and mix thoroughly, e.g. using an Ultra Turrax (see general remarks).
(iv) Pour 25 ml medium portions into appropriate Petri dishes and allow the agar to solidify for at least 15 min.
(v) Plate bacterial clones (see general remarks) and incubate at optimal growth temperature for the specific organism for at least 16 h.
(vi) Positive clones are identified by a fluorescent halo after overnight growth or after prolonged (2–7 days) incubation at 4°C for clones expressing low amounts or less active enzymes. Because of the low solubility of middle- and long-chain fatty acids in aqueous media, clearing halos are barely formed. Hence, esterase/lipase activity is detected by fluorescent complexes that are formed between the cationic rhodamine B and free fatty acids released from the substrate lead to yellow fluorescing colonies and/or halos around active colonies. These can be visualized by irradiation of the plate with UV light, e.g. at 254 nm, for example on a UV table for visualization of ethidium bromide-labelled DNA after gel electrophoresis. Photodocument agar plates (e.g. with a digital camera) and further proceed with selected clones, which were identified as lipolytically active, as appropriate.

Note: If the propagation of the colonies in further experiments is planned, apply UV radiation only for a short period of time (a few seconds) to prevent damaging effects of the UV light. Alternatively, blue light can be used for excitation, e.g. by NGFG15-FastGene Blue/Green LED Gel TransIlluminator XL (460–530 nm). However, background fluorescence of rhodamine B (excitation maximum 580 nm) increases (Fig. S3).

Note: Agar plates containing oils and rhodamine B constitute a frequently applied robust assay to detect lipase activities in different bacteria (Kouker and Jaeger, 1987; Jaeger and Kovacic, 2014). However, the production of fluorescent pigments may interfere with the rhodamine B fluorescence. The fluorescent siderophore pyoverdine leads to a bright fluorescence of many *Pseudomonas* strains under UV light exposure. Production of fluorescent siderophores should not be an issue for libraries within *E. coli* or the strains of the *P. pertucinogena* lineage investigated here because of the absence of respective gene clusters (Bollinger *et al.*, 2018). However, if pyoverdine producers are the strains of interest, supplementing additional iron to the medium decreases the siderophore production and therefore the autofluorescence (Fig. S4). The necessary amount is probably dependent on the physiology of the investigated strain and the applied growth medium. Concentrations described in protocols for appropriate mineral media for the respective strain may offer a suitable starting point.

Polyesterase screenings

Impranil® DLN assay.

(i) Add 4 ml of Impranil® DLN-SD emulsion (COVESTRO, Leverkusen, Germany) per 1 l of sterile molten LB agar (see general remarks) and mix thoroughly, e.g. using an Ultra Turrax (see general remarks).

(ii) Pour 25 ml medium portions into appropriate Petri dishes and allow the agar to solidify for at least 15 min.
(iii) Plate bacterial clones (see general remarks) and incubate at optimal growth temperature for the specific organism for at least 16 h.
(iv) Positive clones are identified by a clearing halo after overnight growth or after prolonged (2–7 days) incubation at 4°C for clones expressing low amounts or less active enzymes. Photodocument agar plates (e.g. with a digital camera) and further proceed with selected clones, which were identified as active, as appropriate.

Note: Impranil® DLN-SD emulsion contains isothiazolones as biocidal supplements to prevent spoilage. In the concentrations used here, we observed no impaired growth of the investigated bacteria.

Note: The anionic Impranil® DLN-SD may become difficult to emulsify into the agar in our experience when a salt-rich growth medium is applied.

Note: Impranil® DLN is also described as a useful substrate to uncover polyurethanase activities. Although this activity, like polyester hydrolysis, is not widespread, the verification of hits from an Impranil® DLN-based screening by determination of sequence homology or additional esterase activity assays is suggested (not described in this article).

Polycaprolactone diol (PCD_{Mn530}) assay.

(i) Prepare a 50% (v/v) PCD (average M_n 530 Da) emulsion: Mix the PCD_{Mn530} (Sigma-Aldrich/Merck) and 50 g l^{-1} gum arabic with sterile distilled water. Homogenize the mixture for at least 1 min to yield a stable emulsion, e.g. using an Ultra Turrax (see general remarks).

