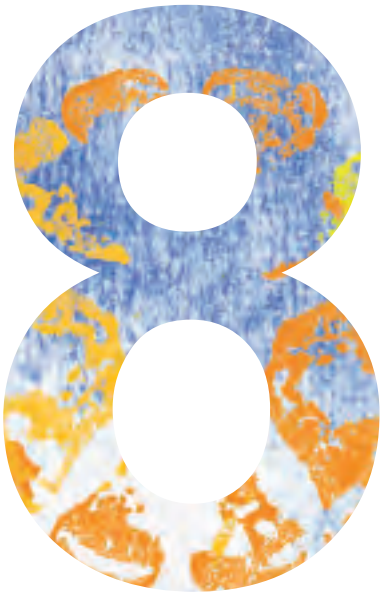
Discovery, characterization and implementation of novel polyester depolymerizing enzymes

Verschoor, J.

General discussion

Jo-Anne Verschoor, Arthur F.J. Ram and Johannes H. de Winde



Over the past 100 to 150 years humans have introduced a plethora of synthetic polymers with high chemical diversity (Horton, 2023). These amazing materials fit in every niche of our lives since there is a plastic for every possible application (Plastic Europe-Association of Plastics Manufactures, 2023). However, the abundant use of plastics and consequently their disposal has now led to an environmental crisis negatively influencing ecosystems, biodiversity, food supply and human health (**Chapter 1**). In this thesis, we have described the identification, conservation and characterization of several polyester hydrolases from different organisms and kingdoms using several different expression systems.

Identification of novel polymer degrading enzymes

In adapting to changing environments, microorganisms have evolved enzymes to degrade complex natural polymers. As a side effect, some of their substrate-promiscuous enzymes are also capable of degrading synthetic polymers to a certain extent.(Chen et al., 2010; Karimi-Avargani et al., 2021; Perz et al., 2016; Zhang et al., 2024). Especially towards synthetic polyesters and polyurethanes, enzymatic degradation by microorganisms has been reported (reviewed in **Chapter 2**). This may be due to the structural similarity between the ester and urethane bonds in synthetic polymers and those found in natural polymers, enabling incidental degradation of synthetic materials. For the identification of polyester and polyurethane depolymerizing enzymes often screening of environmental isolates has shown to be an interesting approach (Gaytán et al., 2020; Skariyachan et al., 2018). However, experimental screening for plastic degradation can be complicated because plastics are intrinsically hard to degrade and difficult to disperse in the medium. Therefore, oligomeric mixtures or dispersions are frequently used as model substrates for screening purposes. To be able to compare studies we suggested a standardized workflow to provide guidelines toward the discovery and optimization of plastic degrading enzymes (**Chapter 2**). Instead of focussing on isolates from polluted areas, it may be interesting to focus on organisms known to degrade specific recalcitrant natural compounds, since this has been a very fruitful approach for the discovery of cutinases that are active on synthetic polyesters. In **Chapter 6**, we have discussed the promise of the unique substrate promiscuity of cutinases, which often also display activity on synthetic polyesters. The *Fusarium* cutinases also showed clear activity on polyester-polyurethane dispersion Impranil-DLN and on BHET. This might also be an interesting approach for exploring enzymes involved in the depolymerization more recalcitrant natural polymers. This may offer a promising avenue for identifying biocatalysts active on more complex synthetic plastics, such as polyolefins with carbon-carbon backbones. To date, no

polyolefin degrading enzymes have been identified, suggesting that other sources need to be investigated (Buchholz et al., 2022). Especially, enzymes related to lignin or lignocellulose degradation such as manganese peroxidases, versatile peroxidases and laccases may be interesting targets for the degradation of the more complex synthetic polymers (Sidar et al., 2024; Zampolli et al., 2023; Zhang et al., 2023; Zhou et al., 2025).

