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ENZYMATIC DEGRADATION OF PLASTIC AND OPTIMIZATION OF MICROBIAL SYSTEM FOR THE PRODUCTION OF ITS DEGRADING ENZYMES

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ABSTRACT

This study outlines existing metabolic engineering techniques for biocycling PET into high value compounds, as well as a number of engineering approaches intended to improve the performance of PET-degrading enzymes. One of the most popular aromatic polyesters in the world, polyethylene terephthalate (PET) has an annual demand of over 29 million metric tons in 2022 and is expected to rise by 40% by 2030. Due to the growing amount of PET waste and the existing insufficiency of recycling techniques, it has accumulated in terrestrial ecosystems, posing serious hazards to world health. These technologies seek to convert recovered PET into more valuable items in order to address energy issues as well as environmental sustainability. One potentially biosustainable technique for handling and recycling plastics is enzyme-mediated biocatalytic depolymerization. Protein engineering developments have been applied to modify and improve the many plastic degrading enzymes that have been discovered from microbial sources. Additionally, microbial metabolic engineering makes it possible to create customized microbial chassis that can break down PET substrates and transform the resulting monomers into compounds that are useful for industry.

Keywords: Enzymatic degradation, Plastic, Optimization, Microbial system

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INTRODUCTION

Once touted as innovative materials with countless applications, plastics—synthetic polymers—have become an

indispensable aspect of contemporary life. They are widely used in many different industries and in common

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consumer goods due to their affordability, durability, and adaptability. However, a major worldwide issue has emerged as a result of the exponential expansion in plastic manufacture and consumption: disposing of plastic garbage [1-6]. Plastic output has increased by 680% during the past 50 years, reaching an estimated 390 million metric tons (Mt). Plastic waste, including microplastics, has accumulated in soil and aquatic environments as a result of this production and inappropriate disposal, endangering both human health and natural ecosystems. One of the most common petroleum-derived plastics used for packaging is poly(ethylene terephthalate) (PET), a semi-aromatic, heteroatomic synthetic polymer composed of terephthalic acid (TPA) and ethylene glycol (EG) linked by ester bonds. Most PET plastic is landfilled or incinerated, processes that do not create economic value and contribute to releasing CO₂ into the atmosphere, posing an additional environmental issue. Mismanagement of PET waste exacerbates plastic pollution, necessitating the exploration of economically viable solutions for its end-of-life management and the embrace of circular economy principles [7-9]. Closed loop recycling of post-consumer PET, which aims to return it to its original material state, is one such option. Just 9% of plastics, including PET, are now recycled mechanically. However, because the final recycled goods have poor mechanical and structural quality, this

procedure frequently results in downcycled material.

If PET's monomers are available in extremely high purity, PET can be repolymerized into high quality PET. A number of thermal processes, including as thermal oxidation and catalytic pyrolysis, as well as chemical processes, such as glycolysis, alcoholysis, and alkaline and acidic hydrolysis, can do this. Due to the usage of hazardous chemicals and possible financial problems brought on by the high energy required to finish the operations, these approaches may be less profitable from an economic standpoint [10, 11]. By using biotechnological microbial agents for PET depolymerization, toxicity concerns could be avoided, and biodegradation may become more economically viable. The subject has been completely transformed by the recent discovery of *Ideonella sakaiensis*, a soil bacteria that can secrete PET-depolymerizing enzymes that cleave the ester bonds of PET. This finding creates new opportunities for the development of bio-based PET recycling methods that are profitable. Researchers have been able to identify new classes of PET-degrading enzymes from various microbial ecosystems (plastisphere) and identify the biochemical pathways supporting the metabolism and bioconversion of PET monomers in microbes thanks to developments in bioinformatics and omics approaches, such as multi-omics-based systems biology, metagenomics, and metabolic pathway reconstruction techniques.

There are still a number of issues with the catalytic efficiency and thermal stability of native PET hydrolases, despite the potentially significant benefits of enzymatic PET biodegradation. Because of these restrictions, PET hydrolases have a turnover rate that is too low for use in modern industrial settings. However, the investigation of structural changes using various protein engineering designs to improve enzyme activity has been made easier by the clarification of the crystal structures and catalytic processes of multiple classes of PET hydrolases [12-15]. The quick synthesis of numerous model and non-model microbial systems for breaking down and absorbing carbon from PET substrates has been facilitated by sophisticated synthetic biology technologies and the optimization of microbial cell factory designs.