Note: Add the gum arabic powder to the respective volume of water. Filling water into a tube or a bottle with a layer of the powder at the bottom should be avoided because it will result in a hard-to-dissolve clot of gum.

(ii) Add 30 ml of PCD_{Mn530} emulsion per 1 l of LB agar and mix thoroughly, e.g. using an Ultra Turrax (see general remarks).
(iii) Pour 25 ml medium portions into appropriate Petri dishes and allow the agar to solidify for at least 15 min.
(iv) Plate bacterial clones (see general remarks) and incubate at optimal growth temperature for the specific organism for at least 16 h.
(v) Positive clones are identified by a distinct halo after overnight growth or after prolonged (2–7 days) incubation at 4°C for clones expressing low amounts or less active enzymes. Notably, the formation of clearing halos by enzymatic activity on PCD agar plates is often accompanied by a grainy accumulation of apparent hydrolysis or transesterification products at the edges of the halo, which is not observed on plates supplemented with tributyrin or Impranil® DLN. However, this even enhances the perceptibility of the halo (Fig. S4). Photodocument agar plates (e.g. with a digital camera) and further proceed with selected clones, which were identified as active on polyesters, as appropriate.

Assay with polycaprolactone (PCL) nanoparticle plates (common polyesterase assay, protocol derived from Jarrett et al. *(1984)).*

(i) Prepare a 5 g l^{-1} PCL solution by completely solving PCL (average M_n ~10 000 by GPC, density 1.146 g ml^{-1}, Sigma-Aldrich/Merck) in pre-heated acetone at 50°C under continuous stirring. Pre-heat an appropriate volume of sterile water likewise to 50°C for the next step.
(ii) Prepare a PCL particle suspension by slowly pouring the PCL solution drop by drop under continuous stirring into the water until a final acetone percentage of ca. 10–15% is reached.

Note: A turbid dispersion should be formed. Pour carefully, because too fast supplementation of PCL solution easily leads to the formation of tiny globular plastic particles instead of a homogenous suspension.

(iii) Add 100 ml of the warm PCL suspension per 1 l of LB agar (see general remarks) and mix thoroughly, e.g. using an Ultra Turrax (see general remarks).
(iv) Pour 25 ml medium into appropriate Petri dishes and allow the agar to solidify for at least 15 min.
(v) Plate bacterial clones (see general remarks) and incubate at optimal growth temperature for the specific organism for at least 16 h.
(vi) Positive clones are identified by a clearing halo on slightly turbid plates after overnight growth or after prolonged (2–7 days) incubation at 4°C for clones expressing low amounts or less active enzymes. Photodocument agar plates (e.g. with a digital camera) and further proceed with selected clones, which were identified as active on polyesters, as appropriate.

Example: Identification of polyesterase activity among members of the *P. pertucinogena* group

The recently established *Pseudomonas pertucinogena* lineage (Peix *et al.*, 2018) consists of several species barely explored until today. The group appears especially interesting for its distinct characteristics with respect to metabolism, genome size and, not least,

