The Application of CRISPR Gene Editing Technology in PET Biodegradation

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Abstract. Plastics used in people's daily life bring convenience on the one hand, and on the other hand, a large amount of plastic waste also brings great pollution to the environment. Polyethylene terephthalate (PET) is a polymer polymerization material, which has become one of the most widely used plastics in the world because of its durability and easy processing. The stability of the ecological environment around it is seriously threatened by the large amount of PET used and its difficult degradation.PET even endangered human health. The traditional methods of PET treatment are physical and chemical methods such as incineration, landfill, and heat cracking, etc. Comoared with the first two generations of gene-editing system, CRISPR/Cas9 system has the advantage of easy design, low cost and high efficiency. Currently, the most favorable method of treating PET to protect the environment is biodegradation. This paper introduces the nature of PET and the research progress of PET degradation enzymes, seeking to improve the activity and thermal stability of degradation enzymes by CRISPR/Cas9 gene editing technology, this paper explores the construction of mutants with high thermal stability and high degradation activity of the fusion dual enzyme of PETase and MHETase by CRISPR/Cas9 gene editing technology.

Keywords: PET; biodegradation; CRISPR/Cas9 gene editing; dual-enzyme mutant.

1. Introduction

Plastic products are widely used in agriculture, clothing industry, construction industry and other areas closely related to people's lives and bring a lot of convenience to our lives. PET is the most popular polyester plastic, and it is consumed annually more than 300 million tons, representing approximately 13% of the total plastic production worldwide [1].

PET has good creep, fatigue and abrasion resistance, and is cost-effective, easy to process and durable, due to the large amount of PET use has spawned research on PET hydrolases. However, according to statistics every year the global production of plastics is growing, while the recycling rate of waste plastics is low [2]. The high hydrophobicity and chemical inertness of plastics make it difficult to degrade naturally in the environment, leading to a large accumulation of plastics, and ultimately resulting in a worldwide "white pollution" problem. Some plastics that are difficult to be naturally degraded are mainly treated by physical or chemical means, but this will cause secondary pollution of the ecological environment, and landfilled plastics will form more hazardous microplastics over time, which will ultimately threaten human health through various ways [3]. At present, the plastic varieties widely used in people's daily life mainly include polyethylene terephthalate (PET), polyethylene (PE), polyvinyl chloride (PVC) and so on. In recent years, the PET biodegradation method has begun to emerge, which is low-cost, mild, and does not require hazardous chemicals and expensive machinery [4]. At the same time, it is in line with the concept of sustainable development and has made some progress and may be an efficient and environmentally friendly solution to the problem of pollution control of waste plastics in the future. However, the low degradation efficiency limits its application, so the preparation of large quantities of efficient PET hydrolases is the key to achieve PET biodegradation on a large scale. In recent years, researchers have made progress in the application of biodegradation using CRISPR-Cas system.

2. Biodegradation of PET

PET is a polymer of oil source (Fig. 1). In the presence of PETase, PET is decomposed into BHET, MHET, TPA, under PETase BHET decomposes to MHET, while MHET decomposes into environmentally harmless small molecular compounds TPA and EG under MHETase (Fig. 2) [5] . Microorganisms that can degrade PET in nature include bacteria and fungi (Table 1) [6]. There are also many types of enzymes associated with PET biodegradation (Table 2) [7].In 2016, PETase and MHETase were identified in the bacterial *Ideonella sakaiensis 201-F6* strain, which uses PET as the sole carbon source, which the investigators identified as involved in PETdegradation [8].PETase is a typical α/β hydrolase fold, containing a center composed of nine β -strands: β -fold, sandwiched in the middle by seven α -helices [9]. PETase has a catalytic triad, an oxyanion hole and two bonds that have disulfide, the preservation of disulfide bond 2 (DS2) is strictly enforced in all enzymes with homologous structures, with the specific disulfide bond 1 (DS1) located close to the active site [10,11]. PETase's active site has a higher degree of flexibility when at room temperature, and this can be attributed to DS1 [12]. In addition, other PET hydrolytic enzymes have been continuously discovered. In 2020, a chimeric dual-enzyme system was established by Knott et al [13].