Important to consider, and at the same time wonderful to realize, is the variety of enzymes expressed by microorganisms that appear to be involved in the depolymerization of plastics. For complex substrates, either a tightly regulated system or cascade of several enzymes is required for successful depolymerization. To identify the specific enzymes and their corresponding reactions, homology searches, transcriptomics and proteomics can be employed. However, another interesting approach not yet mentioned in **Chapter 2** would be the screening of various microbial secretomes (of individual microbes or microbial consortia) with activity-based probes (Figure 1). These probes consist of three components, the “warhead” which reacts with the active site of the enzyme creating a covalent bond between the probe and the enzyme (van Kasteren et al., 2017). The warhead is attached to a linker which links it to a detectable marker which allows for visualisation and can be recognized via mass spectrometry (van Kasteren et al., 2017; Willems et al., 2014). This enables the identification of specific catalytic features and possible isolation of the corresponding enzymes, allowing researchers to further study their activity on the model substrates as well as the actual plastic. This has been a very fruitful approach for the identification of serine hydrolases, glucosidases and many more enzymes (Liu et al., 2015; Wu et al., 2019). This method is not often used for the identification of novel plastic depolymerizing enzymes, but examples can be found in literature for the identification of polyester-polyurethane degrading enzymes (Russell et al., 2011). This approach is promising for the identification of enzymes responsible for the degradation of more recalcitrant polymers (such as PE, PS and PVC) and may shine a light on the variety of enzymes required for successful depolymerization.

Another important aspect mentioned in **Chapter 2** is the lack of standardized methods complicating the identification of active strains and corresponding enzymes. Recently this matter was more extensively discussed suggesting guidelines to improve specifically enzyme screening and reproducibility (Wei et al., 2025). Since these guidelines mostly focus on enzymes and not on microbes or microbial consortia it would be wise to establish similar guidelines for these systems.

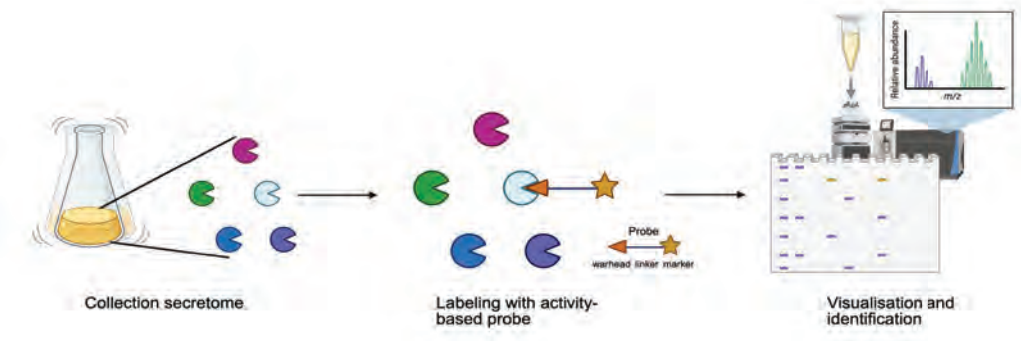


Figure 1: **Schematic overview of enzyme identification with activity-based probes**

Natural response to plastics and induction of enzyme expression

Microorganisms tightly regulate enzyme expression to adapt according to their environment. Therefore, identifying the inducer required for protein production is an essential link between the required environmental ques and enzyme discovery. It also provides insights into the natural response of these microbes towards these polymers and ultimately aid in the identification of novel enzymes. In several studies different elicitors have been utilized to induce the production of plastics depolymerizing enzymes (Bher et al., 2023; Okal et al., 2025; Taxeidis et al., 2023; Vázquez-Alcántara et al., 2021).

In **Chapter 3,** we have identified a novel conserved BHET degrading esterase, LipA, by performing a large screen of 94 *Streptomyces* strains in different conditions. Here we observed that the addition of N-acetylglucosamine (GlcNac) as an inducer induces BHET degradation in *Streptomyces*. Additionally, we observed that interaction between strains may have an important effect on the expression of BHET degrading enzymes in *Streptomyces.* To obtain a complete image of the catalytic activity of the enzymes, the enzymes were examined for their *in vivo* and *in vitro* activity showing activity on both BHET and amorphous PET at ambient conditions. However, since the activity on amorphous PET was low, we expect no discernible activity on post-consumer PET. To obtain higher activity on polymeric PET further enzyme engineering and optimization will be required (**Chapter 3)**.