habitats with very specific conditions including cold, high-salt and chemically contaminated environments (Bollinger *et al*., 2018). Remarkably for the predominantly terrestrial genus *Pseudomonas*, most of the species within this lineage were isolated from marine or saline habitats. Unlike other *Pseudomonas* species, which are well known for their versatile metabolism, bacteria of the *P. pertucinogena* lineage seem to have a more niche-adapted metabolism in common (Bollinger *et al*., 2018). This is indicated by a comparably small genome and a limited spectrum of utilizable carbon sources. However, the current knowledge about the specific ecological and physiological properties of these species is very limited. An *in silico* search for cutinase homologous proteins uncovered a lipase of *Pseudomonas pelagia* (PpelaLip) which was recombinantly expressed in *E. coli* and proven to hydrolyze different artificial aromatic polyesters, among them poly(oxyethylene terephthalate) (Haernvall *et al*., 2017a; Haernvall *et al*., 2018). The strain itself exhibited likewise activity on the polyesters (Haernvall *et al*., 2017a). The occurrence of genes encoding closely related proteins to PpelaLip appeared to be a common feature of this lineage of *Pseudomonas* sp. (Bollinger *et al*., 2018). Therefore, we investigated one terrestrial and four species from different marine habitats differing in temperature, type of contamination and water depth for polyester hydrolyzing properties (Table 1). All strains were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig) and included *P. pelagia* CL-AP6^T as described producer of PpelaLip as a positive control. We further included *P. bauzanensis* BZ93^T isolated from contaminated soil and the marine species *P. litoralis* 2SM5^T, *P. aestusnigri* VGXO14^T and *P. oceani* KX 20^T. *P. putida* KT2440 (Belda *et al*., 2016) was also included as a well-established and frequently applied member of the fluorescent Pseudomonads (Loeschcke and Thies, 2015) with a versatile metabolism, but without previously described polyesterase activity. In addition, we comprised *E. coli* BL21(DE3) (Studier and Moffatt, 1986) as a negative control because *E. coli* is applied as a standard host for metagenomic library screenings and recombinant esterase production, respectively, with negligible background activity.

All strains were streaked on LB agar plates and incubated at 30°C for 24 h. Distinct colonies of each strain were transferred to the indicator plates using sterile toothpicks and grown for 24 h at 30°C. Plates were photodocumented (Fig. 2), incubated further 24 h at 30°C and afterwards stored at 4°C for 4 days before the final photodocumentation (Fig. S5). All strains showed activity on the indicator plates, except *E. coli* BL21(DE3) and *P. putida* KT2440 which appeared as polyesterase-negative. The production of the fluorescent siderophore pyoverdine leads to bright fluorescence of the latter strain under UV light exposure. As halos were formed not only on Impranil® DLN, which may also result from other enzymatic activities (Fig. 1), but also on the two other polyester substrates, the tested strains of the *P. pertucinogena* lineage can be assumed to produce lipolytic and/or polyester hydrolyzing enzymes. Hydrolysis of the applied substrates by polyesterases was furthermore confirmed by clearing halos exhibited by *E. coli* with pEBP18_Cut (Troeschel *et al*., 2012). This strain is able to express the cutinase gene from *Fusarium solani* f.sp. *pisi* which constitutes a well-characterized enzyme known for its polyesterase activity (Wei *et al*., 2016; Wierckx *et al*., 2018), from a shuttle vector applicable to metagenomic library screenings (Thies *et al*., 2016) (Fig. S6).

In conclusion, polyesterase activity that was suggested by the previous identification of respective genes by sequence homology searches (Bollinger *et al*., 2018) could be experimentally confirmed for these strains. The

Table 1. *Pseudomonas* strains analysed for polyesterase activity.

Species	DSMZ No.	Habitat[a]	Origin[a]	References[b]
P. aestusnigri VGXO14^T	103 065	Crude oil-contaminated intertidal sand samples	Spain 42°46′ 29.27″ N 9°7′27.08″ W	Sánchez *et al.* (2014); Gomila *et al.* (2017)
P. bauzanensis BZ93^T	22 558	Soil from an industrial site	Bozen, South Tyrol, Italy	Zhang *et al.* (2011)
P. litoralis 2SM5^T	26 168	Seawater of the Mediterranean coast	Spain 40° 27′ 24″ N 0° 31′ 36″ E	Pascual *et al.* (2012)
P. oceani KX 20^T	100 277	Deep-sea (1350 m)	Okinawa Trough, Pacific Ocean	Wang and Sun (2016); García-Valdés *et al.* (2018)
P. pelagia CL-AP6^T	25 163	Antarctic green algae co-culture	Antarctic Ocean	Hwang *et al.* (2009); Koh *et al.* (2013)
P. putida KT2440[c]	6125	Plasmid free derivative of *P. putida* mt-2, isolated from soil in Japan		Nakazawa (2002); Belda *et al.* (2016)

a. Environment from which the species was isolated (habitat) and geographical origin of the sample (origin) as stated in the type strain description.
b. References for original descriptions and, if applicable, genome announcements.
c. *P. putida* was included as an established representative of the fluorescent Pseudomonads.

