## Chapter 22

# Screening and cultivating microbial strains able to grow on building blocks of polyurethane

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## Abstract

The diverse benefits of synthetic polymers is overshadowed by the amount of plastic waste and its whereabouts. The problem can only be tackled by reducing and recycling of plastics. In this respect, investigating the (microbial) degradation of each type of polymer currently used may provide further understanding that fosters the development of new feasible recycling technologies. Here we present a strategy to isolate bacteria from environmental samples that are able to degrade hydrolysis products and building blocks of polyurethane (PUR). Protocols are presented to enrich bacteria on the primary diamines 2,4-diaminotoluene (TDA) and 4,4'-diaminodiphenylmethane (MDA) as well as an oligomeric PUR (Sigma Aldrich, proprietary composition). For TDA and the oligomeric PUR, methods are suggested to monitor their concentration in bacterial enrichment cultures.

**Key words:** polyurethane, PU building blocks, biodegradation, TDA, MDA, isolation of bacteria

## 1. Introduction

Synthetic polymers (plastics) are typically derived from petroleum oil and are an important pillar of our modern society. Among plastics, polyurethanes (PUR) are used as basic material (often as foams) in medical, textile, packaging and construction industries. In 2018 only, the demand for PU in Europe increased to about 4 million tons (PlasticsEurope, 2019). As the building blocks for polyurethane synthesis are numerous and diverse, there is a huge range of different polyurethane polymers. The properties of PUR highly depend on the structure of the polymer backbone and they can be tailored to have a certain rigidity, strength, flexibility and toughness. However, the common characteristic is that all PUR share is the presence of urethane bonds (also known as carbamate bonds). Precursors used to synthesize PUR are diisocyanates and polyols together with additives such as catalysts, cross linkers, and chain extenders, among others. Besides the mentioned urethane bonds, the polyols used for synthesis can additionally contain polyester (polyester PUR) or polyether bonds (polyether PUR). Regarding the diisocyanates employed for the PUR polymerization process, aliphatic and aromatic compounds are common. Figure 22.1 shows the diisocyanates mainly used for PURs production: 4,4'-methylenediphenyl diisocyanate (MDI) and toluene-2,4-diisocyanate (TDI).

[Insert Figure 22.1 approximately here]

In order to investigate PUR biodegradation, two approaches are conceivable: to investigate the change in properties of bulk polymers or the release of degradation products including oligomers and monomeric building blocks. The first studies of PUR polymer degradation by microbial communities have been reported approximately 50 years ago with the exposure of polyester and polyether PUR to fungal activity (Kanavel, Koons, & Lauer, 1966; Ossefort & Testroet, 1966). The purpose of these initial studies was to improve the durability and quality of the materials rather than solving an environmental concern of plastic waste management. Since the 1990s, many studies focused their efforts on analyzing PUR degradation using Gram-positive and Gram-negative bacteria and are summarized in comprehensive review papers (Cregut, Bedas, Durand, & Thouand, 2013; Mahajan & Gupta, 2015; Wierckx et al., 2018). Some of them aimed to improve PUR properties. Other studies focused on the increasing accumulation of plastic waste in landfills or on the decreasing raw material supply (e.g. crude oil). Regarding the latter, a recycling strategy for polyethylene terephthalate (PET) to produce novel PUR polymers was recently accomplished. Enzymatic hydrolysis of PET, subsequent degradation of the building blocks and hydroxyalkanoyloxy-alkanoates (HAAs) production in a *Pseudomonas* strain led to the chemo-catalytic synthesis of a partially bio-based PUR (Tiso et al., 2020).

Generally, polymer degradation is caused by oxidation and hydrolysis reactions. However, many factors such as the chemical structure of the polymer (crystallinity, cross-linking, chemical groups in the molecular chains, etc.) and the abiotic conditions (pH, redox conditions, humidity, temperature, etc.) have an impact on the biodegradation reactions. The protocols presented in this chapter refer to standardized lab conditions in batch cultures of microbial (pure) cultures isolated from the environment. The following chapter describes the investigation of the biodegradation of building blocks of polyurethane polymers by isolating strains from the environment that are capable to use them as sole source of carbon and energy. However, diisocyanates are not stable in the environment due to their immediate reaction with water and the subsequent formation of the corresponding primary amine after decarboxylation (Kreye, Mutlu, & Meier, 2013; Ozaki, 1972). Therefore, diisocyanates such as TDI and MDI are not suitable as model substances to investigate PUR building block degradation in aqueous environments. Instead, their corresponding primary diamines 2,4-diaminotoluene (TDA) and 4,4'-diaminodiphenylmethane (MDA) were chosen. In addition, they are the environmentally relevant PUR hydrolysis products because these diamines were identified as intermediates in the biodegradation of PUR material (Akutsu-Shigeno et al., 2006; Matsumiya, Murata, Tanabe, Kubota, & Kubo, 2010) and are common precursors in polyurethane production processes. In summary, due to the immediate reaction of PU monomers MDI and TDI with water, these monomers were replaced by MDA and TDA to investigate the PUR monomer metabolism (Utomo et al., 2020). Also, an oligomeric PUR was chosen as a model compound for PUR biodegradation experiments. The isolation of a bacterial strain on the oligomeric PUR was reported earlier (Mukherjee et al., 2011).

