Recent advances in biocatalysts engineering for polyethylene terephthalate plastic waste green recycling

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\textbf{A B S T R A C T}

The massive waste of poly(ethylene terephthalate) (PET) that ends up in the landfills and oceans and needs hundreds of years for degradation has attracted global concern. The poor stability and productivity of the available PET biocatalysts hinder their industrial applications. Active PET biocatalysts can provide a promising avenue for PET bioconversion and recycling. Therefore, there is an urgent need to develop new strategies that could enhance the stability, catalytic activity, solubility, productivity, and re-usability of these PET biocatalysts under harsh conditions such as high temperatures, pH, and salinity. This has raised great attention in using bioengineering strategies to improve PET biocatalysts’ robustness and catalytic behavior. Herein, historical and forecasting data of plastic production and disposal were critically reviewed. Challenges facing the PET degradation process and available strategies that could be used to solve them were critically highlighted and summarized. In this review, we also discussed the recent progress in enzyme bioengineering approaches used for discovering new PET biocatalysts, elucidating the degradation mechanism, and improving the catalytic performance, solubility, and productivity, critically assess their strength and weakness and highlighting the gaps of the available data. Discovery of more potential PET hydrolases and studying their molecular mechanism extensively via solving their crystal structure will widen this research area to move forward the industrial application. A deeper knowledge of PET molecular and degradation mechanisms will give great insight into the future identification of related enzymes. The reported bioengineering strategies during this review could be used to reduce PET crystallinity and to increase the operational temperature of PET hydrolyzing enzymes.

1. Introduction: Plastic waste “A massive environmental crisis”

The massive production of plastics has been started since the year of 1950. Subsequently plastics have been massively utilized in different sectors and started to be absolutely necessary for modern society (Thompson et al. 2009). Recently, the improvement of plastic design and production to facilitate its re-use, repair, and recycling is the major concern, as noted in the Paris agreement on Climate Change, to decouple plastics production from fossil resources and to reduce greenhouse gas emissions (Fig. 1a). Plastic materials, especially PET, provide economic benefits as they are widely spread in our daily life, and its global production is increasing rapidly due to the simple synthesis, low-priced production, robustness, and durability that are beneficial for the packaging industry (Lebreton and Andraudy 2019). The world plastic production, robustness, and durability that are beneficial for the pack

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\textbf{Abbreviations:} PET, poly(ethylene terephthalate); PE, polyethylene; BHET, bis(2-hydroxyethyl) terephthalate; MHET, mono(2-hydroxyethyl) terephthalate; BA, benzoic acid; HEB, 2-hydroxyethyl benzoate ethylene glycol; PHAs, polyhydroxalkanoates; EG, Ethylene glycol; TPA, terephthalic acid; PETG, polyethylene terephthalate glycol; PCA, protocatechuic acid; DCD, 1,6-dihydroxycyclohexa-2,4-diene dicarboxylate; LCC, leaf-branch compost cutinase; IsPETase, \textit{Ideonella sakaiensis} 201-F6 PETase; SEM, scanning electron microscopy; Tg, glass transition temperature; HPLC, high performance liquid chromatography; TPA-Na, disodium terephthalate; 3D, three-dimensional; GFP, green fluorescence protein; AP, alkaline phosphatase; PAA, polyacrylamide.

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Production is increased rapidly and expected to reach over 34 billion metric tons in 2050 (Geyer et al. 2017). In Europe, the main applications of the plastics industry are represented in the automation industry (~9.9%), packaging industry (~39.9%), construction (~19.8%), and electronics (6.2%) (Facts 2019). Utilization of plastic in the packaging industry has risen a massive environmental problem due to the difficult biodegradation of many synthetic plastics such as polyethylene (PE), poly(vinyl chloride), polystyrene, polypropylene, and PET which remain in the environment for hundred years (Eriksen et al., 2014; Lechner et al., 2014). Besides, the plastic fabrication process consumes about 8% of the world’s fossil fuel (Andrady 2015). These environmental problems became more critical because of the massive disposal of plastic wastes, which negatively affects the ecosystems and marine life in rivers and oceans (Chen et al., 2008; Seo and Park, 2020). Driven by the rapid urbanization and the rapid increase in population, the world accumulated plastic waste is predicted to reach 3.4 billion tons by the year of 2050, up from 360 million tons in 2018 (Kaza et al. 2018) (Fig. 1b). During the year of 2015, about 9% of plastic waste had been recycled, while 12% had been burnt, and 79% had been accumulated in landfills (Geyer et al. 2017) (Fig. 1c). Landfilling is a less favored alternative, and currently, it is gradually phased out in Europe. Effective strategies for increasing the recycling rate of plastic waste need to be developed, so this waste could re-enter the economy as valuable goods to retain their economic value and reduce their waste.

Herein, we critically review the bioprocessing of plastic degradation biocatalysts, including the biotechnological processes that have been applied for enhancing plastic waste degradation, with a focus on the most recent strategies for improving the production, catalytic activity, thermal stability, and re-usability of plastic degradation biocatalysts. This could be performed through protein engineering intensification approaches such as mutagenesis strategies, the discovery of new PET biocatalysts candidates, using native and alternative chassis for enhancing the biocatalysts production, incorporation of metal ions to stimulate enzymes activity, and enzyme immobilization. The trends and challenges facing the PET biodegradation process were also summarized in this review. We also detailed the biodegradation mechanism using these biocatalysts, as the major hurdle in PET degradation is the initial cleavage of the insoluble macromolecule into smaller ones to facilitate its uptake by microorganisms for further utilization.

2. Plastic waste: The search for persistence removal and recycling continues

Plastic recycling via chemical, mechanical, and biological processes
are the main conventional methods used until now (Table 1). Elimination of plastics in a landfill is the easiest and common way followed in the world, but it is hazardous and has many disadvantages due to the slow degradation rate of plastic wastes because of the anaerobic conditions surrounding the landfill. Furthermore, UV and solar radiation have no effect on the plastic degradation process (Abdel-Shafy and Mansour, 2018; Cleary, 2014; Qasaimeh et al., 2016). These causes plastic wastes aggregation in landfills, rivers, oceans, and terrestrial environments and contaminate the groundwater (Gewert et al. 2015). This pollution has a lethal effect on marine animals via ingestion or being trapped in plastic debris (Nelms et al., 2016; Wilcox et al., 2015). Countries that suffer from the scarcity of land such as Japan head for plastic incineration as an energy source though this process is usually harmful to the environment due to the liberated toxic materials such as furans and dioxins (Li et al. 2001). Besides, the cleaning of liberated gases from the incineration process is usually challenging (Burnley et al., 2015; Levchik and Weil, 2004).

Plastic recycling, converting plastics waste into reusable materials, is an alternative method to overcome plastics waste contamination. Plastic recycling using the mechanical process is an economical standard method that usually does not change the basic structure of the material and can be performed in two stages. Plastic wastes during mechanical recycling have to be sorted first, shredded, melted, then granulated, and used with the virgin plastic for industry (Grigore, 2017; Ragaert et al., 2017). The primary stage of this recycling process returns the plastic to its original purpose, and the cleanest waste will be chosen for this stage, such as the PET bottles. In the second stage, the low molecular weight recycled PET is utilized for fiber production. The high-density polyethylene, low-density polyethylene, and PET are processed in the second stage via several melt and remould cycles. One of the main disadvantages of mechanical recycling is that it is limited to the monolayer plastics than the multilayer one. Furthermore, temperature-sensitive plastics cannot be handled mechanically (Scalenghe 2018). Chemical recycling is usually performed via alkaline, acidic, and neutral approaches for plastic de-polymerization to its monomers or other useful products using hydrolysis, glycolysis, and methanolysis. This chemical process needs selective and active catalysts that are costly and energy-consuming and require high temperatures to give a product mixture that is difficult to be separated which limits its industrial applications (Garcia and Robertson 2017). Although mechanical and chemical recycling methods are widely used, they are very risky for the environment (Azapagic et al. 2003). The recycling sites are commonly unhealthy as they are the main source for spreading infectious diseases and toxic volatile organic compounds that harm plant and animal life (d’Ambrières 2019).

Environment-friendly recycling strategies for solving the ubiquitous pollution of the environment with plastic waste are in high demand. Biodegradation of plastic waste using microorganisms is a cost-effective eco-friendly strategy that could be applied through microorganisms’ cultivation or via their enzymes extraction (Restrepo-Florez et al. 2014). Herein, the enzymatic activity of these enzymes catalyzes the polymer bond cleavage into monomers. While the hydrophobicity of plastic surface allows microorganisms to form biofilms over their surface and use them as a carbon and energy source, therefore, the degradation process will be enhanced (Kale et al. 2015). Aliphatic polyester showed high biodegradation efficiency when compared with the aromatic one (Cerdà-Cuellar et al. 2004). Intriguingly, a synergistic chemo-enzymatic hydrolysis process for PET depolymerization, was recently reported (Quartinello et al. 2017). Firstly, the chemical pretreatment was conducted in an eco-friendly way without using harsh chemicals and produced powder composed of 85% terephthalic acid (TPA) under neutral conditions (250 °C and 39 bar). After that, the remaining oligomers were furtherly hydrolyzed enzymatically via cutinase producing TPA with high purity of 97%. Subsequently, more research investigations need to be achieved in this field. Recently, biodegradable plastics with a short lifetime in the environment have been developed. This kind of plastic called “oxy-degradable plastics” because they contain some additives that accelerate the oxidation process, but unfortunately, they end up with a rapid fragmentation into massive amounts of micro plastics upon their exposure to the sunlight and oxygen (Ammala et al., 2011; Kubowicz and Booth, 2017). The produced micro plastics take a long time for complete biodegradation, and the environmental problem will remain (Chae et al., 2019; Ruan et al., 2018).

3. Production of PET biocatalysts

3.1. Potential native chassis for PET biocatalysts production

PET is aromatic polyester, and its degradation difficulty owes to the presence of the non-hydrolyzable covalent bonds that have a subunit substrate called diethylene glycol terephthalate (Kawai et al. 2019). Ethylene glycol (EG) and TPA are the main building units of PET.