comparison of the halo sizes as an indicator for the enzymatic activity revealed remarkable differences: (i) Closely related species exhibited very different strengths of activity. *P. litoralis* and *P. oceani* showed large hydrolysis halos already after one night of incubation, whereas the activity of *P. pelagia* became clearly visible only after growth for 48 h and several further days at 4°C (Fig. S5). (ii) The activity on polyester substrates appeared more prominent than on tributyrin and coconut oil while a polyesterase itself should be able to hydrolyze the triglyceride substrates likewise very well (A. Bollinger, unpublished). Both observations may be caused by variances in the specific activities of the different enzymes or attributable to differentially regulated polyesterase production and secretion by the different bacteria in reaction to the substrates. Further studies are necessary to assess whether the enzyme biochemistry or the bacterial physiology is the dominant factor behind the apparently massive differences in polyesterase activity.

Discussion

Lipolytic enzymes with activities on polyesters are already highly interesting for a variety of industrial applications and may become even more important in the future for plastic waste and microplastic removal (Wei and Zimmermann, 2017; Urbanek *et al*., 2018). Currently, many scientific studies on the detection of bacterial polyesterase enzyme production rely on a first screening step utilizing the clearance of media from polymer nanoparticles, often polycaprolactone as a simple aliphatic polyester or PET as prominent industrially applied polyester (Jarrett *et al*., 1984; Nishida and Tokiwa, 1993; Wei *et al*., 2014). These procedures are associated with certain disadvantages as they imply solving of the solid polymer in hazardous organic solvents like acetone, dichloromethane or 1,1,1,3,3,3-hexafluoro-2-propanol and subsequent precipitation by careful addition to heated water or immediately to molten agar.

