New species *Pseudomonas capeferrum* TDA1 as a plastic monomer utilizer and a PHA native producer

R N C Utomo¹, H J Heipieper², C Eberlein², and L M Blank¹*

¹Institute of Applied Microbiology - iAMB, Aachen Biology and Biotechnology – ABBt, RWTH Aachen University Worringerweg 1, 52074, Aachen, Germany
²Department of Environmental Biotechnology, Helmholtz Centre for Environmental Research - UFZ, Permoserstr. 15, 04318 Leipzig, Germany

lars.blank@rwth-aachen.de

Abstract. Over the decades, global plastic production has been exponentially increasing with a significant increase of plastic waste as well. Consequently, our environment has suffered a lot because synthetic plastic is less biodegradable or even not completely biodegradable. On the other hand, the conventional recycling rate and plastic management in the top ten plastic contributors are still low to reduce the contamination and pollution from plastic waste. Particularly, Indonesia, one of the world's most outstanding emerging market economies and has the most contribution on plastic waste in ASEAN, should consider breakthrough and novel technology to fight global plastic waste. Polyhydroxyalkanoates (PHA) might have the closest relation to plastic waste upcycling because this compound can be used as the primary material to synthesize bioplastic, so-called plastic, to the bioplastic process. Many *Pseudomonads* can natively produce PHA as their extracellular product. This study qualitatively shows that the new strain *Pseudomonas capeferrum* TDA 1 natively produces PHA from various sole carbon sources, including plastic monomers. This finding gives significant insight for many improvements to the "plastic to bio-plastic" process on an industrial scale.

1. Introduction

Resin, which is made from crude oil, is the core ingredient for almost all plastic products. High-density polyethylene (HDPE), low-density polyethylene (LDPE), polypropylene (PP), polystyrene (PS), polyvinylchloride (PVC), polyethylene terephthalate (PET), and polyurethanes (PUR) are categorized as polymer resin-based plastics [1,2]. Since plastics are used in many sectors due to versatility, plastic waste has been exponentially accumulated in the environment. The significant increase of plastic waste in the environment is due to their low biodegradability, low recycling rate, and mismanagement, which brings many environmental and health issues, such as water, soil contaminations, and diseases [3–5].

Particularly, polyurethanes (PU), mainly synthesized from polyols, isocyanates, and chain extenders, share a global production of around 27 million metric tons, making them the 6th most abundant polymer worldwide. However, many PU is highly stable, which is apparent that only half of the PU amount product has been generated as waste [2]. Their varied mixture and composition also cause a lack of recycling of PU. Not limited to those factors, 2,4-toluenediamine, a common precursor and putative degradation intermediate of PU, is considered a toxic compound [6,7]. While enzymatic depolymerization of ester-based PU is possible by several enzymes, such as esterase, urease, amidase,
and protease, ether-based PU is quite recalcitrant towards enzymatic depolymerization [8,9]. As alternatives, solvolysis, pyrolysis, and hydrolysis are reported for the chemical recycling of PU [10–12]. To follow up the recent studies on PU depolymerizations, the advanced technology on upcycling PU monomers to high-value products needs further investigation. Therefore, we tried to enable a new strain *Pseudomonas capeferrum* TDA1 to utilize PU monomers in this study [6,7]. Since not only plastic industry contributes annually million tons of waste, in this study, we also investigated the ability of this strain to grow on medium-chain fatty acids, which are possibly derived from oil waste of various industries [13].

2. Methodology

2.1. Chemical and materials

All monomers, namely adipic acid, 1,4-butandiol, ethylene glycol, 2,4-toluenediamine, terephthalic acid, sodium decanoate, and sodium octanoate, were purchased from Sigma-Aldrich Chemie GmbH. Sulfuric acid (98%) for the HPLC buffer was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Dichloromethane for GC-sample preparation was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany).

2.2. Microorganism and culture condition

*Pseudomonas capeferrum* TDA1 was isolated from soil rich in brittle plastic waste in a former landfill (Paunsdorf, Leipzig, Germany).[7] The strain was grown in a mineral salt medium (MSM) [14] using the different plastic monomers or monomer mixture as carbon source(s) as indicated.

