Enhancing PET Degrading Enzymes: A Combinatory Approach

Joho, Yvonne et al. “Enhancing PET Degrading Enzymes: A Combinatory Approach.” Chembiochem. 7 Apr. 2024

Antea Mariani e Alice Vaudi

TYPES OF PLASTICS

Plastics are generally categorised as **polymer with a specific backbone composition;**

The elemental composition of the backbone can either be all-carbon, or other atoms may be present:

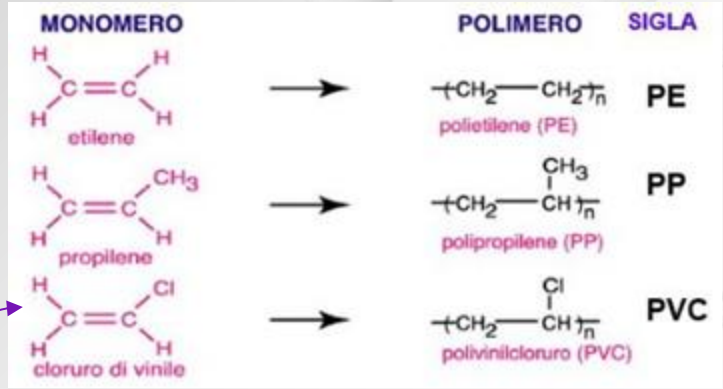
- oxygen
- nitrogen
- silicon
- sulfur

Classified by:

- specific molecular repeating monomer unit and through the attachment of various different functional side-chains

- type of synthetic method used to create them

- physical properties



GLOBAL ISSUE OF PLASTIC WASTE

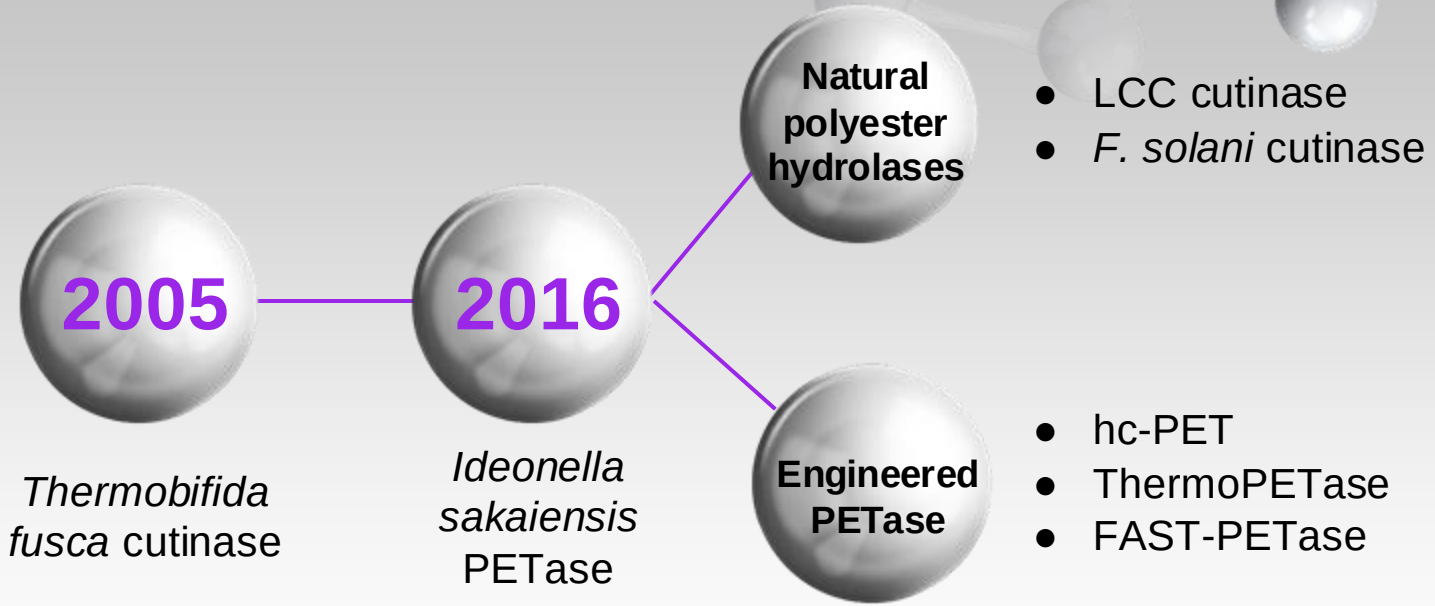
- The escalating global issue of plastic waste accumulation and its improper disposal practices have led to significant environmental and health concerns.
- Only 9% of plastic waste gets recycled.
- The ingestion of microplastics pose potential risks to human health.