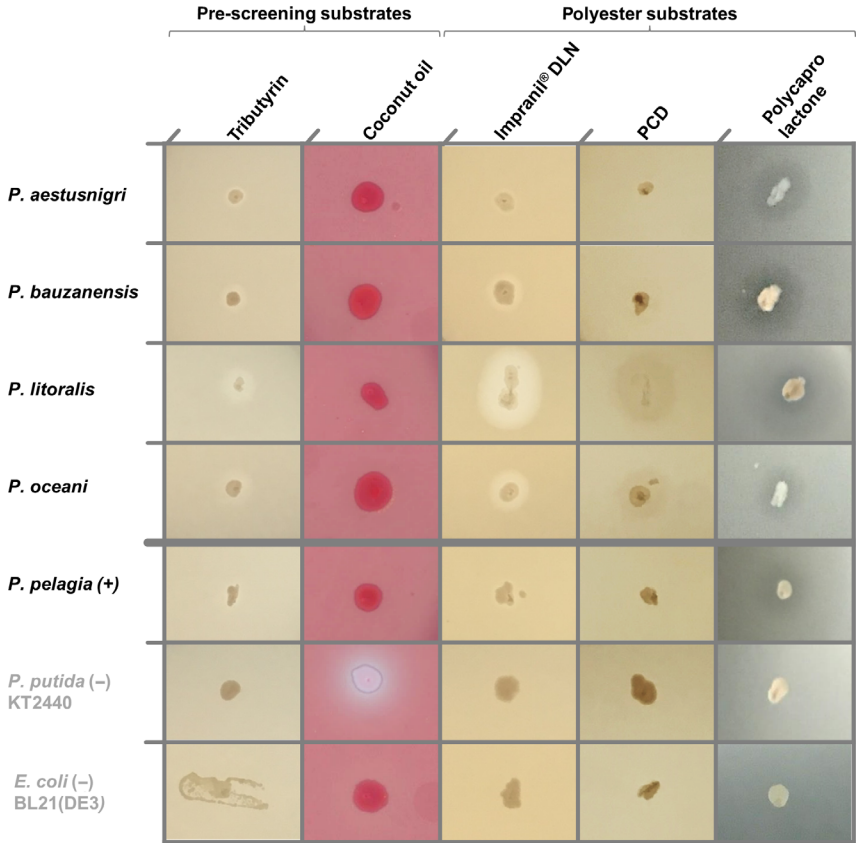


Fig. 2. Polyesterase activities exhibited by *Pseudomonas* species. The colonies were grown for 24 h at 30°C on LB agar plates supplemented with different substrates: Tributyrin (esterase activity); coconut oil + rhodamine B (mid-chain-length hydrolyzing esterase); Impranil® DLN (synthetic polyester polyurethane, polyesterase activity); PCD_{Mn530}, polycaprolactone diol (synthetic polyester, polyesterase activity); and polycaprolactone nanoparticles (current standard for polyesterase screens, polyesterase activity). *P. putida* as an example for a fluorescent Pseudomonad and *E. coli* as a negative control are indicated by grey letters. The white halo around *P. putida* relies on the fluorescence of the siderophore pyoverdine and does not indicate clearance of the substrate. All plates were photodocumented under white light, except coconut oil + rhodamine B-supplemented plates which were exposed to UV light (λ = 254 nm). Shown are exemplary colonies of a set of at least three colonies for each combination on independent plates. Halo formation of the depicted colony is representative for all replicates.

In both cases, a temperature just below the boiling point of the applied solvents is necessary. Hence, the boiling temperature may easily be exceeded and the risk of a sudden evaporation of hot solvents is immanent. Connected to this is the reassembly of larger globular polymer particles because of the sudden exposition of the insoluble polymer to water. Finally, the solvent has to be evaporated by heat or ultrasonication to avoid detrimental effects to the cells which are to be investigated. In addition, this step is necessary for the exchange of solvent shells around the plastic nanoparticles against water which makes them accessible for hydrolases. In our experience, the named procedures not only bear safety hazards but also require considerable handling practice to obtain reproducible results. The application of emulsifiable polyesters like Impranil® DLN or lower molecular weight polycaprolactone derivatives instead appeared in our hands to be a more rapid and straightforward procedure. While PCD–agar was to our knowledge not described to prepare screening plates before, Impranil® DLN-supplemented agar has previously been described and applied to identify and characterize polyurethanases, e.g. in biofilms that degrade coatings in military aircrafts (Howard *et al*., 2001; Biffinger *et al*., 2015, 2018; Hung *et al*., 2016). In our experiences, this substrate is also perfectly suited to identify polyesterases. This observation is in line with studies using this substrate to assess cutinase activities in turbidometric experiments (Schmidt *et al*., 2017). It is further supported by the fact that *F. solani* f.sp. *pisi* cutinase producing recombinant *E. coli* that are able to hydrolyze the polyester polyurethane (Fig. S6). However, Impranil® DLN screening may yield false positive hits constituting protease- or amidase-like enzymes rather than esterases. Hence, hits from these screenings should be verified using esterase activity assays based on the hydrolysis of, e.g., triglycerides or *p*-nitrophenol esters (Jaeger and Kovacic, 2014). Generally, application of inexpensive and easily available triglyceride substrates like tributyrin and coconut oil for screening may be highly useful to pre-select esterolytic organisms or clones in a library in advance to specific polyesterase assays (Fig. S7). PCD_{Mn530} is near twice as expensive as the applied polycaprolactone (source: Sigma-Aldrich); however, the small amounts necessary to prepare one litre medium for screening approaches render this substrate also affordable according to our experience. Impranil® DLN-SD emulsion is conventionally purchased as a bulk product for industrial coating applications. Hence, conditions to obtain small scaled product samples for the laboratory application have to be enquired on an individual basis. The applicability of a two-step strategy combining pre-selection and subsequent polyesterase activity assay was shown by the identification of novel types of polyesterases within a set of hydrolases from metagenomic libraries that were identified by their lipolytic activity in previous studies (Hajighasemi *et al*., 2018).