Table 1

<table>
<thead>
<tr>
<th>Method</th>
<th>Mechanical degradation</th>
<th>Chemical degradation</th>
<th>Biodegradation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Process requirement</strong></td>
<td>Re-use of plastic products without structure alteration</td>
<td>Plastic de-polymerization to monomers via hydrolysis, glycolysis, and methanolysis</td>
<td>Plastic polymer bond cleavage into monomers via enzymes produced by microorganisms</td>
<td>(Grigore 2017)</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>High</td>
<td>High</td>
<td>Not required</td>
<td>(Ragaert et al. 2017)</td>
</tr>
<tr>
<td><strong>Degradation rate</strong></td>
<td>Fast</td>
<td>Fast</td>
<td>Moderate</td>
<td>(Carta et al. 2003)</td>
</tr>
<tr>
<td><strong>Product separation</strong></td>
<td>Applicable</td>
<td>Difficult</td>
<td>Applicable</td>
<td>(Grigore, 2017; Ragaert et al., 2017)</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>Commonly used</td>
<td>Simple method</td>
<td>Environmentally friendly</td>
<td>(Fatima, 2014; Hopewell et al., 2009; Pasann and Spychaj, 1997; Tournier et al., 2020)</td>
</tr>
<tr>
<td><strong>Disadvantages</strong></td>
<td>Environmentally unfriendly</td>
<td>Source of infectious diseases and toxic volatile organic compounds</td>
<td>Environmentally unfriendly</td>
<td>(Al-Sabagh et al., 2016; Karayannis and Achillas, 2007; Pasunn and Spychaj, 1997)</td>
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<td></td>
<td>Source of infectious diseases and toxic volatile organic compounds</td>
<td>Costly &amp; energy consuming</td>
<td>Source of infectious diseases and toxic volatile organic compounds</td>
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<tr>
<td></td>
<td>Deterioration of product characteristics</td>
<td>Uncommonly used</td>
<td>Deterioration of product characteristics</td>
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<td>limited to monolayer plastics</td>
<td>Limited to condensed polymers</td>
<td>limited to monolayer plastics</td>
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<td>multilayer and temperature sensitive plastics are not applicable</td>
<td>multilayer and temperature sensitive plastics are not applicable</td>
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</table>
Plastics biodegradation is a quite slow process and easily affected by temperature, pH, humidity, and ultraviolet rays. After that, microorganisms will be adopted for the complete degradation of the remaining plastics. Biodegradation of PET usually causes shortening of the polymer chain, which subsequently reduces the molecular weight of the polymer. After that, oligomers, dimers, water-soluble monomers will be produced and can pass the microbial cell membrane to be used as a carbon source. PET crystallinity, high molecular weight, strong C-C bonds, and extremely hydrophobic surface play an essential role in the biodegradation process. Crystallites act as barriers to moisture diffusion (Calleja et al. 1994). Generally, amorphous polymers with a linear structure can be degraded faster than crystalline polymers with branched structure (Mierzwia-Henztek et al. 2019). Recently, some fungal and bacterial strains have been discovered for PET biodegradation into low molecular weight oligomers or monomers such as Bis(2-hydroxyethyl) terephthalate (BHET) and mono(2-hydroxyethyl) terephthalate (MHT) (Wei et al. 2016). Saprophytic scavengers are bacteria having the ability to remineralize wastes’ organic carbon and hydrocarbons that are considered the building blocks of plastics. Actinobacteria, Gram-positive phylum, was reported as a promising bacterial strain for whole-cell degradation process. Crystallites act as barriers to moisture diffusion (Calleja et al. 1994). Generally, amorphous polymers with a linear structure can be degraded faster than crystalline polymers with branched structure (Mierzwia-Henztek et al. 2019). Recently, some fungal and bacterial strains have been discovered for PET biodegradation into low molecular weight oligomers or monomers such as Bis(2-hydroxyethyl) terephthalate (BHET) and mono(2-hydroxyethyl) terephthalate (MHT) (Wei et al. 2016). Saprotypic scavengers are bacteria having the ability to remineralize wastes’ organic carbon and hydrocarbons that are considered the building blocks of plastics. Actinobacteria, Gram-positive phylum, was reported as a promising bacterial strain for whole-cell catalysis of PET, especially the genera Thermomonospora and Thermobifida (Herrero Acero et al., 2011; Ribitsch et al., 2012a). PET degradation based on whole-cell biocatalyst has excellent advantages over utilizing the free enzymes by reducing the degradation process time and material-intensive protein purification process (de Carvalho 2017). Comamonas testosteroni F4 and alkali-resistant C. testosteroni F6 were used as whole-cell catalyst for micro sized-PET degradation (Gong et al. 2018). The alkali-resistant C. testosteroni F6 showed better degradation of micro sized-PET at high alkaline condition (pH 12) after 48 h at 37 °C. The degradation products using both strains were composed of BHET, MHT, TPA, and methyl acrylate (MA).

Yoshida et al. 2016 (Yoshida et al. 2016) discovered a bacterial strain called Ideonella sakaensis that belongs to the genus Ideonella and the family Comamonadaceae. This bacterium showed effective biodegradation of PET polyester, which was extracted from the plastic recycling plant in Sakai, Japan, and used the PET as a sole carbon source. I. sakaensis secretes two enzymes, PETase, and MHETase enzymes, that are involved in the conversion of PET polyester into simple and non-harmful recyclable monomers. PETase first hydrolyzed the PET polymer into MHET that was further hydrolyzed into TPA and EG. These final products were furtherly utilized by the bacterium as a food source (Austin et al. 2018). PETase from I. sakaensis whole-cell catalysis showed 5.5, 88, 120 fold higher hydrolyzing activity toward PET polyester when compared with low-crystallinity cutinase, Fusarium solani cutinase, and Thermobifida fusca hydrolyase at low temperature (Müller et al. 2005).

Esterase is an enzyme family that can cleave the ester bond (short chain acyl ester) found in PET monomers with observed surface modification. Bacillus and Nocardia were reported for the initial degradation of PET via esterases (Sharon and Sharon 2012). Esterase from Thermobifida Thh_Est showed effective surface hydrolysis for PET polyester, and its effect is similar to cutinases from the same genus (Ribitsch et al., 2012a).

Lipases are widely known for their catalytic hydrolysis of the long-chain (more than C18) water-insoluble triglycerides, and they are characterized by the interfacial activation phenomenon. Lipases were reported for degradation of PET fabrics to some extent through enhancing their wettability, dye-ability, and absorbent characteristics (Gupta et al. 2015). Different organisms have been reported for lipase production as PET whole-cell catalyst such as Thermomyces lanuginosus (Eberl et al. 2009), Candida antarctica (Vertommen et al. 2005), Triticium aestivum, and Burkholderia spp. (Nechwatal et al. 2006). Lipases showed a significant higher hydrolysis rate toward aliphatic polyester nanoparticles (100 nm) than the polyester biofilm, and the same effect was also observed for the aromatic one (Müller et al. 2005). It is supposed that the low crystallinity of the polyester nanoparticles is the reason for the high degradation rate (Welzel et al. 2002). Bacterial lipases are more effective in the bioconversion of PET to the intermediate product MHET by 50-fold higher when compared with the fungal one which needs the additional presence of plasticizers (Eberl et al. 2009). A consortium composed of three Pseudomonas spp. and two Bacillus spp. were reported for effective PET degradation at 30 °C after six weeks incubation (Leon-Zayas et al. 2019).

Leaf-branched compost cutinases (LCC) are lipolytic esterolytic enzymes which belonging to the lipases family, but the two enzyme families are showing different catalytic behavior toward PET degradation. Cutinases have serine at their catalytic triad which is not buried under an amphipathic loop unlike lipases (Longhi and Cambillau 1999). Cutinases are more active toward the soluble and emulsified substrates (Sulaiman et al. 2012). Bacteria and fungi are the major sources of cutinases. It has been demonstrated that cutinases from Aspergillus oryzae, Aspergillus niger, and Aspergillus sp. (Bermúdez-García et al. 2017), Penicillium citrinum (Liebminger et al. 2007), Humicola insolens (Ronkvist et al. 2009), Thermobifida fusca (Bruseckner et al. 2008), Fusarium solani (Alish-Sarki et al. 2006), F. solani pisi (Vertommen et al. 2005), and Thermobifida cellulolysisitica (Herrero Acero et al., 2011) have hydrolyzing activity toward low-crystallinity PET. Thielavia terrestris was reported for TctuA cutinase production with a molecular weight of 25.3 kDa, showed optimal activity at pH 4 and 50 °C, and effectively degraded PET and polycaprolactone with a hydrolytic rate of 1.1 mg h-1 mg-1 protein and 203.6 mg h-1mg-1 protein, respectively (Yang et al. 2013). Cutinase from Humicola insolens is more active toward PET films than cutinase produced from Thermobifida cellulolysisitica. Crystallinity in the PET films of 10 and 20% sharply decreased the enzymatic hydrolysis efficiency. This drop in enzymatic hydrolysis was not observed when the same enzyme was used for poly(ethylene furanate) due to its less crystallinity (Weinberger et al. 2017). Despite that, the enzymatic hydrolysis of PET oligomers is much faster than the long-chain polymers (Ribitsch et al. 2011). Besides, it has been reported that cutinase from Pseudomonas mendocina and Fusarium solani showed about 10-fold activity on low crystalline PET (7% crystallinity) than on higher crystalline PET (35%) (Donelli et al. 2009).