RECYCLING

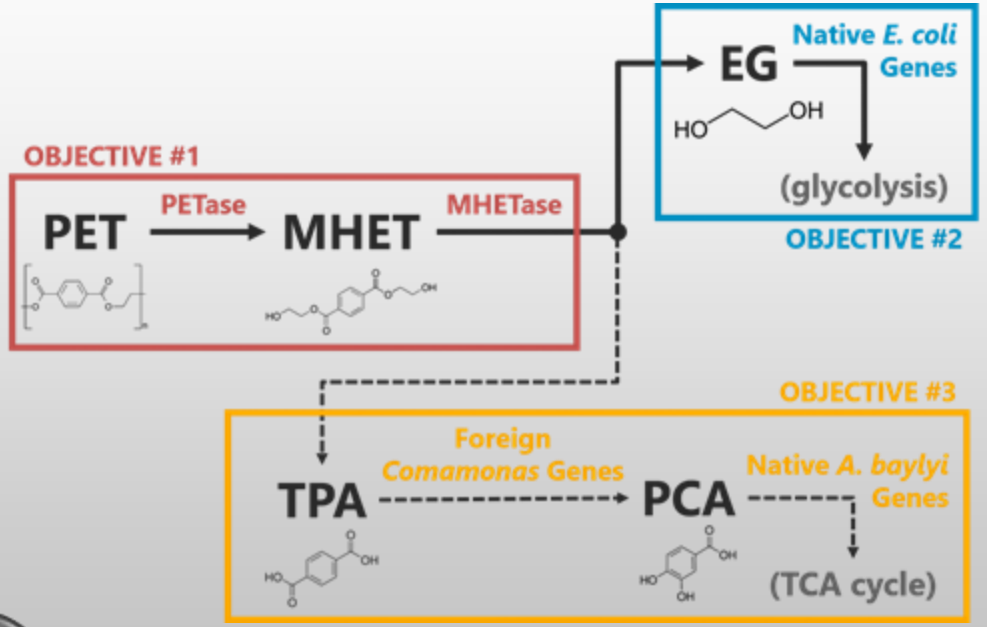
- Advanced recycling is the conversion of plastic waste into monomers or other valuable raw materials by means of cracking, gasification or **depolymerization;**

- **Enzymatic depolymerization** offers a relatively simple and cost-effective solution for easily hydrolysable plastics such as **polyethylene terephthalate** (PET).

PET ENZYMATIC DEPOLYMERIZATION



WHO IS PETase?



PETase is a small protein which consist in 290 aa:

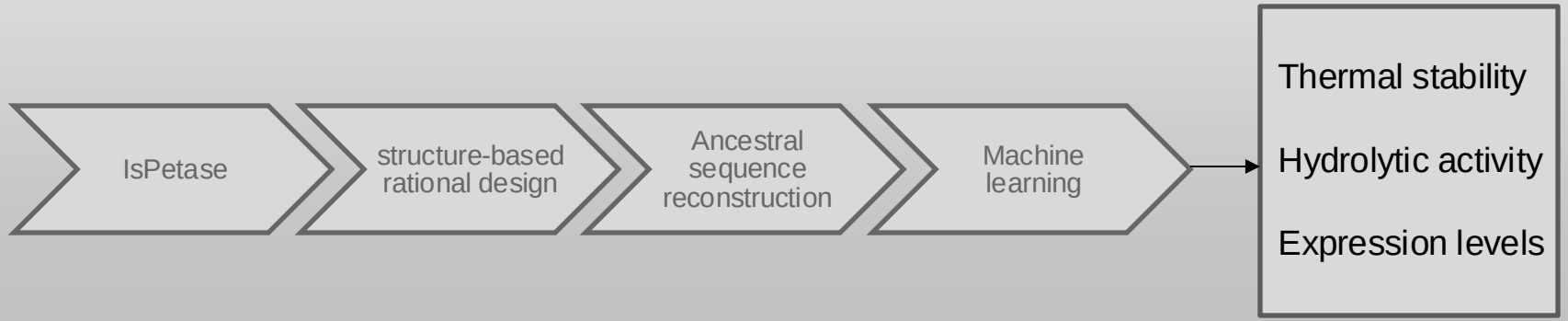
- involved in the degradation and assimilation of PET
- acts synergistically with MHETase to depolymerize PET