Optimization of plastic depolymerizing enzymes

Most enzymes derived from aquatic and soil environments function under ambient conditions, and often require engineering to enhance their activity, thermostability, and robustness for industrial applications (Rigoldi et al., 2018). During the past years

several approaches for enzyme engineering have been utilized to obtain enzymes with higher thermostability and activity closer to the glass transition of PET to promote the number of amorphous regions and stimulating accessibility of the polymer and thereby the depolymerization process (Akram et al., 2024). Protein-substrate docking enables more rational mutation design that might lead to more targeted enzyme optimization. This also allows for the introduction of disulfide bridges that increase thermostability. By rational engineering Tournier and colleagues were able to optimize the LCC cutinase to be more thermostable and have higher turnover rates resulting in an industrial feasible protein providing the basis for the first commercially used PHE by Carbios (Tournier et al., 2020). Another approach is the use of directed evolution, where original or rationally engineered enzymes serve as templates for introducing random mutations at specific sites in the protein. The resulting expression library is then screened using high-throughput methods to identify optimized variants (Bell et al., 2022). Alternatively, the addition of specific domains can increase (thermos)stability of enzymes of interest. Detailed analysis of thermotolerant PET hydrolases and their unique or conserved domains, supports engineering of more thermostable enzymes, by adding specific domains the enzyme of interest (Erickson et al., 2022). Additionally, turn-over rates can be improved by increasing the binding affinity using binding domains. Especially carbohydrate binding domains have shown to not only enhance binding to carbohydrates but often also enhance binding to PET (Dai, Qu, Huang, et al., 2021; Dai, Qu, Hu, et al., 2021; Graham et al., 2022).

Another method to optimize enzyme characteristics and specificities is the use of ancestral sequence reconstruction (ASR). An ASR may lead to more robust enzymes with a broader substrate range which can then be optimized or evolved towards the efficient degradation of the synthetic substrate of choice. In **Chapter 4** we performed an ASR on the *Tf*Cut2 cutinase from *Thermobifida fusca*. The reconstruction yielded 172 ancestral sequences of which five were chosen based on their position in the phylogenetic tree and their relation to the known PET-hydrolases. As a result of the reconstruction, several short domains were introduced resulting in longer protein sequences than in their reference enzymes. While enzyme expression was successful, protein purification was not possible due to solubility issues when expressed in *E. coli*. To overcome the solubility issue, we made several attempts to increase the solubility of the ancestral proteins in **Chapter 5**. The observed insolubility is most likely due to the intrinsic properties of the protein's amino acid sequence. To optimize the structure and remove motifs that might behave disruptively, a Phyre2.2 analysis was performed (Powell et al., 2025). The program predicted disordered regions at the N- and C-termini

of all derived ASR enzymes, suggesting that these regions might contribute to aggregation or misfolding. However, removal of these regions did not result in soluble protein. Fusion of solubility-enhancing fusion tags resulted in purified fractions of MBP-fused variants of enzymes 120 and 143. While the yield of soluble protein was limited, these results highlight the potential of MBP as a solubility-tag. The MBP-tagged 120 and 143 showed enzymatic activity on the shorter para-nitrophenyl (pNp) substrates hinting towards esterase like-activity compared to their corresponding descendants which are more active on longer substrates displaying more cutinase-like activity. This suggests that the enzymes have indeed evolved towards more complex substrates over time. Robustness and stability of these enzymes have yet to be examined. However, these enzymes may be optimized towards their substrate of interest using one of the above-mentioned methods for protein optimization.

The most recent development with a major influence on science, is the introduction of machine learning (ML) and artificial intelligence (AI). These programs can accurately predict which amino acid residues are important for certain enzyme features and make educated decisions on possible targets for mutagenesis (Lu et al., 2022). ML- and AI-based approaches may allow for *de novo* enzyme design, making specific enzymes which have not been seen in nature before (Dauparas et al., 2022; Eisenstein, 2023; Winnifrith et al., 2024). By applying these strategies, it may be possible to refine plastics depolymerizing enzymes, ultimately enhancing their stability and functionality for biotechnological applications. These directions may promote further investigation to achieve optimized, scalable production of these valuable enzymes.

Protein expression for pure enzyme applications

When pure enzymes are required for research or industrial applications, it is important to select a suitable production host that aligns with the enzyme's origin, intended use and purpose. When solely focusing on prokaryotic enzyme production and reaching high yields, several hosts can be utilized. The predominant prokaryotic expression host is *Escherichia coli*. The system is well established, has a rapid growth rate, efficient cloning systems are in use and according to several signal peptides have been identified for possible secretion (Reena Gomes et al., 2016; Tripathi & Shrivastava, 2019). Relatively easy engineering and rapid growth rates allow for high-throughput expression and rapid testing of expression constructs and conditions (Jia & Jeon, 2016). Important limitations of *E. coli* are the lack of post-translational modifications, the formation of inclusion bodies and in some cases accumulation of endotoxins (Reena Gomes et al., 2016). As shown in **Chapters 3, 4, 5 and 7** successful protein expression

in *E. coli* was often achieved irrespective of the protein. However, concentrations and solubility did differ per enzyme. Addition of signal peptides like PelB can result in protein secretion, overcoming the possible accumulation problem in the cell (Soong et al., 2023).

