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Note

# A simple and novel method for the production of polyethylene terephthalate containing agar plates for the growth and detection of bacteria able to hydrolyze this plastic

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ABSTRACT

Polyethylene terephthalate is used in the manufacture of many products. Microbes able to hydrolyze the plastic are known, and offer promise for practical waste management. Screening for hydrolysis usually involves unwieldy culturing in the presence of film. A rapid screening method based on culturing on agar plates is here described.

The accumulation of synthetic polymers in the environment is a serious and growing problem. Polyethylene terephthalate (PET) is an aromatic polyester used in the manufacture of many products, including bottles. Its recalcitrance to degradation has led to its accumulation in the environment. However, a number of bacteria with enzyme systems able to fully or partially degrade the plastic offer promise for practical plastic waste management (reviewed by Carr et al., 2020; Kawai, 2021). The recent discovery of the mesophilic bacterium *Ideonella sakaiensis* which is able to degrade and metabolize PET represents a promising development in sustainable recycling efforts (Yoshida et al., 2016). A novel two enzyme system was found to be involved in depolymerization of PET and the ultimate production of central metabolites, which could be further catabolized as carbon and energy sources (Yoshida et al., 2016). At least as promising in this regard are a number of thermophile-derived cutinases. It has been suggested that these alone can be regarded as *true* PET hydrolases (Kawai, 2021) and thus methods for detecting PET hydrolysis should also enable screening of thermophiles. Among other properties, thermostability is needed for significant degradation of the PET building block (Kawai et al., 2019). The later feature is because appreciable PET-hydrolysis is only achieved at higher temperatures (close to the glass transition temperature, GTT) where the polymer structure fluctuates. In aqueous solution the GTT is lowered to 60–65 °C; water molecules enter between the polymer chains, weaken hydrogen bonds and increase polymer chain flexibility, which in turn increases access to the bulk PET and results in faster rates of PET degradation. Screening for microbes or their enzymes able to hydrolyze PET usually involves time consuming culturing in the presence of a model substrate (eg 3PET), PET-film or in a few cases PET-nanoparticles (see table 1. in Carr et al., 2020). Production of nanoparticles (which can be included in agar) involves precipitation/solvent-evaporation techniques using potentially health-damaging solvents such as 1,1,1,3,3,3-hexafluoro-2-propanol (Wei et al., 2014) and trifluoroacetic acid (Rodríguez-Hernández et al., 2019). Typically these protocols also take several days to complete. Rapid screening methods for detection of PET-hydrolyzing microbes are desirable. Whereas several polyesters have been incorporated as emulsions in nutrient agars, enabling screening of single clones, or populations of microbes with plastic-degrading capabilities (e.g., Charnock, 2021; Urbanek et al., 2017), this does not seem to have been reported for PET. After inoculation with microorganisms, the formation of a halo or in some cases a complete zone of clearing around the colony indicates that the microorganisms are able to depolymerize or even solubilize the polymer - the first step of biodegradation. Owing to the practical and conceptual simplicity of agar-based techniques, they can allow high throughput screening of environmental samples for desired properties. The current work shows one way production of PET-containing agar can be readily achieved.

The method here described for the detection of PET-hydrolysis by colonies of bacteria uses two-layers of agar medium: the bottom layer contains nutrients and this is overlaid with a PET-containing layer. Each layer is made by pouring approximately 20 ml solution into sterile 9 cm diameter petri dishes.

Step 1 – preparation of nutrient agar layer: The bottom layer consists of 50% nutrient strength R2A (Reasoner and Geldreich, 1985). To make the medium, 9.1 g of R2A powder (17209; Sigma-Aldrich, St. Louis, MO,

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USA) and 6.0 g bacteriological agar (A5306, Sigma-Aldrich) in 1000 ml ion exchange purified water were heat sterilized by autoclaving. R2A was chosen as the nutrient base, as it contains a greater variety of nutrients than most general purpose media, and often produces higher plate counts (Reasoner and Geldreich, 1985). Furthermore, the buffering of the medium and the its pyruvate content aid in the recovery of stressed bacteria. We (manuscript in preparation) and others (Kawasaki and Kamagata, 2017; Calabrese and Bissonnette, 1990) have shown that inclusion of pyruvate in a defined media significantly increases plate counts for a variety of samples. As R2A is commercially available, this also circumvents the need for assembling a nutrient base from its components.