2.3. Adaptive laboratory evolution

Initially, adaptive laboratory evolution (ALE) was performed by cultivating *P. capeferrum* TDA1 in MSM with 20 mM adipic acid as a pre-culture. The pre-culture then was used to inoculate 250 mL clear glass Boston bottles with Mininert valves (Thermo Fisher Scientific, Waltham, MA, USA) containing various compositions of monomers for the adaptation on ethylene glycol. Serial transfers were reinoculated several times after the cultures reached an OD600 of at least 0.5, with a starting OD600 of 0.1. After growth was detected (usually overnight), single colonies were isolated from ALE cultures by streaking samples on LB agar plates. After ALE on ethylene glycol, one strain (ALE TDA1) out of 10 strains was selected according to the highest growth rate and biomass in MSM with 30 mM ethylene glycol using the Growth Profiler (R) 960 (Enzyscreen, Heemstede, The Netherlands).

2.4. Analytical methods

2.4.1. Monomer analysis. Three monomers, adipic acid, 1,4-butandiol, and ethylene glycol, were analysed with High-Performance Liquid Chromatography (HPLC) using a Beckman System Gold 126 Solvent Module equipped with autosampler, column oven (Beckman Coulter GmbH, Krefeld, Germany), and a Smartline 2300 refractive index detector (Knauer, Berlin, Germany). Analytes were separated using a 300 x 7.8 mm, Metab-AAC, BF-Series column (Isera, Düren, Germany) and a 40 x 8 mm organic acid resin precolumn (CS Chromatographie, Langerwehe, Germany). The eluent was 5 mM H₂SO₄ at a flow rate of 0.7 ml/min. 2,4-toluenediamine was quantified by Gas Chromatography (GC) equipped with Flame Ionization Detector (FID) (Thermo Scientific-Trace GC Ultra). A ZB-wax plus column was used with helium as a carrier gas. For quantification, 2,4-toluenediamine was extracted using dichloromethane with a sample to dichloromethane ratio of 2:1 (0.4 ml sample + 0.2 ml dichloromethane). Afterward, the solution was vortexed for 30 mins at 400 rpm, centrifuged for 5 mins at 13,000 rpm, and GC-FID analysed the lower phase. The split ratio was set to 20 according to the manual of Dikma Technologies Inc [15].
2.4.2. Polyhydroxyalkanoates staining and microscopy observation. For PHA qualitative analysis by staining, 0.2% of a stock solution of 0.25 mg Nile red (Sigma-Aldrich Chemie GmbH) per ml dimethylsulfoxide (DMSO) was added into the sterilized medium to give a final concentration of 0.5 μg/ml. The agar plates were exposed to ultraviolet light (312 nm) after appropriate cultivation periods to detect the accumulation of PHAs.[16] For qualitative analysis by microscopy observation, the culture from appropriate cultivation periods was observed under Leica DM1000 Led microscope using dark field (DF) contrast with 1,000 folds of magnification

3. Result and discussion

3.1. Plastic monomer and preferable substrate utilization

Previously, P. capeferrum TDA1, which can utilize adipic acid, 1,4-butanediol, and 2,4-toluenediamine, was evolved to utilize ethylene glycol [6,7]. At the end of 19-day adaptive laboratory evolution, this strain can utilize 30 mM of ethylene glycol with OD600 of 1.8 (unpublished data) and now so-called ALE TDA1 strain. It has been proved in many studies that PHA is produced by bacteria when the environmental condition is not optimal due to the limitation of nitrogen or other nutrients and an excess of carbon source. Furthermore, nitrogen limitation plays a vital role in enhanced PHA granule formation in most Pseudomonads. Therefore, we first observed the growth of ALE TDA1 strain on several PU monomers (adipic acid, 1,4-butanediol, and ethylene glycol) and medium-chain fatty acids (Decanoate and octanoate) as a sole carbon source, while one-tenth of the usual amount of ammonium sulfate solution was used as a limited nitrogen source [17,18]. Decanoate and octanoate were used in this study as two of the best alternative substrates in PHA production [17,19]. Each substrate used in this experiment, gave equal C-molar concentration of 0.333 mol/L with 55 mM adipic acid, 83 mM 1,4-butanediol, 166 mM ethylene glycol, 33 mM decanoate, and 42 mM octanoate.

![Figure 1](image_url)

**Figure 1.** The growth of ALE TDA1 in MSM with different single monomers as a sole carbon source. A growth profiler accessed growth in 2 ml medium in 24-square-well plates. Errors depict the deviation of the mean (n = 2).