Additionally alternative expression systems such as *Bacillus subtilis* can be used when enzyme expression is not successful (Schumann, 2007). This bacterium is the main model system regarding the monoderm bacteria and can be preferred over *E. coli* since it is less likely to produce endotoxins (Reena Gomes et al., 2016). Like *E. coli* an extensive genetic toolbox is present including several promotors, signal peptides and terminators were identified for production of recombinant proteins (Liu et al., 2019; Souza et al., 2021). Another possible prokaryotic host is *Pseudomonas.* This bacterium has high metabolic flexibility and similar growth characteristics as *E. coli* allowing for fast growth and production of proteins and can reach similar protein titers (Chen, 2012; Reena Gomes et al., 2016; Tripathi & Shrivastava, 2019;Nikel et al., 2014; Nikel & de Lorenzo, 2018).

When producing proteins from eukaryotes it is wise to select a eukaryotic expression system since the post-translational modifications and modification frequency differ significantly (Macek et al., 2019; Yang et al., 2022). Expression of eukaryotic proteins in bacteria is likely to result in the lack of correct post-translational modifications which often results in inactive or unmatured proteins. For eukaryotic protein expression yeast species are often used. The two most predominant yeast are *Saccharomyces cerevisiae* and *Pichia pastoris*. Both are often regarded as cost effective, easy to manipulate and safe and are therefore often used for expression of biopharmaceutics (Gomes et al., 2018; Tripathi & Shrivastava, 2019). However, the titers that can be achieved with yeast have a limit, when requiring higher titers often the switch to expression in *Aspergilli* is made (Ntana et al., 2020). These filamentous fungi like the other expression hosts are shown to have a wide genetic toolbox allowing for efficient expression of proteins. Several cloning toolkits are available including modular cloning systems and integrative systems (Arentshorst et al., 2023; Li et al., 2022; Mózsik et al., 2021; Van Leeuwe et al., 2019). A common problem in *Aspergillus niger is* the production of proteases which degrade the protein after secretion. By removing the protease coding genes and replacing them with integration sites, for heterologous protein expression, higher protein production can be achieved (Arentshorst et al., 2023; Li et al., 2022). **Chapter 6** highlights the potential of the multicopy expression system in *A. niger* for the production of *Fusarium* cutinase. Expression of the four cutinases in *A. niger* increased cutinase activity and enabled the transformed *A. niger* strains to degrade Impranil-

DLN and tributyrin. This expression was confirmed using SDS-PAGE and Western-blot analysis confirmed the production of all four cutinases. Interestingly, the Cut5 variants were highly produced in *A. niger*, whereas Cut1 variants exhibited significantly lower protein yields despite being under the regulation of the strong glucoamylase promotor. Since the cutinases display a high similarity in the core sequence of the protein but low similarity in the signal and pro-peptide it was hypothesized that lack of recognition of the highly divergent pro-peptides is responsible for the low production of Cut1 (Gemeren et al., 1996; Poulsen et al., 2006; Plíhal et al., 2007; Van Outram et al., 2021).

Production of enzyme cocktails

Especially with mixed plastics the use of enzyme mixtures or enzyme cocktails is an interesting approach to secure efficient and complete degradation. Research has shown that some enzymes exhibit synergistic effects when combined (Carniel et al., 2017). In nature the use of “natural enzyme cocktails” often a variety of enzymes produced by on host or a consortium, often result in more efficient degradation of natural and synthetic polymers (Kang et al., 2020; Negi et al., 2009; Salinas et al., 2023; Skariyachan et al., 2016). Developing these enzyme cocktails and co-expressing the enzymes in the same host could both improve polymer degradation efficiency and feasibility (Carniel et al., 2017; Lopes et al., 2018). The *A. niger* multicopy expression system (Arentshorst et al., 2023) presents an efficient strategy to produce multiple enzymes in one strain and obtain a customized enzyme cocktail. By integrating various enzymes into different landing sites, it is possible to control and vary gene copies and thereby control expression levels and enzyme ratios for specific applications. Since the strain has a relatively clean background, purification of these cocktails would be minimal making the process more feasible. Therefore, we are convinced that development of such enzyme cocktails might contribute significantly to a greener future regarding mixed waste streams.