Step 2 – preparation of a plastic containing top agar layer: a top layer containg PET was poured aseptically onto the solidified R2A layer. The following approach was successful for homogenization of PET in molten agar prior to pouring.

Noble agar (J10907.22, Alfa Aesar, Masachusettes US) at 15.5 g/l, and the anionic surfactant *N*-Dodecanoyl-*N*-methylglycine sodium salt, Sarkosyl NL, (L5777, Sigma-Aldrich) at 0.1 g/l were added to 1× PBS (pH 7.4). This basal medium was autoclaved at 121 °C for 20 min. and maintained at 80 °C prior to addition of PET. Typically small (200 ml) portions of medium were prepared in a wide-mouthed 250 ml pyrex Erlenmeyer flask to improve subsequent dispersion of PET during sonication/stirring. The use of noble agar reduces possible influences by impurities commonly found in lower grade agars, and it provided a clearer medium enabling better detection of zones of PET hydrolysis.

Small pieces of amorphous PET sheet (Goodfellow, ES303010, London, UK) cut with a sterile scissor were added to 5 ml DMSO (Sigma-aldrich, W387520) at about 0.03 g/ml in a heat-stable borosilicate glass (30 ml volume). The glass was covered with foil or a metal cap and heated to about 180 °C with gentle swirling every few minutes until the PET pieces were dissolved (about 10 min). The PET solution was added quickly to the warm agar base as follows: The warm agar was stirred with a bar magnet at a speed adjusted to be just under that where a vortex was created. Mixing of PET solution into the molten agar was achieved using ultrasonic homogenization (Vibra-Cell VCX130, Sonics, Seattle, WA, USA with a standard CV18 converter and 6 mm diameter probe). The tip of the probe previously disinfected with ethanol and dried with sterile paper, was placed about 8 mm below the agar surface. PET solution was added quickly dropwise to the agar during sonication (40% amplitude with 55 s on/5 s off pulses). Drops were released close to the tip of the probe in the region of agitation using a sterile Pasteur pipette. To minimize precipitation of PET before mixing into the agar, the Pasteur pipette was warmed briefly in a Bunsen flame. As the high temperatures used could potentially lead to damaging contact of materials with the skin and eyes, a protective visor was worn during preparation of the PET-containing top agar. A carbon filter face mask was also used as a precautionary measure to reduce inhalation of DMSO vapors and fine particles potentially arising during dispersion of PET in the medium by ultrasonication. Suitable hearing protection was also employed during the ultrasonication step.

Drops of the PET solution appeared to disperse effectively, and typically the process was stopped after the agar took on a slightly cloudy appearance (visual evaluation). For 200 ml agar, about one ml PET-solution was required. The final concentration of PET was then about 0.015 – (0.02)% *w/v*. On cooling the plates had a uniform, slightly cloudy appearance of homogenized PET.

Although the top agar was not sterile, growth of contaminants in the agar has not been observed. Plates have been stored at 4–10 °C for at least a month without losing the ability to reveal PET-hydrolytic activity. By slightly increasing the thickness of the PET-containing layer, it was found that plates packed in plastic bags could be incubated at 50 °C without drying of the top layer for at least 2 weeks, thus allowing detection of PET-hydrolyzing thermophiles. To help maintain a humid environment around the plates and avoid drying, moistened filter paper was placed inside the plastic bags.