## 2. Methods

### 2.1 Isolation and characterization of bacteria from soil samples capable of degrading PU building blocks

#### 2.1.1 Rationale

So far no pure culture was isolated that uses TDA or MDA as sole source of carbon and energy. Thus, nature's diversity was mined for a bacterial TDA/MDA degrader on a site rich in brittle plastic waste. This site was chosen to increase the chances to find a PUR building block consumer. To avoid the availability of an additional carbon source a mineral medium was chosen for isolation (Hartmans, Smits, van der Werf, Volkering, & de Bont, 1989).

#### 2.1.2 Materials, equipment and reagents

UV/visible spectrophotometer

TDA (Sigma Aldrich, molar mass= 122.17 g/mol; 98 % purity)

MDA (Sigma Aldrich, molar mass= 198.26 g/mol; purity ≥ 97.0 %)

PU diol solution (Sigma Aldrich; 88% m/m; proprietary composition), aliphatic, dihydroxy-functional oligomeric PUR

Salts (table 22.1) and trace elements (table 22.2) to prepare mineral medium (table 22.3)

[Insert tables 22.1 to 22.3 here]

#### 2.1.3 Protocol

1. Prepare agar plates (table 22.3) containing 10 mM of TDA or 10 mM MDA as well as agar plates not containing any additional carbon source.
2. Take about 10 g of soil, preferably from a soil rich in brittle plastic waste and fill it into a 50 ml test tube.
3. Proceed directly or store the sample at 4 °C.
4. Resuspend 1 g of each soil sample in 9 mL of 0.9% NaCl (m/v) and then dilute it 1:10 with 0.9% NaCl (m/v), this solution can be stored at 4 °C.
5. Prepare a dilution series ($10^{-1}$, $10^{-2}$ and $10^{-3}$) of the stock solution (prepared in number 4) and use 100 µl for inoculation of agar plates prepared in number 1.
6. Store the plates at 30 °C.
7. Check the plates after five days of incubation, discard the plates completely covered with fungi. From the plates with fungi and bacteria, transfer the latter to fresh plates containing the same carbon source.
8. Check after a few days if the transferred bacteria grew and repeat the transfer two or three times more.

9. Choose bacteria that grew after each transfer and check their growth on agar plates not containing a carbon source to avoid isolation of autotrophic strains.
10. For growth in liquid culture prepare the same media as mentioned above without the addition of agar-agar (table 22.3).
11. Prepare stock solutions of 200 mM for TDA and MDA in 10 mL methanol. These solutions must be prepared and used the same week that you inoculate the cultures. In addition~~Also~~, it should be made sure that methanol cannot be used as a carbon source for the strains isolated beforehand.
12. Use 250 mL sterile flasks with 50 mL mineral medium containing 2 mM of TDA or MDA (5 mL from stock solution prepared in number 11 in 50 mL total culture volume) or 3g/L PU diol solution.
13. Pick a colony of a strain of interest and inoculate a liquid culture. Perform the experiment in triplicates.
14. Incubate the cultures at 30 °C and 106 rpm.
15. Measure optical density (OD) daily in a UV/VIS spectrophotometer at a wavelength of 560 nm during 14 days.
16. To test if TDA or MDA are not only carbon and energy source for the isolated bacteria prepare mineral media as described above but without the addition of $NH_4Cl$.
17. Compare the obtained optical densities between N-deficient media and N-containing media.

### 2.1.4 Safety considerations and standards

TDA and MDA are toxic substances. Avoid contact with skin and eyes as well as formation of dust and aerosols.

### 2.1.5 Analysis and statistics

In Figure 22.2, optical density data from an isolated strain growing on 2 mM TDA in nitrogen-deficient and nitrogen-containing mineral media is shown (Cárdenas Espinosa et al., 2020). The growth yields are the same and from that, it can be derived that TDA not only serves as a carbon and energy but also as a nitrogen source for the isolated bacterial strain.