The promise of microbial cell factories for recycling and upcycling

In contrast to pure enzyme systems, cell factories may utilize the metabolic potential of microorganisms to covert one compound to another and thereby offer the prospect of circularity and upcycling. The first-generation cell factories often focused on the use of simple hosts producing one compound. Nowadays those approaches use more complex microbial systems and often use synthetic biology to achieve production of high value compounds (Lee et al., 2012). Similar cell factories have already been shown to be effective for the conversion of lignocellulosic feedstocks to fine chemicals (Meijnen et al., 2011). Additionally, it has been shown that PET monomers or waste

can be converted to more high-end compounds such as aromatic compounds, plastic monomers or bioplastics like polyhydroxyalkanoate (PHA) (Diao et al., 2024; Nagarajan et al., 2021; Salvador et al., 2019; Tiso et al., 2021; Wierckx et al., 2015; S. Zheng et al., 2024).

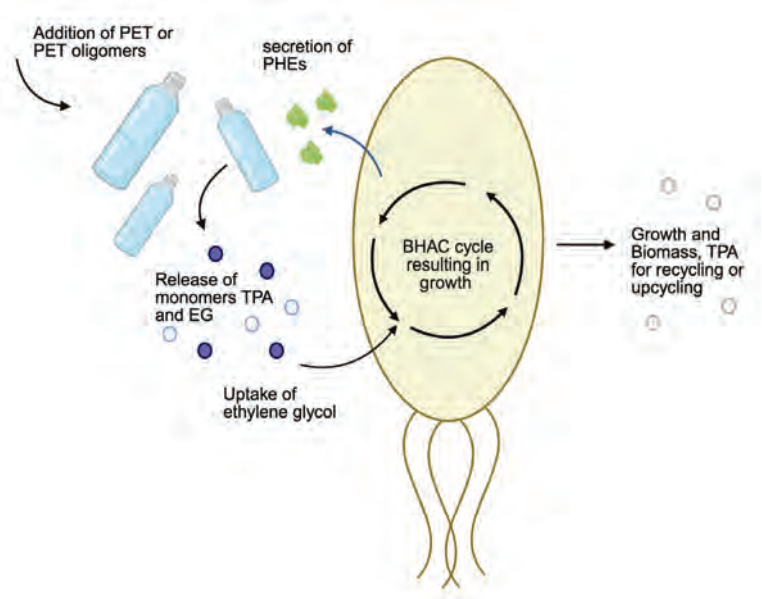


Figure 2: **Schematic overview of *Pseudomonas putida* cell factory after incorporation of BHAC pathway** Polyethylene terephthalate (PET) is degraded by PET-hydrolyzing enzymes (PHE) resulting into the release of monomers ethylene glycol (EG) and terephthalic acid (TPA). EG can be taken up by the *P. putida* which metabolizes it using the β-hydroxyaspertate cycle. TPA will accumulate in the medium and can be used for recycling and upcycling of PET.

A very promising organism for the creation of plastics related cell factories is *Pseudomonas putida*. Especially specific strains such as *P. putida* S12 display high tolerance towards aromatic compounds and solvents (Hosseini et al., 2017; Kusumawardhani et al., 2018). Allowing for survival in the presence of high concentrations of monomers. In Addition, these strains are likely to grow and withstand two phase fermentation which allows for efficient separation of the hydrophilic and hydrophobic compounds during fermentation (Li et al., 2024; Neumann et al., 2006). In **Chapter 7** we set out to create the first part of the cell-factory for the conversion of PET to its monomeric compounds by *P. putida* KT2440. By the integration of PET-degrading enzymes LipA and PET46 in the genome of *P. putida* KT2440, using the Tn7 integration system (Zobel et al., 2015), we attempted to obtain higher degradation levels of BHET. Interestingly, we found endogenous BHETase activity for *P. putida*

KT2440 and no increased BHET degradation for the transformed strain. To engineer a full cell factory, it would be interesting to introduce PHEs into the ethylene glycol utilizing strain created by Schada von Borzyskowski and colleagues which is able to efficiently use ethylene glycol (EG) as carbon source in liquid medium by the introduction of the β-hydroxyaspertate cycle (Schada von Borzyskowski et al., 2023) (Figure 2). The released terephthalic acid could then be upcycled to vanillin, adipic acid, PHA or other interesting compounds (Li et al., 2021; Liu et al., 2023; Valenzuela-Ortega et al., 2023; Welsing et al., 2021). This would result in a cell factory able to grow EG as a monomer released for PET hydrolyzation and the upcycling of terephthalic as valuable compound, monomer of other polymers or even bioplastics.