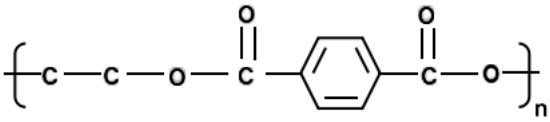

Fig. 1 Molecular formula of polyethylene terephthalate

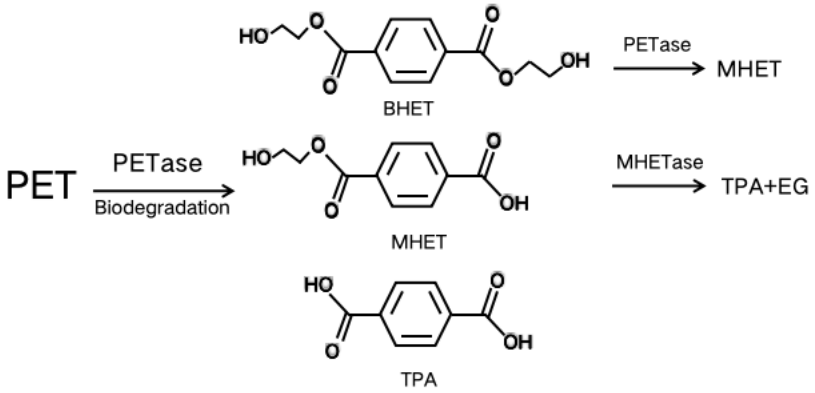

Fig. 2 Schematic diagram of PETase degradation of PET [5]

In this system, MHETase is linked to PETase by various lengths of glycine-serine adaptors (8,12 or 20 amino acid residues), thereby the PET degradation efficiency is raised by a minimum of 2.8-fold [13]. The fusion system produced approximately six times the degradation product from the PET film over 30h at 96 °C [13]. In the same year, the mutant of LCC-ICCG could decompose 200g of PET in 10 hours with an efficiency of up to 90% [14]. Making the decomposition material produced by the mutant enzyme to create a new PET was found that it was as strong as the PET that had been recycled, only higher than that of fossil raw materials PET 5% [14]. In 2022, a structure-based machine learning algorithm was designed called FAST-PETase, which is more stable at a range of temperatures and PH and improves the reaction rate [15]. FASTPETase is mutated from wild-type IsPETase and exhibits excellent PET hydrolysis activity [15]. In January, the researchers demonstrated that the post-translational glycan modification of P. pastoris makes it a good expression host for PETase, and that the glycosylation of the N-junction site in IsPETase greatly boosts the enzyme's catalysis efficiency [16]. In April, researchers isolated a hydrophilic polyester hydrolase, PHL 7, from the metagenome of plant composting, along with six homologues (PHL 1-6) [17]. At 70°C, TPA and EG are produced by rapidly hydrolyzing amorphous, better than all previously reported PET hydrolases and their engineered variants [14,15]. The low activity and heat resistance of PET hydrolase lead to the low efficiency of PET degradation.Therefore, the researchers are still

constantly studying and exploring.The latest study of the fusion duplex of KL-MHETase and FAST-PETase was 2.6 times faster than that of FAST-PETase alone [18].Currently, researchers improve the thermal stability and activity of the enzyme by molecularly modifying the PET-degrading enzyme, and the main modification positions are the enzyme substrate binding pocket, the addition of metal ions such as Ca^{2+} and Mg^{2+} to enhance the enzyme's stability, the reduction of product inhibition of the enzyme, and the enhancement of the effective contact area between the degrading enzyme and PET to increase the efficiency of the degradation [19]. In conclusion, screening the fusion dual enzyme system with better thermal stability and higher enzyme activity can greatly improve the catalytic efficiency of the enzyme.

Table 1. PET degrading microorganisms [6]

Microorganism strain	Substrate	Time(d)	T(℃)	pH	Weight loss(%)
Priestia aryabhattai VT 3.12	PET powder	18	30	/	69.0
Bacillus pseudomycoides VT 3.15	PET powder	18	30	/	66.0
Bacillus pumilus VT 3.16	PET powder	18	30	/	64.0
Microbacterium oleivorans JWG-G2	PET particles	5	25-40	6.0-9.0	1.0
Bacillus cereus	PET granular	40	29	/	6.6
Bacillus gottheilii	PET granular	40	29	/	3.0
Ideonella sakaiensis 201-F6	PET films	42	30	7.0	≥99.0
Penicillium simplicissimum 28f2	Fragments	28	30	/	3.09
Aspergillus fumigatus	PET bottles	42	25-45	7.0-11.0	22.0

Table 2. Enzymes associated with PET degradation [7]

Enzymes	PET Quality(mg)	Time(h)	T(℃)	Degradation rate(%)
TfH	100	21	50	55
HiC	90	96	70	95
FsC	90	96	50	5
LCC	25	24	50	50
Cut190	25	72	63	13.5
TfCut2	45	120	70	97
IsPETase	30	18	30	23
Lipases	200	36	50	35
ThC_Cut2	15	48	50	90
Esterase	10	24	30	66
Hydrolase TfH	25	168	55	54.2
CaL	25	168	55	0.4
PsL	25	168	55	0.3
Carboxylesterase	0.3	1	60	8