In order to build an open loop recycling system for creating higher value molecules (upcycling) from PET depolymerization intermediates as TPA and EG, these systems use synthetic microbe-based biological reactions or integrated biological and chemical reactions. We will first give a summary of various kinds of PET-degrading enzymes in this review, with an emphasis on their mechanistic action on PET. Second, we will look at research on the microbial synthesis and secretion of PET-degrading enzymes as well as engineering methods used to improve their catalytic activity. Lastly, we will examine in more detail the novel metabolic engineering techniques

used so far to rewire microbial metabolism in order to create microbial-based PET upcycling systems.

ENZYMATIC DEGRADATION OF PET

Enzymatic degradation of PET involves the hydrolysis of its ester bonds, yielding BHET (bis(2-hydroxyethyl) terephthalate), MHET (mono-(2-hydroxyethyl) terephthalate), TPA, and EG. Several classes of hydrolases have been identified with hydrolytic capabilities against PET and/or its degradation intermediates. These include cutinases, poly(ethylene terephthalate) hydrolase, mono(ethylene terephthalate) hydrolase, carboxylesterases, and lipases.

Cutinases

The hydrolysis of cutin, an insoluble polyester polymer that forms the plant cuticle through interconnected ester linkages of omega-hydroxy acids, is naturally catalyzed by cutinases, which are α/β superfamily hydrolases. They are frequently found in green plants, saprophytes, and phytopathogenic microbes like bacteria and fungus. In contrast to bacterial cutinases, which have a molecular weight of 30–40 kDa, fungal cutinases have a molecular weight of 22–26 kDa, two disulfide bonds rather than one, and many post-translational modifications [16-18]. Their optimal temperature ranges from 30 to 40 °C (40–75 °C for bacterial cutinases), with an optimal pH between 7.5 and 10 (7–8 for bacterial cutinases). They share a catalytic site comprising a serine, histidine, and

aspartate triad, and notably lack the typical hydrophobic lid found in most lipases and esterases. This lid usually covers the active site and facilitates interfacial substrate activation. The exposed active site of cutinases allows them to accept a broad range of hydrophobic substrates, including PET, making them powerful catalysts for biotechnological applications.

Carboxylesterases

Carboxylesterases are lipolytic hydrolases that catalyze the cleavage and synthesis of short chain carboxylic esters on a variety of substrates. They are attractive biocatalysts for commercial usage because of their great stability in inorganic solvents and wide substrate specificity. They have a catalytic triad made up of S-E-H and the G-X-S-X-G consensus motif, which is characteristic of cutinases and serine hydrolases. Thermophilic actinomycetes' polyesterses have shown hydrolytic activity against BHET and MHET. It has recently been demonstrated that *B. subtilis* nitrobenzylesterases may transform BHET into TPA. Therefore, carboxylesterases can be used in conjunction with other PET-degrading enzymes to consume BHET produced during PET degradation [19-22].

Lipases

Hydrolytic enzymes that work against short chain carboxylic esters are called lipases. They have a lid covering their active site, unlike cutinases, which may decrease their action against PET substrates. As such, in order to start the catalytic process, they need interfacial activation. Promiscuous activity has been demonstrated by lipases against a

variety of polyester substrates. Purified lipase from the green plant *Triticum aestivum* shown partial hydrolysis action on PET as early as 2005. Since then, it has been found that many different lipases from bacterial and fungal origins may break down PET [23, 24]. Although the number of discovered PET-degrading enzymes has significantly increased, their naturally occurring activity is not optimally suited for industrial application as plastic biocatalysts. Thus, protein engineering strategies are being explored to enhance both their efficiency and stability.

IMPROVING PET DEGRADATION BY ENZYME CO-LOCALIZATION

Enzymatic surface erosion of PET substrates begins with the degradation of the exterior surface, exposing the substrate's interior. However, enzymatic activity can only access a limited number of surface areas. This idea depends on engineering enzymes involved in sequential catalysis reactions to collocate them in close proximity through chemical and/or structural interactions, even though spatial clustering of sequentially acting enzymes is an elegant strategy mainly used to improve synthetic metabolic pathways. Enzyme fusion and multi-enzyme complexes are two synthetic approaches that can be used to enhance enzyme-substrate interaction and reduce the long range diffusion of intermediates. The binding of hydrolases to the surface of PET is one of the limiting processes that probably reduces their effectiveness in depolymerizing PET

[25-29]. In nature, enzymes involved in the breakdown of polymers frequently adopt multi-domain designs. These modules generally comprise a catalytic domain and a non-catalytic one. Several non-catalytic binding domains, including carbohydrate-binding modules (CBMs), hydrophobin proteins, and anchor peptides (APs), have been applied to enhance the binding activity of PET-degrading enzymes over PET substrates.