Biological depolymerization, recycling and upcycling; hope or hype?

So far, several aspects of enzyme discovery, optimization, expression and design of cell factories have been discussed. Both enzymatic systems and cell factories have a wide variety of applications that can be utilized to face certain aspects of our current plastics challenges.

When focusing on bioremediation, water purification and composting, the use of microbial consortia and pure enzyme systems is most promising since it does not require the release of GMOs (Anani & Adetunji, 2021; Lokesh et al., 2023; Viel et al., 2023). Microbial consortia can be released into the environment and either catalyze or accelerate the degradation (Zhou et al., 2022). Importantly, this would be most efficient in soils polluted with micro- and nano plastics since these small particles cannot efficiently be removed from the soil or aquatic environments (Zhou et al., 2024). Bioremediation of heavily polluted areas still requires mechanical removal of the larger plastic pieces. Similar approaches can be taken for water purification or the bioremediation of aquatic environments (Zhou et al., 2024). Accelerated biodegradation or composting can also be achieved when plastics are treated with an enzyme (Kitamoto et al., 2023).

When focusing more on composting of plastics new technologies show that the embedding of well-conserved enzymes in the packaging material, allows for more rapid depolymerization and less pollution (Greene et al., 2021; Guicherd et al., 2024; Sun, 2025). This approach is specifically promising for agriculture where plastics are often mixed with the soil. The embedding of enzymes is also interesting to improve compostable plastics and increase their composting rate (Guicherd et al., 2024). The company Carbios is already developing enzyme embedded packaging for more efficient composting (Enzymatic Biodegradation - Carbios).

Carbios has also shown that clean post-consumer PET waste streams can be successfully recycled into new PET material using an enzyme mixture (Enzymatic Recycling - Carbios). This shows the potential of enzymatic plastic recycling in an industrial setting. However, the costs are still relatively high, and the method is still outcompeted by conventional mechanical recycling methods. Moreover, since mechanical pretreatment is still required, to amorphize the plastic, the overall environmental impact of the method is partially compromised (Wei et al., 2025). The method still requires extensive optimization to be a feasible large scale recycling method.

In addition to advances in enzymatic recycling, chemical recycling is also making big leaps in the recycling field and are improving its sustainability (Chemical Recycling in Circular Perspective ; Luo et al., 2024). Chemical recycling requires less pure waste streams than enzymatic recycling, allowing for the use of post-consumer waste. However, this does not mean that enzymatic recycling cannot be incorporated in these methods to create a more efficient and sustainable process. For example, often chemical recycling leads to oligomeric mixtures which cannot be separated easily, a cell factory can be employed to further process these oligomeric mixtures into other chemicals. A representative case is the recently published cell factory which can convert oligomeric nylon mixtures, derived from chemical recycling, into bioplastic PHA (de Witt et al., 2025).

It is important to note that, as discussed in **Chapter 2**, plastics go beyond the polyesters. While the polyesters pose as important proof of concept, enzymatic depolymerization is still not possible or efficient for most other plastics. This is often caused by the high crystallinity, complex chemical characteristics or the overall recalcitrant nature of these plastics. Therefore, it is important that we continue researching and identifying novel enzymes for the depolymerization of plastics. According to research the most progress to battle pollution can be made in waste management and recycling (Lau, 2020; Lebreton & Andrady, 2019; Where Mismanaged Plastic Waste Is Generated?. However, there is more progress to gain, by the development of new more sustainable materials to replace, especially single-use, plastics (Lau, 2020). The amount of mismanaged plastic can also be reduced by reducing the overall number of plastics. This can also be implemented on small scale in homes where people reduce their consumption and reuse what they can. Hopefully the waste management issues will then soon follow and result in more recycling to close the plastics loop and move towards a more sustainable circular use of plastics.