Based on the growth on AA, BDO, EG, decanoate, and octanoate with nitrogen limitation, we could see that growth on EG gave the highest maximum growth rate and the highest biomass among plastic monomers. On the other hand, growth on Decanoate and octanoate outperformed growth on plastic monomers with maximum growth rates of 0.34 and 0.38 h⁻¹, respectively. As preferable and unique alternative substrates, decanoate, octanoate, and a mixture of fatty acids can be utilized by Pseudomonads, for instance, P. putida KT2440 and P. putida mt-2 for mcl-PHAs production [17,20].
3.2. PHA granules formation from plastic monomer and preferable substrate as sole carbon sources

Even though plastic monomers were less preferred as sole carbon sources than the reference substrates (Decanoate and octanoate), we further investigated the growth of *P. capeferrum* TDA1 on the mixture of PU monomers with 10 mM adipic acid, 15 mM 1,4-butanediol, and 30 mM ethylene glycol (60 C-mM of each). Since more 2,4-toluenediamine (TDA) gave a toxicity effect, only 1 mM of TDA was used in this study to see the minimum effect on both 4M and 4M + nitrogen limitation medium. The mixture of PU monomers with- and without nitrogen limitation was used as substrates to see the growth behaviour of *P. capeferrum* TDA1 and consumption rate on those two different conditions.

![Figure 2](image-url)

**Figure 2.** The growth of ALE TDA1 in MSM with 3M (adipic acid, 1,4-butanediol, and ethylene glycol), 4M (adipic acid, 1,4-butanediol, ethylene glycol, 2,4-toluenediamine), 3M + nitrogen limitation, and 4M + nitrogen limitation as sole carbon sources. A growth profiler accessed growth in 2 ml medium in 24-square-well plates. Errors depict the deviation of the mean (n = 2).
It is depicted in figure 2 that nitrogen limitation in 3M and 4M lowered the biomass than the control even though the maximum growth rate between with- and without nitrogen limitation was similar (table 1). The lower biomass showed that nitrogen source plays a vital role in the growth of the ALE TDA1 strain. Nitrogen source is essential for the growth and biochemical synthesis of nucleic acid, lipids, and protein in bacteria. Nitrogen also regulates product formation, in which the biosynthesis of secondary metabolites is limited by the nitrogen sources favouring cell growth, such as ammonium sulfates and certain types of amino acids. Biodegradation of TDA was only observed until 120 h incubation by ALE TDA1, showing better utilization in 4M than in 4M with nitrogen limitation.

**Table 1.** Effect of nitrogen limitation on MSM medium with 3M and 4M as sole carbon sources towards the growth of *P. capeferrum* TDA1 and consumption rates of each monomer.

| Substrate                | µ Max (h⁻¹) | qₘ (mmol g_{dw}⁻¹ h⁻¹) |
|--------------------------|-------------|-------------------------|
|                          |             | AA                      |
| 3M                       | 0.20 ± 0.00 | 1.48 ± 0.24             |
|                          |             | BDO                     |
|                          |             | 2.13 ± 0.04             |
|                          |             | EG                      |
|                          |             | 1.81 ± 0.02             |
|                          |             | AA                      |
|                          |             | 1.29 ± 0.06             |
| 4M                       | 0.23 ± 0.00 | BDO                     |
|                          |             | 1.02 ± 0.24             |
|                          |             | EG                      |
|                          |             | 2.22 ± 0.08             |
|                          |             | AA                      |
|                          |             | 1.02 ± 0.01             |
| 3M with nitrogen limitation | 0.21 ± 0.00 | BDO                     |
|                          |             | 0.99 ± 0.15             |
|                          |             | EG                      |
|                          |             | 1.68 ± 0.11             |
|                          |             | AA                      |
|                          |             | 1.51 ± 0.09             |
| 4M with nitrogen limitation | 0.21 ± 0.01 | BDO                     |
|                          |             | 0.55 ± 0.04             |
|                          |             | EG                      |
|                          |             | 1.19 ± 0.06             |

It is also interesting to see the consumption rates of adipic acid, 1,4-butandiol, and ethylene glycol in those various mixtures. While the consumption of adipic acid and ethylene glycol was relatively similar in 3M and 4M, the consumption rate of 1,4-butandiol was lower in 4M and further lower in 3M with nitrogen limitation and 4M with nitrogen limitation, respectively. Even though few studies of bioconversion of plastic into valuable products, the prominent studies are from upcycling of PET to PHA and PU to rhamnolipids. From Kenny et al., initially, PET was pyrolyzed at 450°C resulting solid fraction of terephthalic acid, which *P. umsongensis* GO16 used as a substrate for PHA production [21]. In a further study, it was genetically identified that responsible genes involved in ethylene glycol metabolism also exist in *P. umsongensis* GO16. After the strain was validated to use terephthalic acid and ethylene glycol as sole carbon sources, the PHA production from both monomers was also proved [22]. From Utomo et al., the mock PU hydrolysate consisting of adipic acid, 1,4-butandiol, ethylene glycol, and 2,4-toluenediamine combined with and without reactive extraction 2,4-toluenediamine was used by defined mixed culture of engineered *P. putida* KT2440 strains as sole carbon sources for rhamnolipid production [6].