[Insert Figure 22.2 approximately here]

### 2.1.6 Alternative methods/procedures

In order to support the hypothesis that a bacterial strain was successfully enriched on a specific compound, a substrate concentration dependent growth yield test can be performed additionally to the protocol above. Here, the correlation between the concentration of TDA or MDA and the growth yields (OD values) of the isolated bacteria in the same phase of growth were compared. For example, when comparing 1 mM and 2 mM of TDA as substrate concentrations, the growth yield on 2 mM should roughly be twice the yield on 1 mM. Take into account that concentrations much higher than those

used for isolating the strain could be toxic for the cells. For example lower OD values on high concentrations (≥ 5 mM) of TDA or MDA in liquid cultures indicate that these concentrations are already toxic for the isolated bacteria.

## 2.2 Quantification of TDA degradation

### 2.2.1 Rationale

A further way to confirm the degradation of the PUR model compounds in the isolated strains is to measure their consumption via chromatographic methods. Here, a protocol is presented where TDA degradation was monitored by high performance liquid chromatography (HPLC) once the media were inoculated with the bacterial strains.

### 2.2.2 Materials, equipment and reagents

TDA (Sigma Aldrich, molar mass= 122.17 g/mol; 98 % purity)

Methanol, Trimethylamine

Equipment for cultivation of bacterial isolates on TDA (see 2.1)

0.2 µm filters, for example polyethersulfone membrane from Whatman™-GE Healthcare

Shimadzu HPLC with CBM-20A (communication bus module), SPD-M20A (photodiode array detector), CTO-20AC (column oven), DGU-20A$_3$ (degasser), LC- 20AB (liquid chromatograph) or equivalent

C18 column (LiChroCART® 125-4, RP-18e, 5 µm, Merck KGaA) or equivalent

### 2.2.3 Protocol

1. Inoculate 50 mL of 2 mM TDA media with a selected strain (see 2.1) and also prepare a sterile control.
2. Collect 1 mL of the cultures daily for 14 days and perform $OD_{560\ nm}$ measurements as well to monitor the biomass growth.
3. Prepare standards with TDA concentrations between 0.1 mM and 3 mM in mineral media to obtain a calibration curve.
4. Centrifuge all samples (7 minutes at 15700 × *g*) and filter through a 0.2 µm polyethersulfone membrane.
5. Quantify TDA concentrations by HPLC using a C18 column.

6. HPLC program: Isocratic elution within 5 minutes with a mixture of 39.5% methanol, 59.5% distilled water, and 1% trimethylamine (flow rate: 0.65 mL/min; injection volume: 75 µL); temperature of the column constant at 25 °C. Detection was done with a photodiode array detector at 278 nm.
7. Calculate TDA concentrations according to the calibration curve.

#### 2.2.4 Safety considerations and standards

TDA is a toxic and carcinogenic substance. Avoid contact with skin and eyes as well as formation of dust and aerosols.

#### 2.2.5 Analysis and statistics

An example for TDA consumption in course of the cultivation is depicted in Figure 22.3 and shows a depletion of 85% of the initial TDA concentration within 4 days. In contrast to that, the sterile control shows only a minor decrease in TDA concentration.

[Insert Figure 22.3 approximately here]

### 2.3 Quantification of PU diol degradation

#### 2.3.1 Rationale

The proprietary composition of the commercially available PU diol solution makes it difficult to assess changes in its concentration during the course of cultivation with isolated bacteria. However, two methods were reported to solve this problem to a certain extent (Howard, Vicknair, & MacKie, 2001; Mukherjee et al., 2011), the determination of the concentration of the oligomeric PUR with high performance thin layer chromatography (HPTLC) and an agar plate assay.

#### 2.3.2 Materials, equipment and reagents

PU diol solution (Sigma Aldrich; 88% m/m; proprietary composition), aliphatic, dihydroxy-functional oligomeric PUR

Ethyl acetate

Hexane

5% Ethanolic sulfuric acid

Equipment for cultivation of bacterial isolates on PU diol solution (see 2.1)

Aluminum-backed HPTLC sheets of 0.2 mm layers of silica gel 60 $F_{254}$ (Merck) or equivalent

CAMAG® Linomat 5 (semi-automatic sample application system for bandwise spray-on application of the sample onto the chromatographic layer, including one dosing syringe 100 µL, 90–230 V) or equivalent