## 1. Quality control

### 1.1. Mesophilic bacteria

*Ideonella sakaiensis* (NBRC 110686) was inoculated onto PET-agar using the streak plate method to achieve well-isolated colonies, and plates were incubated for 72 h at 30 ± 1 °C (Fig. 1). Fig. 2 shows the results of a colony-spotting method: a portion of a colony of *I. sakaiensis* and 74 other bacteria strains grown on primary R2A plates, were transferred to PET-agar using an inoculating needle. Fig. 1 shows zones of complete clearing (indicating complete or extensive hydrolysis of PET) around colonies. Fig. 2 shows similar zones around *I. sakaiensis* growth. Complete zones of clearing might be explained by extensive mineralization of PET through the activity of the two-enzyme system, mentioned above, which deconstructs PET to its constituent monomers (Yoshida et al., 2016). None of the other 74 strains produced zones in the agar, suggesting little or no activity on PET (Fig. 2). The legend to Fig. 2 provides a complete overview of the mesophilic strains tested on PET-agar. None of the plastic-degrading isolates (including multiple plastic degraders) reported by Charnock (2021) produced halos or zones of clearing in PET-agar. Several of these may produce an enzyme with structural similarity to known PET hydrolyzing enzymes (Charnock, 2021). Forty-five polycaprolactone(PCL)-degrading bacteria isolated from terrestrial and aquatic environments in Norway, were also tested.

### 1.2. Thermophilic actinomycetes

The first reported PET hydrolase was TfH (BTA-1) produced by *Thermobifida fusca* (DSM 43793) (Müller et al., 2005). Since then, other thermostable hydrolases and their homologs from the cutinase group EC. 3.1.1.74 have been identified. A complete list as of 2020 of proven and proposed PET hydrolases and their source organisms, together with original references is given in Table 1 of Kawai (2021). In order to investigate if the present method could be used to detect the activity of thermostable PET-hydrolases, the following culture collection strains were inoculated onto the described PET-containing agar and incubated in plastic bags as described above for 2 weeks at 50 ± 1 °C: *T. fusca* (DSM43793), *Thermobifida alba* (AHK119) and *Saccharomonospora viridis* (AHK190). *Geobacillus stearothermophilus* (ATCC 7593) and 3 endospore-producing thermophiles isolated from a commercially available semi-crystalline PET powder (unpublished studies) were also grown. Although copious growth of all strains was observed, no zones of complete or partial clearing were obtained with any. Gouda et al. (2002) originally reported that the production and activity profile of a

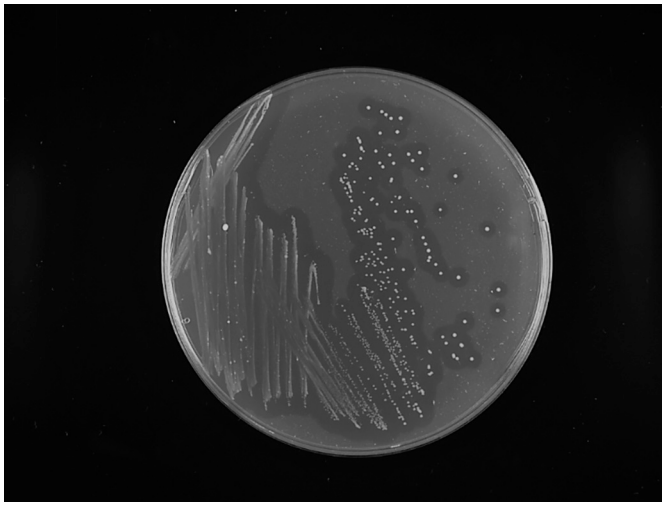

**Fig. 1.** *Ideonella sakainesis* grown in pure culture (30 °C; 72 h) on PET-agar. Zones of complete clearing are visible around individual colonies.