GAP OF KNOWLEDGE

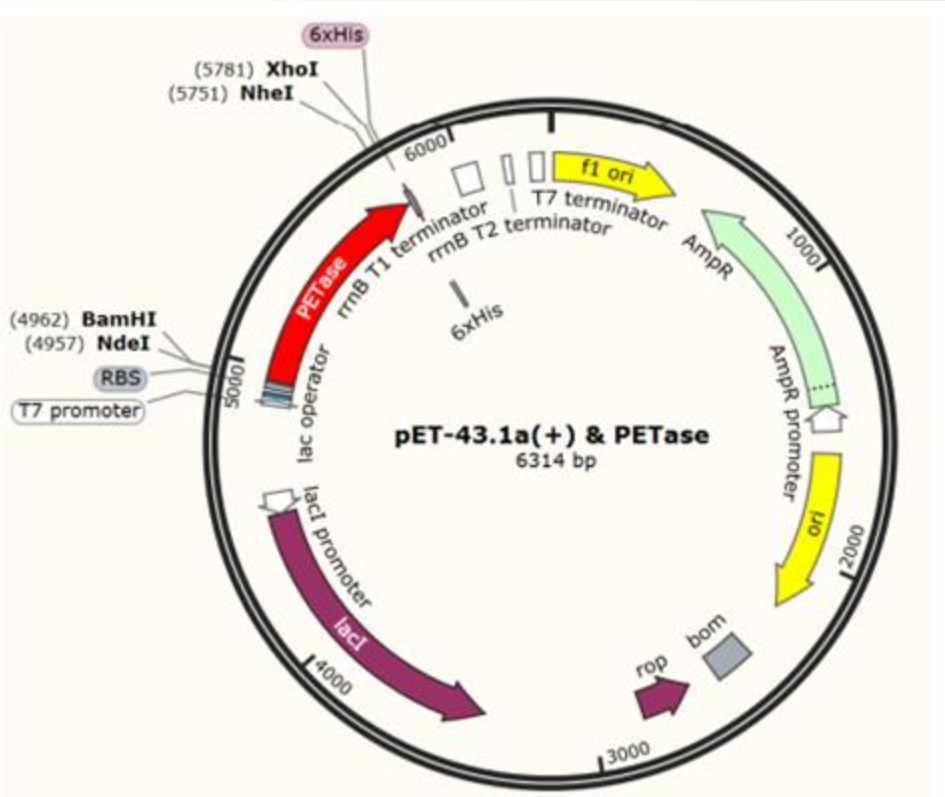
- Improve biophysical and biochemical properties.
- Activity with high-crystalline PET.

ARTICLE GOALS

- Engineering a new variant of PETase that has the following parameters to allow the up-scaling enzyme-based PET depolymerization.