Recently, it has been reported that some native microorganisms producing PET biocatalysts could be found in invertebrates such as insects, which can hydrolyze PET polymers via mechanical grinding and shredding (Yang et al., 2014, 2015b). Thus, will produce smaller plastic pieces that will increase the surface area and allow better attachment of the microorganisms with the surface. The high degradation rate of PE by wax moth’s larvae, Galleria mellonella, was reported via producing holes in PE film (Bombelli et al. 2017). The genera Citrobacter was found to be responsible for PE degradation in Tenebrio molitor’s gut (Brandon et al. 2018). While Bacillus sp. Strain YPI was found to be the responsible bacterium for PE degradation in mealworms (Yang et al. 2015a). In 2011, Shen et al. (Shen et al. 2011) made a preliminary plastic degradation study on the mechanism of mealworms, and isolated 8 strains of bacteria with degradation characteristics from the intestinal tract of mealworms. In 2017, Zhang et al. (Yang et al. 2018) raised yellow mealworms with the plastic film A (30% starch, 70% PE and auxiliary) and B (more than 95% PE and A small amount of auxiliary) as the only food, and yellow mealworms could completely degrade both plastic films. The above studies showed that there were PE degrading bacteria in the intestinal tract of insects, which provided a new way for the biodegradation of PE. In the oligotrophic deep-sea environment, microorganisms in the intestines of marine mollusks, that can degrade plastics, attracted a great concern. More discoveries of invertebrate species will help in plastic waste reduction through the ingestion and degradation of plastics in their guts via enzymes. Even though the discovery of native strains, the finding of a robust strain that can replicate and produce PET biocatalysts in high yield under harsh conditions, such as high temperature and pH, is challenging. Therefore, the over-production of these enzymes via dynamic alternative chassis would be an effective and promising way to promote the large-scale production.
biodegradation process of PET polyester.

3.2. Potential alternative chassis for PET biocatalysts production

3.2.1. Escherichia coli

Recently, the use of model organisms for the overexpression of industrial enzymes has shown an effective and economical outcome. *E. coli* is the most desired fermentative microorganism for industrial biocatalysts production on a large scale due to its easy handling and genetic manipulation (Olayiuyo et al., 2016). Recombinant protein production using *E. coli* imparts a metabolic burden on the microorganism, and this decrease the duplication time, within 24 h, with a high cell density (Samak et al., 2018a). Besides, *E. coli* growth condition requires inexpensive, available components and the target DNA transformation protocol is very fast and handy (Rosano and Cecarelli, 2014). In the last four years, *E. coli* was used extensively for PET biocatalyst production, which helped in solving the crystal structure of these enzymes such as PETase, MHETase, and LCC and provided great insight about the degradation mechanism and substrate affinity toward PET (Almeida, 2019; Fecker et al., 2018; Han et al., 2017; Joo et al., 2018; Sowdharmi et al., 1989; Tournier et al., 2020). Two esterases encoding genes amplified from *Clostridium botulinum* ATCC3502 and expressed in *E. coli* were able to hydrolyze PET at 50 °C, and their catalytic activity was further improved at same temperature via mutation introduction in the zinc-binding domain (Perr et al., 2016). Table 2 summarizes all PET biocatalysts heterologously expressed in *E. coli*.

*E. coli* can also be used for whole-cell catalysis by expressing PETase extracellularly via secretion systems. Sec-dependent and SRP-dependent signal peptides were fused with IsPETase and heterologously expressed from *E. coli* using pET22b-SPMalE:IsPETase and pET22b-SPamE:IsPETase expression systems. The extracellularly secreted IsPETase successfully degraded the PET film after 72 h at 30 °C and the degradation process can be directly applied in the culture medium (Seo et al., 2019).

3.2.2. Pichia pastoris

The eukaryotic microorganism *P. pastoris*, a methylotrophic yeast, is characterized by its easy genetic manipulation and high cell density growth. *P. pastoris* is very robust in the production of soluble and correctly folded recombinant proteins. Furthermore, the cloning procedure of the target DNA gene depends on the linearization of the DNA, followed by its transformation into *P. pastoris* via homologous recombination. This process facilitates the replication of the target gene and allows its overexpression. Expression of PET biocatalysts via *P. pastoris* provides a dynamic growth rate with high cell density, and facilitate the purification process of the produced biocatalyst from the growth medium directly due to the extracellular expression. *P. pastoris* also have the ability to perform post-translational modifications such as acylation, glycosylation, and methylation. Intriguingly, the expression of recombinant LCC in *P. pastoris* leads to the production of correctly-folded glycosylated LCC that showed a higher thermostability by 10 °C when compared with the native one (Shirke et al., 2018). Glycosylated *Thermobifida fusca* cutinase, Thc,Cut1, mutant strains expressed in *P. pastoris* showed effective degradation of poly(butylene succinate) and poly(3-hydroxybutyrate-co-3- hydroxyvalerate) within 96 h (Gamerith et al., 2017).

3.2.3. Microalgae

Microalgae is a promising potential economic cell factory for PET biocatalyst production. Recently, algal genome data and transformation protocols became widely available with rapid development of their genetic tools, and this can facilitate their usage for biocatalysts production enhancement. Over 40 different microalga strains such as *P. tricornutum* and *C. reinhardtii* became very easy and successful for genetic manipulation so far (Gang et al., 2015). Recently, the photosynthetic microalga *P. tricornutum* was used as chassis for PETase extracellular expression and the expressed PETase showed 80-fold higher turnover of low crystallinity polyethylene terephthalate glycol (PETG) when compared with the bottle-grade PET at 30 °C. Furthermore, the expressed PETase was active toward shredded pieces of PET at the high saline condition and mesophilic temperatures, 21 °C resulting in a hydrolyase mainly consists of TPA and MHET (Moog et al., 2019). The green algae, *Chlamydomonas reinhardtii* CC-124, was recently reported for the effective heterologous production of PETase. The produced PETase was able to degrade PET film at 30 °C after incubation of 4 weeks (Kim et al., 2020). The main advantages of microalgae for biocatalyst production can be summarized as follows: (1) the phototropic growth in photo-bioreactors, which hinders the transgenes from releasing into the environment (Gong et al., 2011). (2) Expression of the recombinant biocatalysts through the chloroplast genomes (Lauersen et al., 2013a). (3) The ability to perform post-translational modifications, which lead to the production of glycosylated biocatalysts extracellularly (Lauersen et al., 2013b). (4) Robust cell duplication in a short time, within 24 h, and can reach to 3 h as in the case of *Chlorella sorokiniana* (Mayfield et al., 2007). To date, there is a big gap in using these advantages of microalgae for PET biocatalysts production. Using all the available data tools of microalgae bioengineering will open the field toward cost-effective production of PET biocatalyst with catalytic performance enhancement.

3.2.4. Clostridium thermocellum

* C. thermocellum has attracted considerable attention in recent years as a heterologous expression chassis due to its thermophilic and anaerobic characteristics even though the genetic manipulation difficulties. The optimal growth conditions of *C. thermocellum* can reach 60 °C. Scientists put great efforts to facilitate the genetic manipulation of *C. thermocellum* and avoid gene disruption by developing efficient transformation tools for this bacterium. A transformation protocol for *C. thermocellum* via electroporation has been developed and enhanced the transformation efficiency (Olson and Lynd, 2012). This allowed *C. thermocellum* to be utilized as a whole-cell biocatalyst in biofuel production and lignocellulose saccharification. (Yan et al., 2020) used *C. thermocellum* as a microbial chassis for the extracellular expression of LCC enzyme to degrade PET films at 60 °C. This study showed that the expressed LCC enzyme converted 60% of PET film into soluble monomers after 14 days incubation. An important direction for future expression of PET biocatalyst using *C. thermocellum* is desperately needed.