3. CRISPR/Cas9 gene editing technology

Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) is an adaptive immune system formed by bacteria or archaea to resist viral invasion, which can memorize the invading nucleic acids and target them for cleavage. The CRISPR/Cas system consists of 2 classes, 6 types and 19 subtypes, the first class is sgRNA (composed of crRNA and tracrRNA) and multiple Cas protein subunits. The CRISPR/Cas system consists of 2 types, 6 types and 19 subtypes, the first type is the effector complex composed of sgRNA (consisting of crRNA and tracrRNA) and multiple Cas protein subunits, including type I, type III and type IV; the second type is the effector complex composed of

sgRNA and a single protein subunit, including type II, type V and type VI, of which type II CRISPR/Cas9 is more widely used. Cas9 protein and guide RNA (gRNA) [20], Cas9 protein has two structural domains with cleavage functions, namely, the HNH nuclease domain that recognizes and cleaves exogenous DNA sequences complementarily paired with sgRNA bases and the RuvC nuclease domain that recognizes and cleaves another exogenous single-stranded DNA that is not complementarily paired with sgRNA bases. structural domain.

The sgRNA is obtained by the combination of tracrRNA and crRNA, a single-stranded guide RNA that includes a stem-loop structure, a duplex of repeats and antirepeats, contains a 20-bp region complementary to the target sequence (N20), and contains a specific PAM site adjacent to N20 (5'-NGG-3') [21]. During gene editing, Cas9 will recognize the PAM sequence site for cleavage to introduce a DSB in the DNA, causing gene editing repair at a specific site. Cellular repair mechanisms include non-homologous recombination end-joining (NHEJ) repair and homologous recombination repair (HDR). In the absence of a repair template, the ubiquitous error-prone non-homologous end-joining (NHEJ) pathway is activated, leading to random insertions and deletions at double-strand break sites (DSBs), often resulting in disruption of gene function. The homology-directed repair (HDR) pathway, which can generate the desired mutation N through homologous recombination, provides the basis for performing precise gene modifications including knock-ins, deletions, corrections, or mutations [22]. The CRISPR/Cas9 gene editing system has demonstrated promising potential in many areas. Gene knockout has been realized in human in vitro cultured cells, mice, zebrafish, maize, wheat and other species; the two structural domains of Cas9 with cleavage function, HNH and RuvC, are inactivated to become dCas9, which only has the ability to bind to the target site and can be used for gene expression regulation, and dCas9 coupled to acetyltransferase can be used for editing epigenomes; in the field of medicine, CRISPR/Cas9 has shown good potential in many fields. In medicine, CRISPR/Cas9 can be used to construct animal tumor models and gene therapy. In addition, CRISPR/Cas9 has certain advantages in antiviral research and treatment of monogenic and polygenic diseases [23]. The hotspot of recent research is about the vectors delivered by CRISPR/Cas9 system, which provides the possibility of applying CRISPR/Cas9 gene editing technology to more species, more diseases, and more transgenic plants [24].

4. Application of CRISPR/Cas9 on PETase and MHETase

With the continuous development of CRISPR gene editing technology, we can use CRISPR/Cas9 technology to establish a database for constructing PETase and MHETase dual-enzyme mutants [25, 26], so as to find fusion dual-enzyme mutants with high degradation activity and thermal stability, and can realize industrial-scale biodegradation and efficient recycling of PET. The construction of mutant libraries should pay attention to the following points: i) screening for optimal sgRNAs; ii) selecting high-quality genomes of target species as reference genomes in the design; iii) designing sgRNAs and evaluating the information of potential off-target sites; iv) controlling the concentration of Cas9/sgRNA complexes; and v) improving the genome editing system of CRISPR/Cas9 [27,28]. There are few reports related to the use of CRISPR/Cas9 technology to screen efficient fusion dual enzyme systems, and we have only made a preliminary discussion here.

5. Conclusion

At present, researchers have a certain research base on PET hydrolases, and the hydrolases have been found to be able to degrade PET effectively, but these hydrolases also have many shortcomings. For example, the hydrolase PETase is substrate specific and requires a low reaction temperature but has a high potential for the degradation of PET, the hydrolase can be further studied. We can further study this hydrolase and screen the proteins and genes related to the degradation activity and thermal stability of the hydrolase, and link it with CRISPR technology to realize the complete degradation and recycling of PET. PET biodegradation will become one of the most effective means of

degradation of PET and even other plastics, and more research is needed to explore the potential of these enzymes to provide a theoretical basis and research solutions for the complete degradation of plastic pollution and the creation of a green ecological future. More research is needed to explore the potential of these enzymes and to provide theoretical basis and research programs for the complete degradation of plastic pollution and the creation of a green future.

Author Contributions

All the authors contributed equally, and their names were listed in alphabetical order.

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