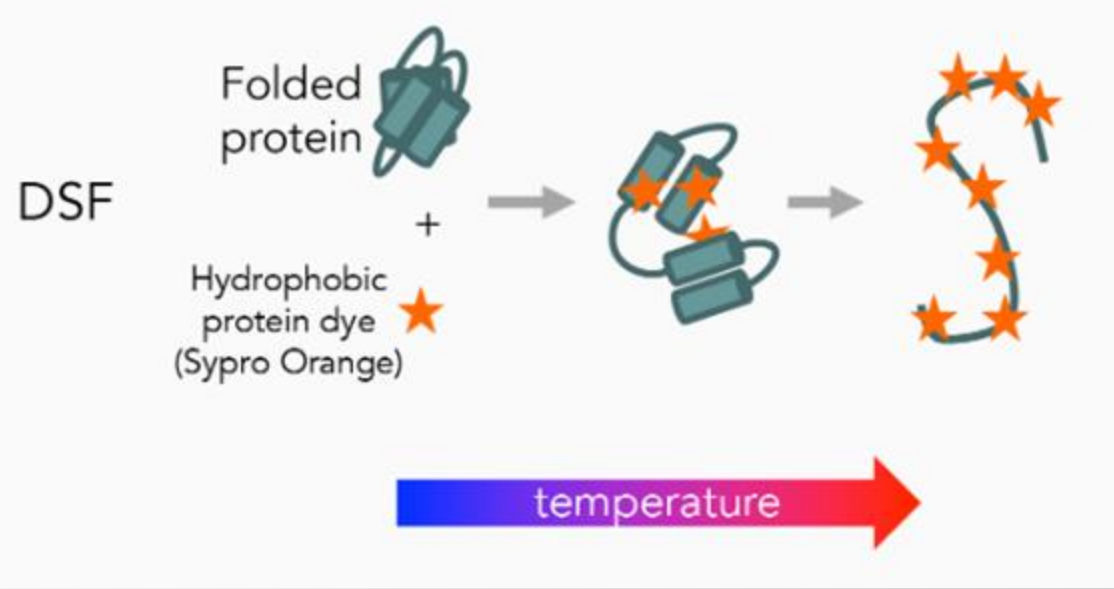


Protein Expression and Purification



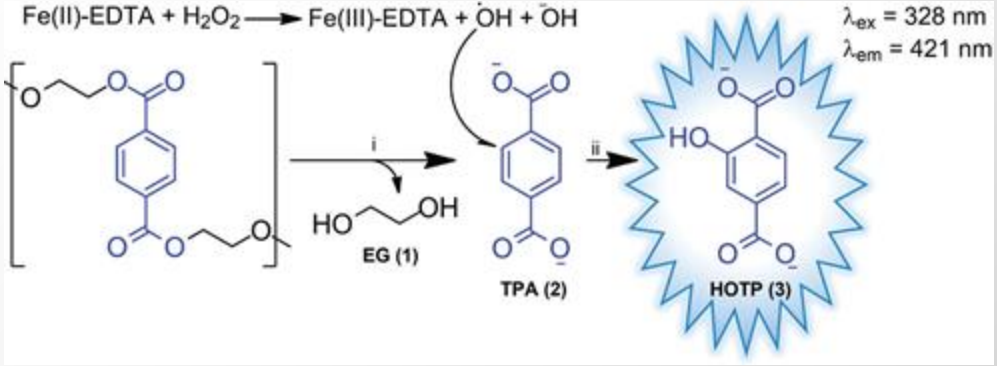
- Expression in *E. coli*
- Signal peptide is excluded (PETase is normally a secreted protein)
- C-Terminal 6xHis
- HisTrap column
- SDS-PAGE

Thermostability Measurement



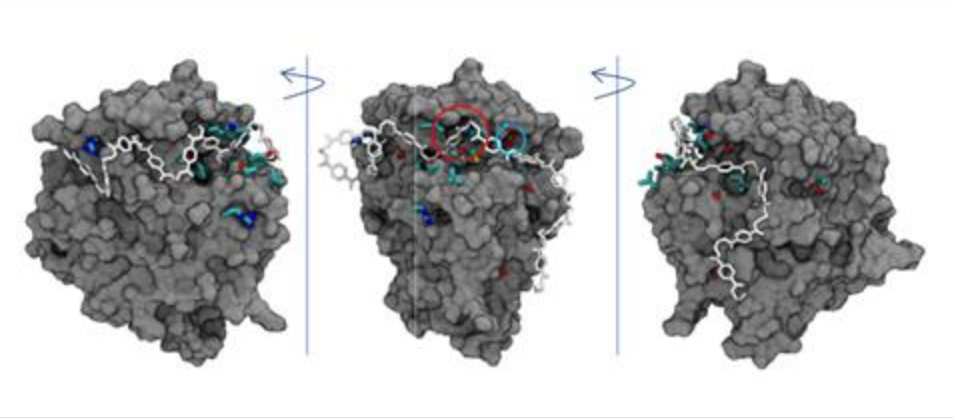
Conventional DSF uses a hydrophobic fluorescent dye that binds to proteins as they unfold.

Enzymatic Activity of Engineered PET Hydrolases



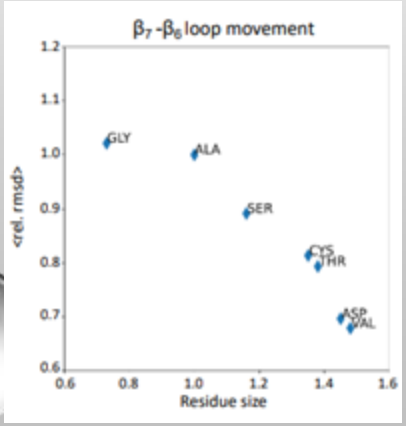
- To assess the efficiency of enzyme activity, the fluorescence of the HOTP;
- HOTP is 2-hydroxy terephthalate;
- TPA convertion in HOTP is obtained by an iron autoxidation ($FeSO_4$)