3.3. Biocatalysts for the recycling of PET hydrolysates

TPA and EG, the main constituent monomers of PET, are produced as the main degradation products of PET with esterases. Microorganisms having the metabolic pathway for these compounds could degrade these monomers. TPA could be degraded to protocatechuic (PCA) by microorganisms pathway encoded by the tph enzymes. First, TPA dioxygenase TphA1A2A3 converts TPA to 1,6-dihydroxybicyclohexa-2,4-diene dicarboxylate (DCD). After that, DCD will be converted to PCA by the action of 1,2-dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylate dehydrogenase TphB (Alvador et al., 2019). These biocatalysts were found in β-proteobacteria *Comamonas testosteroni* YZW-D, actinomycete *Rhodoquadus* sp. strain DK17, and *Comamonas* sp. strain E6 (Choi et al., 2005; Saso et al., 2006; Wang et al., 1995). Acetogens, organisms that utilize the acetyl-CoA pathway to synthesize acetate, can oxidize EG to ethanol and acetaldehyde that will be converted after that to acetate via acetyl-CoA like in *Acetobacterium woodii* (Trifunovic et al., 2016). Microorganisms such as *Pseudomonas aeruginosa* and *Pseudomonas putida* can convert EG to glyoxylate via the action of the periplasmic alcohol dehydrogenase funnelled directly to the Krebs cycle via isocitrate or via proxanediol oxidoreductase in case of *E. coli* (Luo and Lee, 2017; Mückschel et al., 2012). It has been reported that most organisms that have the ability to degrade TPA are also able to degrade EG, which means that effective usage of PET hydrolysis products could be performed using the same microorganism. Recently, the bioconversion of
Table 2
Summary of PET biocatalysts heterologously expressed in E. coli and their microbial sources. Biocatalysts’ gene bank code, protein data bank (PDB) code, molecular weight (MW), substrate, degradation products and temperatures, specificity toward p-nitrophenyl esters, and kinetic parameters toward p-nitrophenyl butyrate (pNPB) were indicated.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Microbial source</th>
<th>Genbank code</th>
<th>PDB code</th>
<th>MW (kDa)</th>
<th>PET used</th>
<th>Degradation product</th>
<th>Degradation temperature(°C)</th>
<th>pNP-Ester Specificity (k_{cat}/K_{m})</th>
<th>Kinetic parameters (pH, K_{m}, thermostability, t_{1/2})</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ideonella sakaiensis PETase</td>
<td>Ideonella sakaiensis</td>
<td>GAP38373.1</td>
<td>5XG0,5XY, S5XZ,5XH2, 5XH1,5XH, SYN68AN</td>
<td>28.6</td>
<td>lcPET (1.9% ct.) and bottle-grade hcPET</td>
<td>TPA, MHET, EG</td>
<td>20-45</td>
<td>ND</td>
<td>ND</td>
<td>(Han et al., 2017; Yoshida et al., 2016)</td>
</tr>
<tr>
<td>Thc_Cut1</td>
<td>Thermobifida cellulolytica DSM44535</td>
<td>ADV92526.1</td>
<td>S5UI</td>
<td>29.4</td>
<td>PET film (37% ct.)</td>
<td>MHET, TPA, BA, HEB</td>
<td>50</td>
<td>C_{2}</td>
<td>K_{m} (pNPB):1.48 mM</td>
<td>(Herrero Acero et al., 2011)</td>
</tr>
<tr>
<td>Thf42_cut1</td>
<td>Thermobifida fusca DSM44342</td>
<td>ADV92528.1</td>
<td>4CG3</td>
<td>29.6</td>
<td>PET film (37% ct.)</td>
<td>MHET, TPA, BA, HEB, MHET</td>
<td>50</td>
<td>C_{2}</td>
<td>K_{m} (pNPB):2.10 mM</td>
<td>(Herrero Acero et al., 2011)</td>
</tr>
<tr>
<td>Tha_Cut1</td>
<td>Thermobifida alba DSM43185</td>
<td>ADV92525.1</td>
<td>ND</td>
<td>28.1</td>
<td>3PET</td>
<td>TPA, BA, HEB, MHET</td>
<td>50</td>
<td>C_{2}</td>
<td>K_{m} (pNPB):1.53 mM</td>
<td>(Ribitsch et al., 2012b)</td>
</tr>
<tr>
<td>Thb_Est</td>
<td>Thermobifida halotolerans DSM44931</td>
<td>AFA45122.1</td>
<td>ND</td>
<td>3PET</td>
<td>MHET, BA, HEB, MHET</td>
<td>TPA, BA, HEB, HEB, MHET</td>
<td>50</td>
<td>ND</td>
<td>K_{m} (pNPB):213 μM</td>
<td>(Ribitsch et al., 2012b)</td>
</tr>
<tr>
<td>Thh</td>
<td>Thermobifida fusca WP_011291330.1</td>
<td>ND</td>
<td>Bottle-grade PET (10% ct.)</td>
<td>ND</td>
<td>Bottle-grade PET</td>
<td>ND</td>
<td>65-70</td>
<td>ND</td>
<td>ND</td>
<td>(Müller et al. 2005)</td>
</tr>
<tr>
<td>BTA-1</td>
<td>Thermobifida fusca DSM43793</td>
<td>AJ810119.1</td>
<td>5ZOA</td>
<td>28.3</td>
<td>Bottle-grade PET</td>
<td>ND</td>
<td>65-70</td>
<td>ND</td>
<td>ND</td>
<td>(Kawai et al. 2014)</td>
</tr>
<tr>
<td>cutinase Cut190</td>
<td>Saccharomonospora viridis AHK190</td>
<td>AB0728484</td>
<td>4WFI</td>
<td>30.3</td>
<td>amorphous PET</td>
<td>TPA, MHET, EG</td>
<td>65</td>
<td>ND</td>
<td>Stable at 50-65 °C for 24 h</td>
<td>(Thumarat et al. 2012)</td>
</tr>
<tr>
<td>cutinase Est119</td>
<td>Thermobifida alba AHIK119</td>
<td>BAK48590.1</td>
<td>3VIS</td>
<td>30</td>
<td>PET film</td>
<td>TPA</td>
<td>50</td>
<td>C_{6}</td>
<td>pH: 6, K_{m} (pNPB): 3.41 mM, stable at 60 °C Stable at 50 °C for 48 h t_{1/2} (85 °C) = 1 h</td>
<td>(Ribitsch et al., 2012b)</td>
</tr>
<tr>
<td>HIC</td>
<td>Humicola insolens 4OYY</td>
<td>lcPET (7% ct), PET (35% ct.)</td>
<td>40YY</td>
<td>TPA, BA, HEB, MHET</td>
<td>TPA, BA, HEB, HEB, MHET</td>
<td>50</td>
<td>C_{6}</td>
<td>pH: 8.5, T_{1/2} 86.2 °C, K_{m} (pNPB): 2.10 mM</td>
<td>(Ribitsch et al., 2012b)</td>
<td></td>
</tr>
<tr>
<td>TfCut1</td>
<td>Thermobifida fusca KW3 CBY05529.1</td>
<td>PET nanospheres</td>
<td>35.8</td>
<td>PET nanospheres</td>
<td>MHET, BA, TPA, HEB, MHET</td>
<td>55-65</td>
<td>ND</td>
<td>ND</td>
<td>(Herrero Acero et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>TfCut2</td>
<td>Thermobifida fusca KW3 CBY05530.1</td>
<td>4CG1, 4CG2, 4CG3</td>
<td>30.8</td>
<td>PET nanospheres</td>
<td>MHET, BA, TPA, HEB, MHET</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>(Sulaiman et al. 2014)</td>
<td></td>
</tr>
<tr>
<td>LCC cutinase</td>
<td>Leaf-branch compost metagenome</td>
<td>HQ704839</td>
<td>4 EB0</td>
<td>28</td>
<td>Amorphous PET film</td>
<td>MHET, TPA, EG</td>
<td>50-70</td>
<td>C_{4}</td>
<td>pH: 8.5, K_{m} (pNPB): 88.8 ± 12.8 μM, stable at 55 °C for 60 min Stable at 50 °C Activity at 50 °C after 60 min ND</td>
<td>(Wei et al. 2014)</td>
</tr>
<tr>
<td>Tcur1278</td>
<td>Thermomonospora curvata DSM43183</td>
<td>ACY96861.1</td>
<td>ND</td>
<td>LePET Nanoparticles</td>
<td>ND</td>
<td>60</td>
<td>ND</td>
<td>pH: 8.5, K_{m} (pNPB): 83.1 ± 11.1 mM, lost 60% of its activity at 50 °C after 60 min ND</td>
<td>(Wei et al. 2014)</td>
<td></td>
</tr>
<tr>
<td>Tcur0390</td>
<td>Thermomonospora curvata DSM43183</td>
<td>ACY959991.1</td>
<td>ND</td>
<td>LePET Nanoparticles</td>
<td>ND</td>
<td>50</td>
<td>ND</td>
<td>pH: 8.5, K_{m} (pNPB): 2500 ± 100 μM</td>
<td>(Ribitsch et al. 2011)</td>
<td></td>
</tr>
<tr>
<td>Clostridium botulinum Cbotu_EstA</td>
<td>ATCC35502</td>
<td>KP859619</td>
<td>5AH1</td>
<td>ND</td>
<td>PET film</td>
<td>MHET, TPA, EG</td>
<td>50</td>
<td>ND</td>
<td>ND</td>
<td>(Biundo et al., 2016)</td>
</tr>
<tr>
<td>Bacillus subtilis BeStB</td>
<td>ATCC35502</td>
<td>ADH43200.1</td>
<td>3PET</td>
<td>TPA, BA, MHET</td>
<td>TPA, BA, HEB, MHET</td>
<td>40-45</td>
<td>ND</td>
<td>K_{m} (pNPB):2500 ± 100 μM</td>
<td>(Ribitsch et al. 2011)</td>
<td></td>
</tr>
</tbody>
</table>

Ct., crystallinity; hcPET, high crystallinity PET; 3PET, bis(benzoyloxyethyl) terephthalate.
re-cycled PET monomers to value-added bioplastic poly-
hydroxalkanoate by *P. putida* KT2440 was demonstrated (Wierckx et al. 2015). This opens the field toward the production of bio-plastic and other value-added products from PET’s enzymatic hydrolysates.

### 4. Challenges in PET biodegradation process

One of the main bottlenecks in PET biodegradation is the hydrophobic force between the PET surface and substrate which hinders this process (Kawai et al. 2019). Therefore, surface hydrophilization of PET is a significant process required for enzymatic PET degradation. Enhancement of rapid degradation rate, anti-pilling behavior, wettab-
ility, and dyeability could be performed via PET surface modification. The ends of polymer chains on the PET surface are expected to protrude, or part of it may form a loop (Oda et al. 2018). Surface hydrophilicity could be increased through the hydrolysis of these loops to carboxylic acid and hydroxyl residues. PET hydrolyzing enzymes could be divided into PET hydrolases and PET surface modifying enzymes (Müller et al. 2005). PET inner block can be significantly degraded via PET hydrolases with a definite change using Scanning Electron Microscopy (SEM). While surface hydrophilization level of PET using PET surface modifying enzymes cannot be observed by SEM. In brief, PET biocatalysts can be used for surface degradation while the PET building block cannot be significantly degraded via PET surface modifying enzymes (Kawai et al. 2019).

Crystallinity, hydrophobicity, PET molecular weight, the flexibility of the polymer chain, surface topography, and hydrolysis reaction temperature are considered the most aspects affecting PET degradation (Tokiwa et al., 2009; Wei and Zimmermann, 2017). The stiffness of PET, produced by the aromatic TPA building blocks, is a primary reason of PET low biodegradability. The glass transition temperature value (Tg) of PET at which polymer chain can dissociate is about 80 °C (Kikkawa et al. 2004). The Tg value of PET during the enzymatic hydrolysis reaction is about 65 °C (Kawai et al. 2014) due to the involvement of water molecules between polymer chains; this will cause flexible, random, and enzyme accessible polymer chains. Therefore, it is believed that faster PET degradation rates could be performed by increasing the tempera-
tures of the enzymatic hydrolysis reaction (Oda et al. 2018). For the previous reasons, strategies for producing thermos-stable PET hydrolyzing enzymes are required. Furthermore, the minimal reactive C-C bonds in the backbone of polyesters are considered as one of the major obstacles of the biodegradation process. Recently, many PET hydrolyzing enzymes have been discovered, but their ability to utilize PET monomers as a carbon source for the microbial microorganisms had not been approved.

Improving PET surface properties via its functionalization using PET biocatalysts is very promising for plastic waste management. These could be achieved by introducing functional groups to PET surface in order to increase its hydrophilicity and subsequently can be used for adhesion with specific materials or covalent binding with specific mole-
cules. Single and double *T. fusca* cutinases were used for surface hy-
drolysis of PET fabrics. One of these mutants created ample space around the active site of cutinase while the other mutant increased the hydrophobicity around the binding site with improvement in the sub-
strate attachment. This PET modification enhanced the degradation rate, and the TPA concentration was elevated in the reaction medium when compared with the activity of the wild type (Silva et al. 2011).