CAMAG® Twin Trough Chamber for 20 x 20 cm plates or equivalent

CAMAG® TLC Scanner 4 (densitometric evaluation of HPTLC plates, spectral range 190 to 900 nm, plate sizes up to 20 x 20 cm, absorbance and fluorescence mode, Software: CAMAG visionCATS) or equivalent

Separatory funnel

### 2.3.3 Protocol

1. Inoculate 50 mL of mineral media containing 3 g/L PU diol solution with a selected strain (see 2.1).
2. Collect 1 mL of the culture at different time points.
3. Centrifuge the samples (7 minutes at 15700 × *g*).
4. Mix the supernatant equivoluminar with ethyl acetate in a separatory funnel, shake well, separate and continue with the ethyl acetate phase.
5. Apply the extract of the supernatant (10 µL) on aluminum-backed HPTLC sheets of 0.2 mm layers of silica gel 60 $F_{254}$ using the CAMAG® Linomat 5 sample applicator.
6. Perform the chromatographic run in a CAMAG® Twin Trough Chamber, saturated with ethyl acetate and hexane (3:1).
7. Evaporate the solvent from the plates and incubated into freshly prepared solution of 5% ethanolic sulfuric acid and heat for 3 min at 150 °C.
8. Wait for color development on the plates and then scan them in absorbance/transmittance mode at 450 nm with a scan speed of 40 mm/s using CAMAG® TLC Scanner 4.

### 2.3.4 Analysis and statistics

In case of a successful PU diol degradation silica plates with samples of a later time point in the course of cultivation show lower values at 450 nm than those at the beginning of the cultivation. Examples can be seen in Mukherjee et al., 2011.

### 2.3.5 Alternative method

1. Prepare agar plates containing 50 mM phosphate buffer (pH 7), PU diol solution (3 g/L) and 0.001% (w/v) rhodamine B (results in an opaque, pink coloured, solid medium).
2. Cut wells of 1 cm diameter in the agar plates and load either with cell suspensions or cell extracts.

3. Incubate the agar plates at 37 °C for up to 72 h.
4. Irradiate plates with UV light at 350 nm. PU cleavage activity can be detected as orange fluorescent halos surrounding the wells.

## 3. Outlook

Further studies on the isolated strains like differential gene expression studies and transcriptomics as well as proteomics should be conducted to elucidate the degradation pathway of PUR monomers as well as the cleavage of PUR oligomers and polymers. Information on the (extracellular) enzymes and metabolites involved~~,~~ could provide valuable information to foster biotechnical approaches to treat plastic waste.

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## Tables

Table 22.1 Mineral salts solution, 10×

| Substance | Concentration (g/L distilled $H_2O$) |
|---|---|
| $Na_2HPO_4$ x $2H_2O$ | 70 |
| $KH_2PO_4$ | 28 |
| NaCl | 5 |
| $NH_4Cl$ | 10 |

Table 22.2 Trace elements solution, 500×

| Substance | Concentration (g/L distilled $H_2O$) | Volume (mL/L) |
|---|---|---|
| $MgSO_4$ x $7H_2O$ | 50.0 | |
| $FeSO_4$ x $7H_2O$ | 5.0 | |
| $MnSO_4$ x $H_2O$ | 2.5 | |
| $ZnCl_2$ | 3.2 | |
| $CaCl_2$ x $6H_2O$ | 0.5 | |
| $BaCl_2$ | 0.3 | |
| $CoSO_4$ x $7H_2O$ | 0.18 | |
| $CuSO_4$ x $5H_2O$ | 0.18 | |
| $H_3BO_3$ | 3.25 | |
| EDTA | 5.0 | |
| HCl (37%) | - | 73 |

Table 22.3 Mineral medium used to isolate bacteria. Carbon source was either TDA or MDA (10 mM in agar plates, 2 mM in liquid cultures) or PU diol solution (3 g/L)

| Substance | Quantity |
|---|---|
| Mineral salts, 10x | 50 mL |
| Trace elements, 500x | 1 mL |
| Distilled water | 449 mL |
| Agar-agar (3.5% final concentration) | 17.5 g |

## Figure Legends

Figure 22.1: Common aromatic diisocyanates used for PU synthesis and their corresponding diamines.

Figure 22.2: Example for growth of isolated strain on 2 mM TDA in mineral medium containing an additional nitrogen source (filled circles) and in nitrogen-deficient mineral medium (empty squares). n=3

Figure 22.3: Consumption of TDA measured via HPLC during the cultivation of an isolated strain on TDA as sole source of carbon and energy in mineral medium (filled squares) and in a sterile control containing TDA (filled circles). n=3