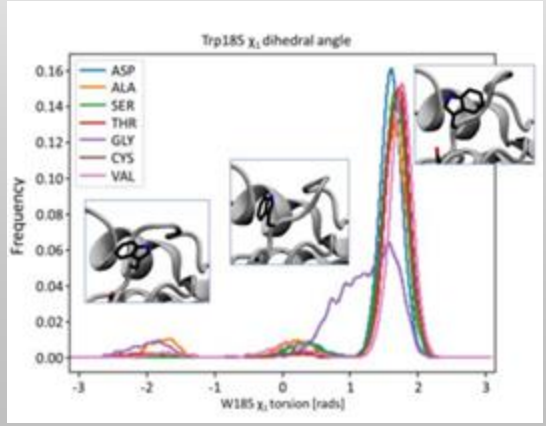
PETase^A: Generation 1



The 8mer-PET interacts with:

- **W185**
- **Residues in the β6-β7 loop** (preceded by W185)





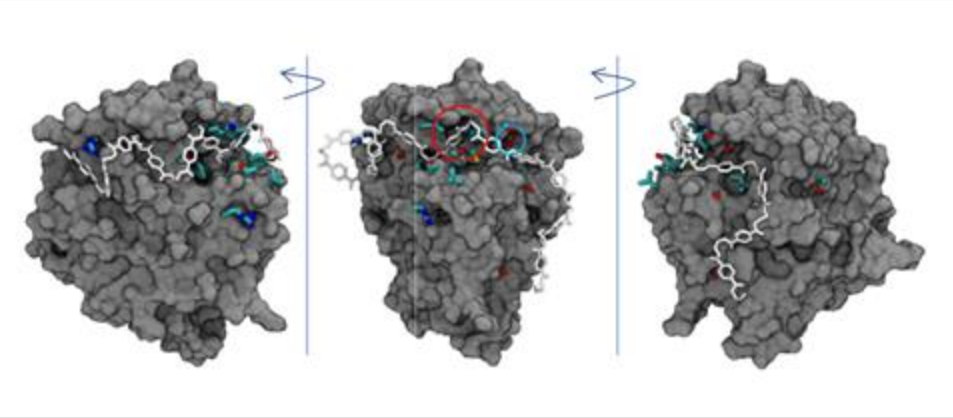
Molecular dynamics simulations of various substitutions at position 186;

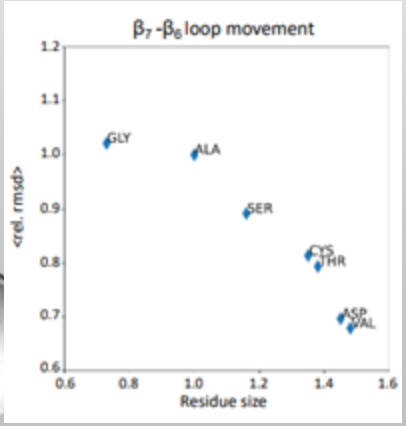
strong dependence of the flexibility of:

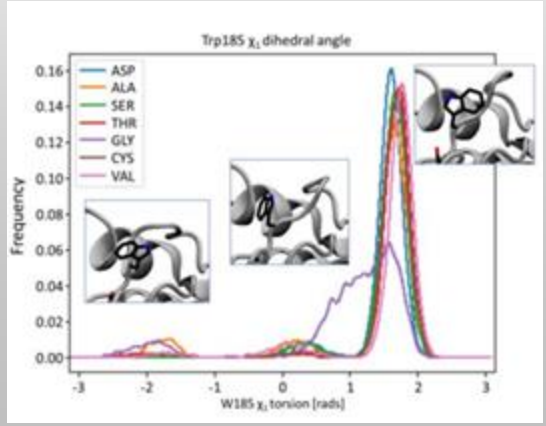
- the side chain of W185
- the β6-β7 loop