### 5. Bioengineering intensification strategies for improving production and catalytic activity of PET biocatalysts

#### 5.1. Protein engineering-Based PET biocatalysts improvement

Protein engineering is playing an essential role in enhancing and constructing biological systems using modern biological tools to in-
crease our knowledge in basic life sciences that consequently will widen its use in the industrial applications such as bioremediation of polluted sites, degradation, and recycling of plastics waste. This could be achieved by enhancing enzymes expression level, increasing the *Km* of the enzyme, discovering more biosynthetic pathways and reaction mecha-
nisms. In fact, this is not an easy task as the low expression level; poor solubility and thermal stability are the main properties of the hetero-
logously expressed enzymes. Furthermore, the synthetic gene circuit components and the genomic context need to be optimized and modi-
fied, respectively. Mutagenesis strategies (i.e. random mutagenesis and site-directed mutagenesis), genome editing, simultaneous amino acid substitutions, computational genomics, structural biology and directed evolution are the recent protein engineering strategies that have been adopted to address a wide range of enzyme engineering. Artificial scaffolding is another promising tool for enhancing the catalytic activity of enzymes by creating enzyme clustering and substrate channels. Furthermore, proteins fusion is essential for enhancing the catalytic activity of PET biocatalysts. Herein, we will critically discuss furtherly how protein engineering tools could enhance plastic degradation en-
zymes expression, catalytic activity, and thermostability (Fig. 2).

#### 5.2. Experimental approaches for PET biocatalysts candidate discovery

The discovery of enzyme candidates and microorganisms with PET biodegradation abilities and strategies for developing an efficient system to enhance the enzyme’s catalytic activity is in need. The PET bottle-recycling factories and landfills are enriched sites with PET polymers and could be a primary source for the discovery of PET degrading mi-
croorganisms. Applying microbial biotechnology strategies are prom-
ising for identifying novel PET biocatalysts from microbial biodiversity-
rich environments. Herein, the microbial communities that utilize the PET as the main carbon source will stimulate the microbial cell to ex-
press enzymes for PET degradation and reaction intermediate hydroly-
sis. Whole-genome analysis, RNA-Seq analysis, heterologous expression of hypothesized genes, phylogenetic analysis, protein purification, and identification are the main approaches used to discover the enzymes responsible for PET biodegradation. The PET degradation process can then be monitored using SEM and Reverse-phase high-performance liquid chromatography (HPLC). If successful, the identified microor-
ganisms could be promising candidates for a future sustainable closed-
loop PET waste recycling.

Yoshida et al. (Yoshida et al. 2016) collected two hundred fifty environmental specimens from plastic bottles recycling site. The research team screened the microbial communities from the enriched samples using PET film as a major carbon source. SEM showed morphological changes in PET film after twenty days with the microbial communities isolated from the environmental samples. *Idonella sakaiensis* 201-F6 was the responsible microorganism for degrading and assimilating PET. Transcriptomic analysis based on homologous en-
zymes to identify the responsible enzymes of PET degradation was performed by growing *I. sakaiensis* 201-F6 on different substrates such as disodium terephthalate (TPA-Na), BHET, PET film, or maltose. TPA catabolic genes were efficiently expressed when the substrates, TPA-Na, BHET, or PET film, were used in the culture medium, separately. However, the highest transcriptional level was observed for PETase encoding gene when PET film used in the culture medium.

The discovery of PETase produced by *Idonella sakaiensis* 201-F6 (IsPETase) (Yoshida et al. 2016) encourages scientists to search for more potential homologs genes due to its efficient and environmentally friendly characteristics. Almeida et al., (Almeida et al. 2019) developed an in silico-based screening approach to search for PETase homologs among some terrestrial and marine isolates. Three potential PETase-like enzymes from the marine-sponge associated Streptomyces isolates were successfully identified. The amino acid and biochemical properties similarities between SM14est-PETase and IsPETase were 41% and 19%, respectively. The three-dimensional (3D) structure of SM14est-PETase was compared with IsPETase. This revealed that IsPETase has a
Bioengineering of PET degrading enzymes

Fig. 2. Scheme of the workflow of bioengineering approaches role in the discovery, degradation mechanism elucidation and catalytic properties improvement of PET degrading enzymes. PET biocatalysts’ discovery depends on the clustering analysis of genes isolated from enriched PET cultures, rational design, and the heterologous expression of these genes for enhanced production and purification steps. The degradation mechanism elucidation includes directing different mutations to target biocatalyst gene, protein crystallization, and molecular docking. Mutagenesis, insertion of Disulfide Bridge or metal ions, and protein fusion are well developed to enhance biocatalysts’ catalytic activity. Direct enzyme immobilization or whole-cell immobilization is necessary for biocatalyst re-usability and its cost reduction.

Fig. 3. Schematic diagram showing complete PET degradation mechanism using PETase and MHETase enzymes. PET interacts with the active site of the biocatalyst via PET’s carbonyl group and its aromatic ring that stacks with tryptophan residue of the biocatalyst. This interaction causes the release of TPA, BHET, and MHET. After that, MHET can be hydrolyzed by the MHETase enzyme giving EG and TPA hydrolysates.
disulfide bond between Cys273 and Cys289 and another disulfide bond between Cys203 and Cys239, which is responsible for the enzyme activity because it is very adjacent to the enzyme’s active site (Liu et al., 2018), while SM14est-PETase did not have any of them. These disulfide bonds are very characteristic for IsPETase and could not be observed at other PETase-like cutinases, which already showed high thermostability and catalytic activity without those bonds (Kawai et al. 2014). Conversely, the SM14est-PETase and IsPETase sharing a similar mode of action toward plastic as their substrate.

5.3. Mutagenesis: An effective approach for degradation mechanism elucidation of newly discovered PET biocatalysts

The discovery of PET hydrolyzing enzymes recently draw researchers’ interest in establishing effective strategies for enhancing the catalytic activity and thermostability of these biocatalysts. The strong binding between the active site of PET hydrolyzing enzymes that have a substrate-binding cleft, and PET surface using its flat hydrophobic surface is the crucial point for effective PET biodegradation. IsPETase follows the same mechanism of cutinases for PET degradation. However, the difference comes from the presence of Trp185 residue near the catalytic triad, which facilitates the binding of the enzyme with PET and the release of the degradation product. The π-staking force of Trp 185 in B conformation stabilizes the hydrophobic interaction between PETase and PET surface. The degradation process occurs in two steps, nick generation step and final digestion step (Fig. 3). During the reaction of the enzyme with the PET substrate, the PET carbonyl group will be adjacent to the catalytic cleft of the enzyme. This will stimulate the nucleophilic attack between them. At the same time, the oxyanion hole stabilizes the tetrahedral intermediate. One ester bond will be cleaved during the nick generation step causing the formation of a nick in PET. After that, a strong binding between the benzoic group of PET and the Trp 185 residue of the enzyme will occur via π-stacking interaction, causing the release of the product from the active center. Furthermore, two PET chains will be generated with different edges, one with TPA-edge liberated from substrate I and MHET-edge liberated from substrate II. After that, the two PET chains will be degraded to MHET monomers during the final digestion step. Continuous degradation of TPA-edge and MHET-edge will produce the four degradation products: MHET, TPA, BHET, and EG. BHET will be furtherly degraded into MHET and EG, and finally, a significant amount of TPA will be accumulated beside the MHET and EG due to PET degradation. After that, MHET can be degraded by the MHETase enzyme.

PETase enzyme belongs to the α/β hydrolase superfamily, and its active site consists of Ser160, His237, and Asp206 residues. Herein, during the degradation process, the nucleophilic attack occurs between Ser160 and the carbonyl group of PET, which offer a more binding affinity with PET plus the first binding with Trp 185. In addition, the core structure of PETase consists of seven α-helices and nine β-strands (Joo et al. 2018). This was elucidated by replacing Ser160, His237, and Asp206 residues with Ala that interestingly showed a complete loss of the catalytic activity of the three variants (Joo et al. 2018).

The ability of two intramolecular disulfide bridges formation (DS1 and DS2) is distinguished for the PETase enzyme. Mutagenesis studies revealed that the integrity of PETase catalytic triad is correlated with DS1 for improving the activity of the enzyme. Furthermore, DS1 allows loop close proximity to the PETase binding site (Austin et al., 2018; Fecker et al., 2018). This loop is very flexible and three residues longer than other PET biocatalysts causing the superior activity of PETase at room temperature and the formation of subsite for the effective binding with PET surface (Han et al., 2017). On the other hand, the DS2 disulfide bond is adjacent to the C-terminus of the enzyme and far from its active site causing the biocatalyst’s structural stability. Therefore, modifying amino acid residues which are not adjacent to the enzyme’s active site could significantly enhance PET-hydrolytic activity by more than 30% (Joo et al. 2018).

As mentioned before, the PETase enzyme is characterized by the existence of Trp185 adjacent to the active site, and this adopts the B conformation of the biocatalyst as Trp 185 movement opens the active site cleft (Austin et al., 2018). The serine residue in PETase was replaced with histidine residue in other PET biocatalysts, causing block for the tryptophan residue in the C conformation. The replacement of the native serine to a histidine residue in PETase confirmed the importance of this residue in this enzyme’s catalytic activity as the histidine variant showed a significant loss of the activity (Austin et al., 2018; Han et al., 2017). Therefore, residues that are not directly attached to the substrate could also play a crucial role in enzyme catalysis, such as S185 (Chen et al. 2018).

5.4. Mutagenesis: An effective approach for dynamic and thermostable PET biocatalysts production

Enzyme design is a powerful tool depend on the structural similarity between enzymes to take the favorable characteristics from one enzyme to another using site-directed mutagenesis. Mutagenesis has several effective approaches for enhancing the production, thermostability, and catalytic activity of PET hydrolyzing enzymes. Directing specific mutations or new catalytic residues at the catalytic active site of enzymes or replacing their divalent metal binding site with a disulfide bridge can alter the enzyme mechanism and function. Furthermore, this process became very handy due to the availability of computer-aided enzyme engineering. Solving the crystal structures of LC cutinases (LCC), Tf cutinase, and IsPETase opened the field toward thermostable-engineered biocatalysts (Fig. 4). Tournier et al. (Tournier et al. 2020) investigated the mode of binding of LCC enzyme with 2-HE(MHET)3 substrate via molecular docking and enzyme contact surface analysis. Subsequently, he discovered that the substrate prefers the binding with an elongated and predominantly hydrophobic groove. In the case of IsPETase enzyme, the substrate binds to a shallow cleft of the active site of the biocatalyst via hydrophobic interaction that is stabilized by the π-staking force with the Trp 185 residue present near the catalytic triad. To increase the thermostability of the LCC enzyme and reduce the reaction cost of the enzyme, the divalent metal binding site could be replaced with Disulfide Bridge. This replacement was performed by Tournier et al. (Tournier et al. 2020) and found that the D238C/S283C variants are showing a melting temperature (94.5 °C) higher by 9.8 °C than the wild type. Furthermore, to enhance the specificity of the LCC enzyme, the mutations F243I and F243W were separately introduced to the enzyme causing activity to restore by 122% and 98%, respectively, compared with the wild type. After that, LCC enzyme scale-up production and PET degradation rate were enhanced by introducing the Y127G mutation to the previously mentioned variants causing 90% PET degradation in 10.5 h and 9.3 h for F243W/D238C/S283C/Y127G and F243I/D238C/S283C/Y127G variants, respectively.