As proof of concept, we continued to investigate the PHA granule formation in nitrogen-limited medium with plastic monomers and medium-chain fatty acids as sole carbon source(s) via qualitative analysis, namely plate fluorescence and microscopy analysis.
Figure 3. Proof of concept of *P. capeferrum* TDA1 as a native PHA producer. A: Biosynthetic pathway of PHA production in *P. putida* KT2440 as a reference [23]. B: BLAST results of PHA encoding genes between *P. putida* KT2440 and *P. capeferrum* TDA1. C: Plate fluorescence staining for PHA production from 3M (adipic acid, 1,4-butanediol, and ethylene glycol), 3M+2,4-TDA (4M), alternative substrate namely Decanoate and octanoate as sole carbon source with nitrogen limitation with different pre-cultures, namely A: glucose, B: glucose + little N, C: Decanoate + limited N, D: 3M + limited N. D: Microscopy observation of PHA granule formation from the different substrate.

Qualitative measurement for PHA production was conducted with a viable-colony staining method using Nile red for direct screening of PHA accumulation. From figure 3 C, pre-culture in MSM with different substrates were used, namely glucose without nitrogen limitation (A), glucose with nitrogen limitation (B), decanoate with nitrogen limitation (C), and 3M with nitrogen limitation (D). After that, the growing cultures were streaked out on Nile red agar plate with 3M (10 mM adipic acid, 15 mM 1,4-butanediol, and 30 mM ethylene glycol), 4M (3M + 1 mM 2,4 TDA), 55 mM glucose, 33 mM decanoate, and 42 mM octanoate as sole carbon sources. The growth and fluorescent emission were then observed after 1-day incubation.

The PHA production on 3M and 4M mediums was observed when glucose was used as a sole carbon source in the pre-culture, while PHA production on Decanoate and octanoate was shown from all pre-culture conditions. It can be concluded that the PHA production from PU monomers needs more favorable conditions in pre-culture. The PHA granule-forming cells were found from all conditions, even after adding 1 mM 2,4-TDA, but the granules inside the cell were relatively less than the other conditions. 2,4-TDA might inhibit not only the growth but also relatively too little carbon to be taken up [16].

According to the study from Beckers et al., several genes are responsible for PHA granule formation in *P. putida* KT2440, namely *phaC1* and *C2*, for encoding pha polymerases, *phaZ* for encoding pha depolymerase, *phaD* for encoding transcriptional regulator, *phaG* for encoding acyltransferase, and *phaI* and *F* for encoding PHA-forming enzymes. By aligning the local similarity between two molecules (*P. putida* KT2440 and *P. capeferrum* genomes) using clone manager (Sci-Ed software), it is confirmed that the responsible genes for PHA synthesis are the PHA synthesis are also present in *P. capeferrum* TDA1 [23].
4. Conclusion
From this study, ALE TDA1 strains can be used to utilize PU monomers and the other alternative substrates, such as decanoate and octanoate, making this strain a great candidate for cell factory in the production of the high-value product, namely polyhydroxyalkanoates (PHA). Under nitrogen limitation, the biomass from cultivation on a mixture of plastic monomers was lower than that without nitrogen limitation. However, the maximum growth rates among both conditions were relatively similar. As proof-of-principle, the PHA granule formation was qualitatively observed from a mixture of PU monomers with nitrogen limitation, demonstrating its applicability as a carbon source for a biotechnological conversion. This study brings to further promising investigation, such as strain, fermentation, and process engineering, and to get closer to the so-called plastic to bioplastic process. It is also shown in this study that strain *Pseudomonas* TDA 1 can also natively produce PHA. For sure, many improvements are still needed to bring the “plastic to bio-plastic” process on an industrial scale. For diversification of products, no doubt that this strain also can be used for producing varied high-value products, such as rhamnolipids and phenazine, which have many applications.