Carbohydrate Binding Modules

Carbohydrate Binding Modules, first discovered in microorganisms degrading chitin polymer chains, promote substrate accessibility for hydrolytic enzymes. They can be divided into three types: A (planar-type), B (endo-type), and C (exo-type) based on substrate specificity and binding mechanism [30, 31].

Hydrophobin Proteins

Fungi release hydrophobins, which are low molecular weight, self-assembling proteins that can facilitate contact with hydrophobic leaf surfaces. Therefore, PET hydrolytic enzymes may be able to target highly hydrophobic PET surfaces with the help of hydrophobic binding modules (HBMs). Enzyme concentration on the PET surface should rise as a result of the covalent fusing of HBMs to PET hydrolytic enzymes, improving hydrolysis. For example, the cutinase Cut1 from *T. cellulolytica* was C-terminally fused to the hydrophobins HFB4 and HFB7 from the saprophytic fungus *Trichoderma* sp. On the PET film's surface, the resultant chimeric protein

showed increased adsorption activity [32-35].

Anchor Peptides

Anchor peptides can encourage adhesion and interaction with different macromolecules, depending on their physical characteristics and architectures. They have been evaluated as adhesion facilitators to PET substrates because of their adhesive qualities. The target protein can be genetically coupled with surface specific anchor peptides [36-40]. It has been demonstrated that the biocatalyst phenolic acid decarboxylase adheres to the PET surface more readily when the dermaseptins DSI, host defense peptides generated by frog skin, are present. The high hydrophobicity of the PET surface presents a challenging obstacle to overcome by relying solely on enzymatic activity. Additional thermal pretreatments are still necessary for modifying the PET hydrophobic surface and reducing its crystallinity rate. However, similar to cellulose degradation enzymatic systems, the construction of synthetic scaffolds comprising multi-modular PET-degrading hydrolases may offer alternative solutions for synergistically increasing enzymatic activity, minimizing product inhibition, and improving substrate adhesion [41-43].

OPTIMIZATION OF MICROBIAL SYSTEM FOR THE PRODUCTION OF PET-DEGRADING ENZYMES

Industrial scale enzymatic degradation necessitates the production of substantial quantities of functional

enzymes, traditionally purified through laborious processes from microbial cultures grown in fed batch or continuous fermenters. Simplifying industrial enzyme production can be achieved by selecting microbial sources with efficient, naturally occurring or synthetic protein secretion systems. PET-degrading enzymes have been successfully expressed in various microorganisms, and numerous metabolic engineering strategies have been employed to boost their production and catalytic efficiency. This section outlines approaches focused on increasing enzyme secretion from host cells, enhancing protein three-dimensional structures, and developing whole cell PET biocatalysts [44-46]. PET polymers' large molecular weight physically prevents cells from internalizing them. Although PET hydrolases have been expressed and secreted heterologously in a number of microbes, the secretion efficiency has frequently been inadequate for efficient PET breakdown. Both enhancing the host's protein secretion mechanisms and tweaking the endogenous signal peptides (SPs) of PET hydrolases have been used to overcome this restriction [47, 48]. *Yarrowia lipolytica* and *B. subtilis*, two naturally occurring microorganisms with complex protein secretion machinery, have also been used as hosts for extracellular PETase synthesis. Further advances in metabolic engineering are hampered by the lack of knowledge on the metabolic pathways controlling protein translocation and secretion, despite notable progress in manipulating

microbial secretory systems to improve recombinant protein recovery. Optimizing intracellular chaperones is another tried and true method for increasing the synthesis of PET-degrading enzymes because protein secretion significantly depends on proper protein folding and secreted proteins fold more slowly than non-secreted ones [49-52].

CONCLUSIONS

A very promising, scalable, and sustainable way to manage PET waste is through the use of synthetic microbial biocatalysts or hybrid biochemical techniques for PET upcycling. Microbial biocatalysts can be scaled up from lab bioreactors to commercial-level PET upcycling by utilizing cutting-edge synthetic microbiology and metabolic engineering techniques. This development would greatly help shield the environment from the damaging impacts of PET waste and promote a circular material economy.

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