The replacement of Arg280 with Ala enhanced the degradation rate of PET using IsPETase by 22.4% and 32.4% in 18 h and 36 h, respectively (Joo et al. 2018). Mutations in IsPETase binding pockets have different influences on BHET and PET polymers due to the high accessibility of the PET polymers toward the binding pocket of IsPETase when compared with BHET. Directing the specific mutations, Y58A, W130A, W130H, and A180I, to widen the binding center space in IsPETase or enhancing the aromaticity on the edge of the binding cleft using the specific mutation S185H showed a significant enhancement in IsPETase catalytic activity at 30 °C and pH 9 (Liu et al. 2018). Mutations around the substrate-binding groove, R61A, L88F, and I179F, were elucidated to be effective for enhancing PETase catalytic activity by 1.4, 2.1, and 2.5 fold higher than the PETase wild type, respectively. In another study, the authors enhanced the thermal stability of PETase enzyme by directing S121E/D186H/R280A mutations to stabilize β-β connecting loop and to extend substrate Ic. These mutations enhanced the thermal stability by 8.81 °C and the PET degradation process was more effective by 14 times at 40 °C when compared with the wild type (Son et al. 2019).
Fig. 5 indicates the mutation effect of replacing the Isoleucine amino acid (Ile179) by the more hydrophobic phenylalanine residue (Ile179/Phe) that significantly enhanced the catalytic efficiency of PETase enzyme toward the 2PET substrate at 30 °C. The phenylalanine residue allowed stronger binding between the PETase and the substrate by enhancing the aromaticity on the edge of the binding pocket (Ma et al. 2018).

One of the most effective strategies in protein engineering is the active site modification via site-directed mutagenesis. This strategy showed a significant effect in substrate reshaping, co-factor specificity, and reactivity (Cedrone et al. 2000). Active site modification of TfU_0883 elevated the enzyme hydrophobicity and enhanced the biodegradation of PET (Silva et al. 2011). Herrero Acero et al. (Herrero Acero et al. 2013) have found that site-directed mutagenesis has increased the catalytic activity of T. cellulosilytica cutinase by three-fold higher than the wild type. Mutagenesis not only enhanced the catalytic activity of cutinase, from T. fusca, 2.7 fold higher than the wild type but also mitigated the simultaneous product inhibition by MHET (Wei et al. 2016). Table 3 summarizes the recent achievements in enhancing the catalytic properties of PET hydrolyzing enzymes via mutagenesis.

5.5. Rational design of biocatalyst mutants

The rational design of biocatalysts is a promising future trend in the enzyme-engineering field. It depends on the in-depth knowledge of enzyme structure, computational simulations, and enzyme’s catalytic performance. Recently, different tools were developed to engineer PET biocatalysts rationally and to re-shape enzyme specificities such as homology modelling and molecular docking. Hydrophobic interaction between the enzyme’s surface and the space around the active pocket of the enzyme is crucial for enzyme attachment and reaction activity (Araújo et al. 2007). PET fiber degradation rate was improved by subjecting double mutation (Q132A/T101A) to the active pocket of TfU_0883 cutinase due to the affinity enhancement of cutinase toward the hydrophobic substrate (Silva et al. 2011). Moreover, PET degradation was enhanced by tailoring the fungal polyester hydrolase for wider substrate active pocket and hydrophobicity improvement (Araújo et al. 2007). To achieve maximum exploitation of PET biocatalysts, conserved amino acid residues, and electrostatic surface are other rationales that need further investigations. The replacement of amino acid residues in the active pocket of TfCut2 cutinase with the highly conserved amino acid residues of LCC cutinase significantly improved PET degradation at 65 °C (Wei et al. 2016). Intriguingly, after the modelling and comparison of Tfcut2 and LCC cutinases from Thermobifida fusca and L. cellulosilytica DSM44535, Acero et al. (Herrero Acero et al. 2013) discovered that the electrostatic and hydrophobic properties of the surface near the active pocket of both enzymes are different and this is the main reason of the variation in their degradation efficiency. The replacement of A19 and A29 amino acid residues, positively charged, with uncharged Ser and Asp amino acid residues improved the degradation efficiency for 3PET and PET. At the same time, the replacement of the uncharged Glu65 by the negatively charged glutamic acid caused a massive loss in the enzyme’s catalytic activity. Pretreatment strategies of PET using anionic surfactants have been recently studied (Furukawa et al., 2018; Sagong et al., 2020). The activity of PETase was enhanced 120 times after the treatment of the low-crystalline PET film with anionic surfactants. The thickness of the PET film was decreased by 22% after 36 h degradation process at 30 °C. During this condition, surfactants enhanced the binding of the hydrophilic PETase with the hydrophobic surface of PET as shown in Fig. 6. The surfactant concentration used through this method of degradation was extremely low (0.005%). Mutagenesis also played an important role in the previous mentioned reaction. Furukawa and his team (Furukawa et al. 2018) introduced single mutations in the substrate binding pocket of PETase by replacing the single cationic amino acid residue adjacent to Serine residue. The catalytic triad of IsPETase constitutes of Ser131, His208, and Asp176. (b) Substrate binding site in IsPETase is open and broad with an extended loop but very narrow and deep in Tfcut2 and LCC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
acid by an anionic one such as R53E, R90E, and K95E. These mutations effectively enhanced and accelerated PET hydrolysis by the aid of anionic surfactant. The surfactant helped in the alignment of PET surface toward the active site of the enzyme. Although the degradation process was performed at 30°C, the degradation result was equal to those that were performed with thermostable enzymes at 70°C or greater. So, designing more surfactant molecules is urgently needed for enhancing PET biodegradation at ambient temperatures.

5.6. Enzymes fusion for higher catalytic performance

Despite of the recent advances in genetic engineering and enzyme fusion strategies, using them in enhancing the catalytic performance of PET hydrolyzing enzymes is not developed so far. Fusion enzymes is a promising strategy for reducing substrate diffusion and eliminating the toxicity of reaction intermediates. This strategy can be performed through linking a set of enzymes covalently at the same macromolecular scaffold or via direct cross-linking (Ricca et al., 2011; Schoffelen and van Hest, 2013; Sharshar et al., 2019). Moreover, PET hydrolyzing enzymes fusion with signal peptides, short peptides directing recombinant proteins toward the secretory pathway, is a significant strategy for enhancing their production and catalytic activity. NusA, *E. coli* transcription-termination anti-termination factor, maltose-binding protein, and glutathione-S-transferase (GST) are solubility enhancing fusion proteins, which were reported for their significant effect in improving the expression, and solubility of a large number of insoluble recombinant proteins (Shib et al. 2002). NusA was found to improve, especially the solubility of more abundant passenger proteins located at organelles (Korf et al. 2005). This strategy can solve the problem of biocatalyst expression in the form of inclusion bodies, one of the main obstacles in the heterologous expression in *E. coli* (Schilling et al. 2007).

Intriguingly, the secretion amount of the recombinant protein differs significantly according to the signal peptide fused with the recombinant protein (Peng et al. 2019). Moog et al. (Moog et al. 2019) used protein engineering to convert the diatom *Phaeodactylum tricornutum* into a beneficial alternative chassis for PET biodegradation. PETase R280A enzyme was fused with a signal peptide of *P. tricornutum* alkaline phosphatase (AP) for PETase expression in photosynthetic microalga *P. tricornutum* diatom into the surrounding culture medium. This fusion enhanced the secretion and production rate of PETase R280A significantly from the diatom. The produced PETase R280A enhanced the degradation rate and decreased the crystallinity level of PETG by 80 fold compared with the PET bottle at 30°C and salt water-based environment. These findings promote the generation of microbial cells capable of using PET as a carbon source. It can also develop further robust biocatalysts for PET degradation in the ocean.

5.7. Dual enzyme system for complete PET degradation

Complete degradation of the crystalline PET thick layer in an environment-friendly manner needs further investigations. MHET is a strong and competitive enzyme inhibitor, and its accumulation in the reaction medium during the degradation process is the main obstacle hindering the complete degradation of PET film. Physical methods to solve this problem by using an ultrafiltration membrane reactor was performed for the repeated elimination of the produced MHET (Andrici et al., 2010; Barth et al., 2015; Kim and Song, 2006). However, this method was disadvantageous due to the massive demand of medium buffer to keep a constant dilution rate in the reactor, and the degradation rate was still unsatisfactory (Obhion et al. 1984). Thus, finding an alternative solution to avoid the inhibitory effect and to enhance enzyme thermostability via protein engineering is urgently needed. The dual
enzyme system has shown to be a beneficial approach for complex polymer hydrolysis (Schoffelen and van Hest 2013). The recent discovery of the bacterial strain *Ideonella sakaiensis* 201-F6, indicates that PET film can be degraded completely via two steps using PETase and MHETase enzymes together. The synergistic activity of the two enzymes is needed for efficient PET biodegradation yielding TPA and EG, which can be used as a building block for a new round of PET synthesis (Palm et al. 2019). Tfcut2 from *T. fusca* KW3 and LC cutinase together showed 2.4 fold higher degradation rate of PET compared with the degradation rate from Tfcut2 only. Tfcut2 in the dual system continuously degraded the inhibitory product MHET proving the synergistic effect of this enzyme with LC cutinase for efficient PET degradation (Barth et al. 2016). Lipase B from *Candida antarctica* (CALB) and cutinase from *Humicola insolens* (HiC) together showed a complete PET degradation to TPA with high thermostability at 70°C. In contrast; HiC showed a restriction for the last step of the reaction when used alone (Carniel et al. 2017).