The halo formation on the opaque white or yellowish Impranil® DLN agar (in dependence of the used medium) and the dark framed halos on PCD plates appeared to facilitate visual recognition of poor activities in comparison with semi-transparent nanoparticle plates. This straightforward readout might also be useful in applications using cutinases as reporter proteins in high-throughput approaches. Examples include transcriptional fusions confirming the successful transcription of target operons to identify promising expression strains (Domröse *et al*., 2017), or as a model protein for studies on protein secretion, e.g. using signal peptide libraries (Knapp *et al*., 2017). In addition, both substrates generally expand the set of polymers applicable to screenings. They may be used in combination with nanoparticle-based screenings to increase hit rates and to detect a broad variety of enzymatic activities in mixed samples as it can be assumed that different enzymes are differentially active on diverse unnatural substrates. Certainly, the aim of the screening was an important determinant for the selection of the substrate. Both assays described here apply aliphatic polyesters (Fig. S1), whereas many of the widely used polyesters like PET contain aromatic building blocks. However, previous studies showed that a large portion of enzymes is active on both types of substrates (Wei *et al*., 2014; Danso *et al*., 2018). This suggests that aliphatic polyesters might still serve as a useful substrate to pre-select candidates for further investigation even if aromatic polyester hydrolysis is the activity of interest. However, evolutive development of respective specificities towards a separation of both activities is discussed (Austin *et al*., 2018); the presented assays are probably not suitable to indicate activity of such enzymes that are selective for aromatic polyesters.

In conclusion, the presented assays are suitable for high-throughput screening applications and may not completely replace but functionally complement the existing nanoparticle-based activity assays to exploit novel organisms and biocatalysts with polyesterase activity. For optimal results, these methods need to be interlinked with appropriate *in silico* strategies to exploit the available DNA sequence information. By using a hidden Markov Model-based search strategy to screen sequence data sets, Danso and co-authors showed that a surprisingly large variety of potential polyesterases is still to be discovered, in particular in bacteria which are currently not considered as a prime source for cutinases (Danso *et al*., 2018). *Pseudomonas* species may constitute an example; in the context of polymer hydrolysis,

they appeared as a source for enzymes hydrolyzing polyurethane (Wilkes and Aristilde, 2017) for a long time, but some very recent reports by the Guebitz group indicated also polyesterase activity in Pseudomonads (Haernvall *et al*., 2017a,b; Wallace *et al*., 2017). The here reported confirmation of polyesterase activity of bacteria from the *P. pertucinogena* lineage, that was already suggested by sequence homology searches (Bollinger *et al*., 2018), underlines the biotechnological potential of this group of bacteria. The predominantly marine Pseudomonas lineage, which includes psychrophilic, halophilic, as well as hydrocarbonoclastic, and heavy metal-tolerant species, may harbour many more intriguing biocatalysts with extraordinary properties.

Agar plate-based assays are a frequently applied tool for the activity-based screenings of metagenomic libraries, in particular for lipolytic enzymes (Popovic *et al*., 2015, 2017; Peña-García *et al*., 2016; Thies *et al*., 2016), also with special emphasis on pollutant degrading enzymes (Ufarté *et al*., 2015). The functionality of this assay with the typical host for metagenomic libraries, *E. coli*, expressing a cutinase encoding gene was indicated here (Fig. S6). In this light, the here presented assays may also prove useful to identify polyester-hydrolytic biocatalysts within metagenomic libraries containing DNA, e.g. from microplastic-polluted habitats. In the future, this may contribute to the exploitation of novel biocatalysts for biotechnological and environmental applications and shed light on natural plastic degradation processes in microbial communities.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Molecular structures of the discussed substrates.
Fig. S2. Growth and polyesterase activities exhibited by *Pseudomonas* species on agar plates based on artificial sea medium and MME minimal medium.
Fig. S3. Coconut oil/rhodamine B agar plates exposed to different light conditions.
Fig. S4. Effect of the additional supplementation of Fe^{2+} on the autofluorescence of *P. putida* on coconut oil +rhodamine B agar plates.
Fig. S5. Polyesterase activities exhibited by *Pseudomonas* species after prolonged incubation at 4°C.
Fig. S6. Polyesterase exhibited by *E. coli* BL21(DE3) expressing the *F. solani* f.sp *pisi* cutinase gene.
Fig. S7. Schematic workflow for agar plate-based screening for polyesterase active clones within a (meta-)genomic library.