Acknowledgement
All authors contributed equally to this work. All authors have read and approved the final version of this manuscript, analyzed and interpreted the data. RNC Utomo gratefully acknowledges the financial support of the Indonesia Endowment Fund for Education (LPDP). Under grant agreement no, LM Blank and HJ Heipieper received funding from the European Union's Horizon 2020 research and innovation program. 633962 for the project P4SB. LM Blank acknowledge funding from the European Union's Horizon 2020 research and innovation program under grant agreement no. 870294 for the project MIX-UP.

References
[1] Plastics Europe 2020 *Plastics – the Facts 2020*
[2] Geyer R, Jambeck J R and Law K L 2017 *Sci. Adv.* 3 25–9
[3] Modak P, Pariathamby A and Seadon J 2017 *Asia Waste Management Outlook*
[4] Barnes D K A, Galgani F, Thompson R C, and Barlaz M 2009 *Philos. Trans. R. Soc. B Biol. Sci.* 364 1985–98
[5] Wierckx N, Narancic T, Eberlein C, Wei R, Drzyzga O, Magnin A, Ballerstedt H, Kenny S T, Pollet E, Avérous L, O'Connor K E, Zimmermann W, Heipieper H J, Prieto A, Jiménez J, and Blank L M 2018 *Plastic Biodegradation: Challenges and Opportunities Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation* (Cham: Springer International Publishing) pp 1–29
[6] Utomo R N C, Li W-J, Tiso T, Eberlein C, Doeker M, Heipieper H J, Jupke A, Wierckx N and Blank L M 2020 *ACS Sustain. Chem. Eng.* 8 17466–74
[7] Cárdenas Espinosa M J, Blanco A C, Schmidgall T, Atanasoff-Kardjalieff A K, Kappelmeyer U, Tischler D, Pieper D H, Heipieper H J and Eberlein C 2020 *Front. Microbiol.* 11 1–10
[8] Magnin A, Pollet E, Perrin R, Ullmann C, Persillon C, Phalip V, and Avérous L 2019 *Waste Manag.* 85 141–50
[9] Magnin A, Pollet E, Perrin R and Avérous L 2020 *Biotechnol Adv* 39 107457
[10] Xanthos M and Patel S H 1998 Solvolyisys *Frontiers in the Science and Technology of Polymer Recycling* (Dordrecht: Springer Netherlands) pp 425–36
[11] Shakorflow A M 2016 *Int J Sci Res* 5 13–25
[12] Garrido M A and Font R 2015 *J Anal Appl Pyrolysis* 113 202–15
[13] Guneser O, Demirkol A, Yuceer Y K, Topay S O, Hosoglu M I and Elibol M 2017 *Brazilian J Microbiol* 48 275–85
[14] Hartmans S, Smits J P, van der Werf M J, Volkering F and De Bont J A M 1989 *Appl Environ Microbiol* 55 2850–5
[15] Dikma Technologies Inc 2018 ProElut™ LLE+ (Liquid-Liquid Extraction)
[16] Spiekermann P, Rehm B H A, Kalscheuer R, Baumeister D, and Steinbüchel A 1999 *Arch Microbiol* **171** 73–80
[17] Poblete-Castro I, Escapa I F, Jäger C, Puchalka J, Chi Lam C, Schomburg D, Prieto M, and Martins dos Santos V A 2012 *Microb Cell Fact* **11** 34
[18] Ciesielski S, Możejko J and Przybylek G 2010 *J Ind Microbiol Biotechnol* **51** 11–20
[19] Borrero-de Acuña J M, Aravena-Carrasco C, Gutierrez-Urrutia I, Duchens D and Poblete-Castro I 2019 *Process Biochem* **77** 23–30
[20] Fontaine P, Mosrati R and Corroler D 2017 *Int J Biol Macromol* **98** 430–5
[21] Kenny S T, Runic J N, Kaminsky W, Woods T, Babu R P, Keely C M, Blau W and O’Connor K E 2008 *Environ Sci Technol* **42** 7696–701
[22] Narancic T, Salvador M, Hughes G M, Beagan N, Abdulmutalib U, Kenny S T, Wu H, Saccomanno M, Um J, O’Connor K E and Jiménez J I 2021 *Microb Biotechnol*
[23] Beckers V, Poblete-Castro I, Tomasz J and Wittmann C 2016 *Microb Cell Fact* **15** 73