### Table 3

Examples of different directed mutations to improve the catalytic performance and productivity of PET hydrolases.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Chassis (ligand)</th>
<th>Substrate</th>
<th>Product</th>
<th>Mutation(s)</th>
<th>Biological effects (results)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>IsPETase</em></td>
<td><em>E. coli</em></td>
<td>PET</td>
<td>MHET, EG, TPA</td>
<td>S160A, D206A, H237A, Y87A, W185A, N241A, S238A, R280A, C203A, C239A</td>
<td>R280A increased PETase catalytic activity by 22.4% and 32.4% in 18 h and 36 h, respectively compared with <em>IsPETase</em> wild type</td>
<td>(Joo et al. 2018)</td>
</tr>
<tr>
<td><em>IsPETase</em></td>
<td><em>E. coli</em></td>
<td>PET</td>
<td>MHET, EG, TPA</td>
<td>W185A, S238F/W159H</td>
<td>The absolute crystallinity loss is 4.13% higher than <em>IsPETase</em> wild type</td>
<td>(Austin et al. 2018)</td>
</tr>
<tr>
<td>leaf-branch compost cutinase</td>
<td><em>E. coli</em></td>
<td>PET</td>
<td>TPA, EG</td>
<td>F243I/D238C/S283C/Y127G, F243W/D238C/S283C/N246M, F243W/D238C/S283C/Y127G, F243W/D238C/S283C/N246M</td>
<td>Achieved 90% of PET degradation into monomers, with a productivity of 16.7 g of terephthalate/L/h over 10 h. Improved melting temperatures by 9.8 °C higher than wild type LCC</td>
<td>(Tournier et al. 2020)</td>
</tr>
<tr>
<td><em>IsPETase</em></td>
<td><em>E. coli</em></td>
<td>PET</td>
<td>MHET, EG, TPA</td>
<td>Y58A, W130A, A180I, S185H</td>
<td>Enhanced the catalytic activity of <em>IsPETase</em> at 30 °C and pH 9.</td>
<td>(Liu et al. 2018)</td>
</tr>
<tr>
<td><em>Saccharomonospora viridis</em></td>
<td>AHK190 Cut190</td>
<td>PET</td>
<td>TPA, EG</td>
<td>R61A, L88F, I179F</td>
<td>Enhanced PETase catalytic activity by 1.4, 2.1, and 2.5 folds higher than the wild type. 179F mutant showed the highest degradation rate (22.5 mg.mol/L)</td>
<td>(Iia et al. 2018)</td>
</tr>
<tr>
<td><em>Saccharomonospora viridis</em></td>
<td>AHK190 Cut190</td>
<td>PET</td>
<td>TPA, EG</td>
<td>Q138A: D250C-E296C/Q123H/N202H</td>
<td>Enhanced the melting temperatures of the mutant enzyme and the degradation rate by more than 30% at 70 °C</td>
<td>(Oda et al. 2018)</td>
</tr>
<tr>
<td><em>Saccharomonospora viridis</em></td>
<td>AHK190 Cut190</td>
<td>PET</td>
<td>TPA, EG</td>
<td>Q138A, I224A</td>
<td>Q138A: Significantly increased the enzyme affinity (lower Km) and activity (higher Vmax and kcat) I224A: Significantly increased the enzyme activity (Vmax), but decreased its affinity (increased Km)</td>
<td>(Kawabata et al. 2017)</td>
</tr>
<tr>
<td><em>MHETase</em></td>
<td><em>E. coli</em></td>
<td>MHET</td>
<td>TPA, EG</td>
<td>S419G, S419G_F424N, W397A</td>
<td>Enhanced the enzyme affinity and activity toward BHET, W397A: enhanced the enzyme activity toward MHET Re-arranged the active site conformation.</td>
<td>(Palm et al. 2019)</td>
</tr>
<tr>
<td>carboxylic ester hydrolase</td>
<td><em>Pseudomonas aesterugini</em> VGX014</td>
<td>PET</td>
<td>BHET, MHE, TA</td>
<td>Y250S</td>
<td>Enhanced the enzymatic activity toward amorphous PET</td>
<td>(Bollinger et al. 2020)</td>
</tr>
<tr>
<td><em>IsPETase</em></td>
<td><em>E. coli</em></td>
<td>PET</td>
<td>MHET, EG, TPA</td>
<td>S214H-I168R15-W159H-S188Q-R280A-A180S-G165A-Q119Y-L117F-T140D</td>
<td>Enhanced <em>IsPETase</em> thermostability at 60 °C for 3 days.</td>
<td>(Cui et al., 2019)</td>
</tr>
</tbody>
</table>

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Fig. 6. Scheme showing the effect of PET surface hydrophilization by anionic surfactant for enhancing the binding with PETase enzyme. Updated from (Furukawa et al. 2018).
5.8. Incorporation of metal ions

Divalent metal-binding sites play a crucial role in improving hydrolases thermostability and catalytic behavior. The presence of calcium ion improves large conformational changes in the enzyme structure, which maintain the substrate-binding groove in an open conformation, and facilitates the enzyme binding with its substrate (Miyakawa et al. 2015). Furthermore, molecular dynamic simulations demonstrated that the higher thermostability of calcium-bound enzymes is due to less structure fluctuation of the bound enzyme than the unbound enzymes (Numoto et al. 2018). The side chains of three amino acid residues in cutinases usually form these binding sites with calcium ions. However, this binding site in LC-Cutinase enzyme is formed of one neutral residue (S283) and two acidic residues (E208 and D238). The thermal stability of the LC-Cutinase enzyme was significantly enhanced by 9.3 °C after adding 35 mM CaCl₂ (Sulaiman et al. 2014). The presence of calcium ions enhanced the $T_{1/2}$ value of LC-cutinase by 8.4 °C and reached 98 °C by increasing calcium ion concentration (Sulaiman et al. 2014). In the case of Cut190 cutinase from Saccharomonospora viridis AHK190, Ca$^{2+}$ ions bind with acidic amino acid surfaces and not with the active-site amino acids. Ca$^{2+}$ ions were very crucial for enhancing the thermostability of the wild-type and mutant Cut190. 300 mM Ca$^{2+}$ showed enzyme stability at pH 9 and 65 °C for 24 h and efficiently degraded different aromatic PET films (Kawai et al. 2014). Calcium ion, 10 and 100 mM, stabilized the tertiary structure of Bacillus stearothermophilus lipase (Kim et al. 2000). The presence of calcium ion significantly enhanced the catalytic activity and showed improvement in protein refolding of lipase from Pseudomonas Sp. MIS38. The calcium ion-binding motifs are located at the C-terminal domain of the enzyme (Amada et al. 2001). Two aspartic acid residues, Asp354 and Asp357, are also required for calcium ion binding in the case of lipase from Staphylococcus hyicus (Simons et al. 1999). TiCut2 enzyme from Thermobifida fusca showed a significant loss of its catalytic activity after incubation at 65.6 °C for 60 min, which hinders its application for PET hydrolysis at temperatures near to the $T_g$ of amorphous PET (Alves et al., 2002; Roth et al., 2014). Then et al. (Then et al. 2015) confirmed that Asp174, Asp204, and Glu253 residues are the binding resides for Ca$^{2+}$ and Mg$^{2+}$ cations in TiCut2 enzyme. Moreover, the melting points of TiCut1 and TiCut2 enzymes were increased by 11 and 14 °C after adding calcium and magnesium ions. Semi-crystalline PET films were efficiently degraded by showing a weight loss 12 and 9% after adding 10 mM calcium ion and 10 mM magnesium ion at 65 °C. Polyester esterase Est119 from Thermobifida alba showed thermo-stability enhancement in the presence of 500 mM calcium ion and Glu292, Glu213, and Asp243 residues were responsible for major Calcium ion binding site (Kitadokoro et al. 2012). Further investigations are required to examine whether metal ions are relevant to PET degradation by other hydrolases.

5.9. Enzyme immobilization strategies for stable and re-usable industrial PET biocatalysts

Immobilization technology is a powerful tool for the biocatalysts industry due to its significant advantages in the industrial applications (Samak et al. 2018b). Immobilization tools are very effective for enhancing the catalytic activity, stability, specificity, and selectivity of enzymes. This enhancement in enzyme characteristics is related to the alteration in enzyme structure, causing favorable structural modifications. Furthermore, the immobilization process can significantly lower the production cost due to the re-usability of the immobilized biocatalysts, as shown in Fig. 7. Immobilization methods are variants according to the type of immobilization support, linkers, and immobilization strategies such as entrapment, adsorption, covalent and non-covalent interaction.

To date, the immobilization supports and techniques used for PET biocatalysts are very limited, which hindering taking the benefits of these biocatalysts for PET waste green recycling. Immobilization supports such as reduced and non-reduced graphene oxide nanosheets, Fe₃O₄ nanoparticles, and nanoflowers and linkers such as...
Polyethylenimine, Na, No-Bis(carboxymethyl)-L-lysine hydrate (NTA-NH₂) and carbodiimide 1-ethyl-3-(dimethylaminopropyl) carbodiimide (Kumar et al., 2020; Samak et al., 2020, 2018b; Xia et al., 2016) could be used as an effective support and linkers for single or double enzyme immobilization for complete PET bioconversion into value-added products such as TPA.