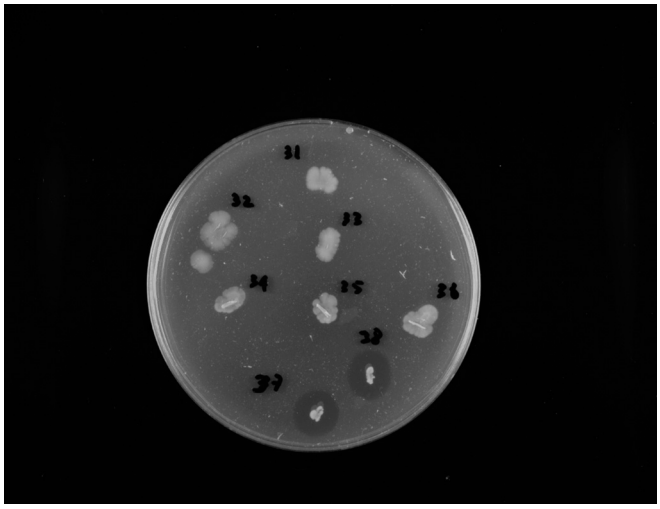


**Fig. 2.** Small portions of colonies grown on primary R2A plates were spotted using an inoculating needle onto PET-agar, and incubated at 30 ± 1 °C. *I. sakaiensis* showing zones of complete clearing in PET-agar is located at positions 37 and 38. In total 75 strains were tested: these included culture collection strains (*S. aureus* DSM 1098, *Burkholderia cepacia* DSM 9241 and *Pseudomonas aeruginosa* DSM 22644) and all the 11 plastic-degrading isolates reported in Charnock (2021). Forty-five mesophilic PCL-degrading environmental isolates from a parallel study and15 randomly chosen soil isolates were also tested.

polyester-degrading hydrolase from *T. fusca* DSM 43793 was controlled in a complex manner involving the induction of enzyme excretion and a number of other factors. Specifically, it was reported that the use of an aromatic-aliphatic copolyester (Ecoflex®, BASF AG, Germany), which was a substrate for the hydrolase, induced its production. Polycaprolactone (PCL), which is an aliphatic polyester, has previously been used as a 'model substrate' to assess PET hydrolase and cutinase activities (Danso et al., 2018). PCL (Powder/flakes, MW 50,000; Polysciences 25090, Warrington, PA, USA) was available in the laboratory, and to investigate if induction of PET hydrolase activity might be achieved using PCL, a few grains of powder were sprinkled in the vicinity of the growth of 2 week cultures on PET-agar. Plates were then incubated for a further 3 days. Already after 24 h, complete zones of clearing were seen around growth of both *T. fusca* and *T. alba*, whereas even after one week of incubation no clearing was obtained around the growth of *S. viridis* or the other thermophiles tested. Fig. 3 shows the growth response of *T. alba* and *S. viridis* on PET-agar after 3 days of additional incubation in the presence of PCL.

It is beyond the scope and intent of the present, methodology article to explore the underlying cellular mechanisms behind these results. However, by including the additional steps described, the PET-agar can also be used to detect PET hydrolase activity in at least some thermophiles. Modifications of the approach that could be investigated are the inclusion of trace amounts of PCL in the PET-agar and further studies on how activity of *S. viridis* PET hydrolase can be promoted.

To summarize, this paper presents details on the production and testing of a PET-containing agar. The agar has advantages over previously used techniques for screening for PET-hydrolyzing activity, including ease of production and price. The practical and conceptual simplicity of agar spread-plate methods, allows the high throughput screening of environmental samples for clones with a desired property. The agar might also be used to check activity of PET-hydrolyzing enzymes after cloning in expression systems. As PET-hydrolyzing genes are extremely rare in the environment (Danso et al., 2018), the possibility of using a simple agar-based screening approaching should be a useful aid in their detection.

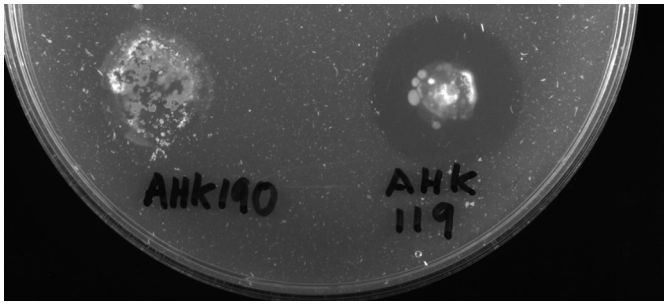


**Fig. 3.** After incubation of *S. viridis* (AHK190) and *T. alba* (AHK119) for two weeks on PET-agar (50 °C) a few grains of PCL were sprinkled on the plate in the vicinity of the growth, and plates were re-incubated for a further 3 days. A zone of clearing in the PET-agar, indicating PET hydrolase activity, was obtained with AHK119 and *T. fusca* DSM 43793 (result not shown), but not with *S. viridis* (AHK190).

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## Declaration of interests

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.

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