Table 4 indicates the enhancement of PET degrading enzyme’s catalytic activity, thermal stability, storage stability, and re-usability via enzyme immobilization. Glutaraldehyde-activated chitosan beads were used to covalently immobilize T. fusca cutinases with 74% adsorption capacity and showed optimal activity at 55 °C, pH 8. The immobilized enzyme showed operational stability in the range of 45 °C to 70 °C, reused for ten consecutive cycles with 80% efficiency, and the catalytic activity was decreased after 13 days storage to reach 50% (Ilegde and Veeranki 2014). Thielavia terrestris cutinase was physically immobilized on the Lewatit VP OC 1600 cross-linked polymer, showed high thermal stability at 80 °C and retained about 64% of its catalytic activity at 90 °C (Su et al. 2018). T. cellulosa cutinase was covalently immobilized on Amber chelated with ferric ions via His-tag binding. The immobilized enzyme showed high catalytic activity toward the cleavage of C₆ - C₄ ester-diel bond with a monomer conversion rate up to 78% and the reusability reached to 24 h cycles with more than 94% residual activity (Pellis et al., 2017). Dual enzymes, TICut2 from Thermohibidida fusca KW3 immobilized on SulfoLink coupling resin, and free LC-cutinase were used for complete PET biodegradation. The dual enzymes showed a 2.4 fold higher degradation rate of PET films at 60 °C compared to the degradation rate using the free TICut2 enzyme. The immobilized enzyme enhanced further degradation of the produced MHET to produce TPA and EG (Barth et al. 2016).

5.10. Whole-cell immobilization

Whole-cell viable microorganism immobilization is another method to enhance plastic biodegradation. Viable bacterial or fungal cells could be immobilized in or on the surface of the polymerized matrix via physical confinement or chemical binding with cellular adherence mechanisms for a more effective plastic degradations process. Immobilization here will control microorganisms’ growth by controlling the porosity degree, reticulation size, hydration rate in organic and inorganic solutions (Huang et al., 2012; Li et al., 2011; Mu et al., 2017; Shan et al., 2005). Herein, the immobilization of pure strains is favorable, viable strains have to be isolated first, then purified and immobilized to produce long-lasting viable biocatalysts as their catalytic activities will be well-preserved.

Whole-cell immobilization has many advantages such as long-term metabolic activity, high cell mass loading capacity, economical viable cells re-usability, genetic stability improvement, cost-effective, sustainability, easy separation of immobilized cells from degradation medium, resistance to high pH, temperatures, and solvents and high operational stability (Gardin and Paus 2001).

Effective immobilization of live cells depends on the inner structure of the matrix; the spherical matrix, for example, is more favorable or rapid production with control of particle diameter. Adsorption, Entrapment inside different polymeric supports, encapsulation within spherical permeable membranes, and covalent binding are different methods for whole-cell immobilization (Bayat et al. 2015). Adsorption method depends on binding the viable cells with water-insoluble particles, this produces electrostatic interaction between the particles and microorganisms’ cellular surface (Zur et al. 2016). Entrapment is the most common method used for whole viable cell immobilization, and polymeric hydrogels, such as polyacrylamide gel (PAA) and calcium alginate, are the widely used carriers so far (Trelles and Rivero 2013). PAA polymeric hydrogels protect the immobilized cells from any hazardous shocks produced by chemical or mechanical environmental factors, so they prevent the loss of cellular activity and facilitate the diffusion of substrate and product (Trevors 1992). Fungi spores also could be entrapped using PAA to induce germination in situ in the presence of cultural nutrients (Favel et al., 1985; Wang et al., 2018). Entrapment using calcium alginate may be unfavorable even though it is a mild process with no toxic and heating requirements, but the enzymatic cellular activity usually depleted through its re-usability (Buscher et al. 1995). In this regards, using the whole-cell immobilization method for PET biocatalyst will open the field for more dynamic and robust enzymes that could enrich the industrial applications of these enzymes and fill the gap to move forward into reality.

6. Economic analysis

The economic feasibility of PET waste green recycling depends on cost-effective large-scale productivity of PET hydrolases, the required time for PET bioconversion, and the useful applications of degradation byproducts. As noted earlier, the mechanical and chemical degradation

<table>
<thead>
<tr>
<th>Name of Enzyme</th>
<th>Immobilization Support</th>
<th>Immobilization method</th>
<th>Thermal stability</th>
<th>Optimum pH</th>
<th>Re-usability</th>
<th>Storage stability</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. fusca Cut1</td>
<td>glutaraldehyde-activated chitosan beads</td>
<td>Covalent</td>
<td>Optimum temperature 55 °C</td>
<td>8</td>
<td>80% after 10 reuse cycles</td>
<td>50% after 13 days</td>
<td>(Ilegde and Veeranki 2014)</td>
</tr>
<tr>
<td>carboxylesterase TICa from T. fusca used with LCC enzyme</td>
<td>SulfoLink resin</td>
<td>covalent</td>
<td>Retained 94% of activity at 60 °C</td>
<td>8</td>
<td>ND</td>
<td>ND</td>
<td>(Barth et al. 2016)</td>
</tr>
<tr>
<td>Aspergillus oryzae Cutinase</td>
<td>Lewatit VP OC 1600</td>
<td>Physical</td>
<td>Retained full activity for 1 h at 80 °C</td>
<td>5–9</td>
<td>ND</td>
<td>ND</td>
<td>(Su et al. 2018)</td>
</tr>
<tr>
<td>Humicola insolens Cutinase</td>
<td>Lewatit VP OC 1600</td>
<td>Physical</td>
<td>Activity decreased after 2 h by 50% at 80 °C</td>
<td>5–9</td>
<td>ND</td>
<td>ND</td>
<td>(Su et al. 2018)</td>
</tr>
<tr>
<td>Thielavia terrestris Cutinase</td>
<td>Lewatit VP OC 1600</td>
<td>Physical</td>
<td>Retained 77% of enzyme activity after 2 h at 80 °C</td>
<td>5–9</td>
<td>ND</td>
<td>ND</td>
<td>(Su et al. 2018)</td>
</tr>
<tr>
<td>Thermohibidida cellulosa lycyta cutinase 1</td>
<td>Opal</td>
<td>covalent</td>
<td>57% monomer conversions at 21 °C</td>
<td>7</td>
<td>Conversion decrease 42% after 24 h reaction (3 cycles)</td>
<td>ND</td>
<td>(Pellis et al., 2017)</td>
</tr>
<tr>
<td>Thermohibidida cellulosa lycyta cutinase 1</td>
<td>Coral</td>
<td>covalent</td>
<td>76% monomer conversions at 21 °C</td>
<td>7</td>
<td>Increase 58% after 24 h reaction (3 cycles)</td>
<td>ND</td>
<td>(Pellis et al., 2017)</td>
</tr>
<tr>
<td>Thermohibidida cellulosa lycyta cutinase 1</td>
<td>Amber</td>
<td>covalent</td>
<td>78% monomer conversions at 21 °C</td>
<td>7</td>
<td>58% after 24 h reaction (3 cycles)</td>
<td>ND</td>
<td>(Pellis et al., 2017)</td>
</tr>
</tbody>
</table>

ND, not detected.
can cost ~250 and 500 €/ton plastic, respectively. In comparison, Tournier et al (Tournier et al. 2020) stated that the production cost of 1 kg enzymes required to recycle PET waste is 21 €. The recycling of 1-ton PET waste needs ~3 kg protein which costs ~63 €, and this process produces sodium sulfate by 0.6 kg/kg-recycled PET and 863 kg terephthalic acid, which was purified after that to 99.8%, during the whole process. In addition to the produced amounts of phosphorus, antimony, and ethylene glycol. Thus, it is estimated that the recycling process of 100,000 tons of PET/year will yield 60,000 tons of sodium sulfate, which represents 0.28% of the global market need. Moreover, the produced terephthalic acid could be used for the manufacturing of PET bottles to close the gap of the circular economy. By contrast, given that all the available bioengineering strategies for PET biocatalysts that were mentioned during this review to enhance their productivity and catalytic activity and followed by immobilization on suitable support, this will enable the biocatalysts re-usability for at least 10 cycles. This supposed to reduce the estimated actual production cost of PET biocatalysts and PET waste green recycling by 10 fold. Further studies of genetic improvement, large-scale production, and immobilization of PET biocatalysts will bring the process of PET waste green recycling closer to practical and commercial reality. Besides, it will fill the global need to solve the hazardous problem of plastic waste.

7. Concluding remarks and future perspectives

The continuous and rapid development of the plastic industry has risen a global warning because of their environmental impact, which is represented in the massive PET waste accumulations in the landfills, seas, and oceans. Traditional methods for PET waste recycling still problematic because of the lethal effect on marine animals and humans. Finding an effective and environment-friendly strategy for PET waste green recycling is in high demand. The discovery of new PET biocatalysts and degrading microorganisms is an excellent movement toward a green recycling scheme for PET waste. Besides, studying their molecular mechanism extensively via solving their crystal structure will widen this research area to move forward the industrial applications. This will require further assistance from computational and structural biology, biochemistry scientists, and expertise of material sciences. Utilization of alternative and more dynamic chassis for enhancing PET biocatalysts production needs further investigations due to the severe shortage in this field. Utilization of the reported bioengineering strategies during this review can potentially dramatically boost the productivity in large scale industrial processes, the enhancement of catalytic performance, and the improvement in thermostability and re-usability characteristics for sustainable applications. More potentially, it may lead to the reduction of PET crystallinity. It can be concluded that protein engineering strategies performed by (Tournier et al. 2020) were highly effective to make the engineered LCC enzyme the best candidate for petrochemical PET hydrolysis so far as 90% depolymerization of PET was achieved within 10 h with 16.7 g TPA productivity/L/h. However, it is recommended to find alternative biodegradable plastics such as polyhydroxalkanoates (PHAs) that are safe and non-toxic biopolymers produced by different microorganisms to facilitate the biodegradation and recycling processes. PHAs are similar to the petrochemical-based plastics, but they are completely biocompatible and biodegradable (Ong et al. 2017). PHAs are easily degradable by microorganisms under limited carbon and energy sources (Chen and Patel 2012). Bacteria such as Bacillus, Nocardiosis, Burkholderia, and Cupriavidus and fungi like Micromycetes and Mycobacterium have been reported for their PHA assimilation under aerobic and anaerobic conditions (Boyandin et al. 2013). Other examples of the bio-based and biodegradable alternatives of PET are aromatic-aliphatic polymers based on diethyl-2,5-furandicarboxylate, the petroleum-based diethyl terephthalate, and diethyl isophthalate which were produced via enzymatic catalysis and the furan-aromatic polymers prepared from biomass-based HMF (Pellis et al., 2019; Zhang et al., 2019).

Declaration of Competing Interest

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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