on the size of residue 186

PETase^A: Generation 1

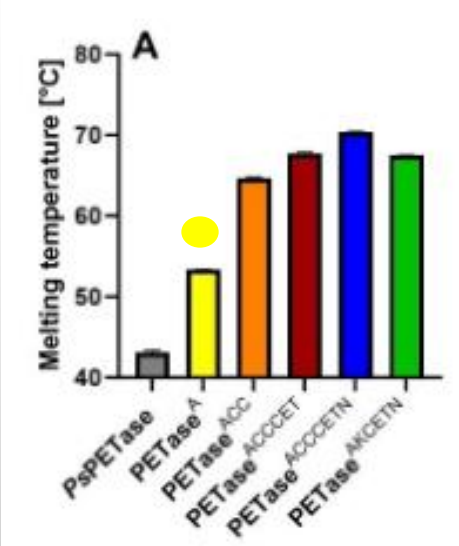


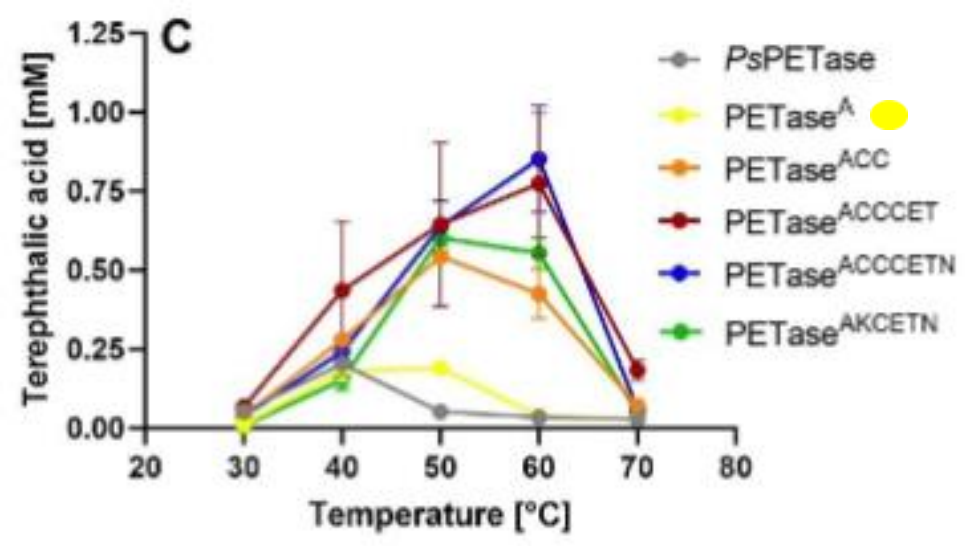




- The beneficial effect of the flexibility of W185 and the loops in the active site has been highlighted
- **D186** is not conserved in other cutinase enzymes and is most **commonly a histidine in thermostable homologs**
- **The substitution D186H alone does not provide better activity**

PETase^A: Generation 1



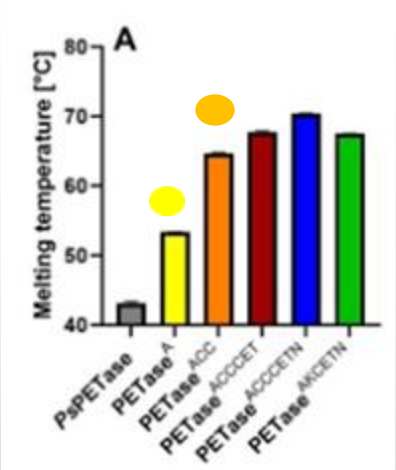


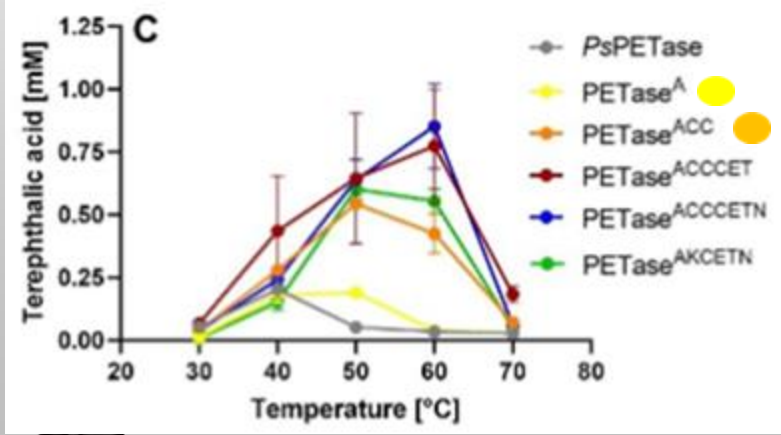
D186A substitutions based on MDS;

exhibites an increase in:

- **flexibility**
- **melting temperature** of~10°C
- Enzyme activity at 50°C

PETase^{ACC}: Generation 2





To improve the thermal stability of the first PETase variant:

- sequence homology studies (TfCut2)
- bibliography studies (Cut190, LCC, ThermoPETase)

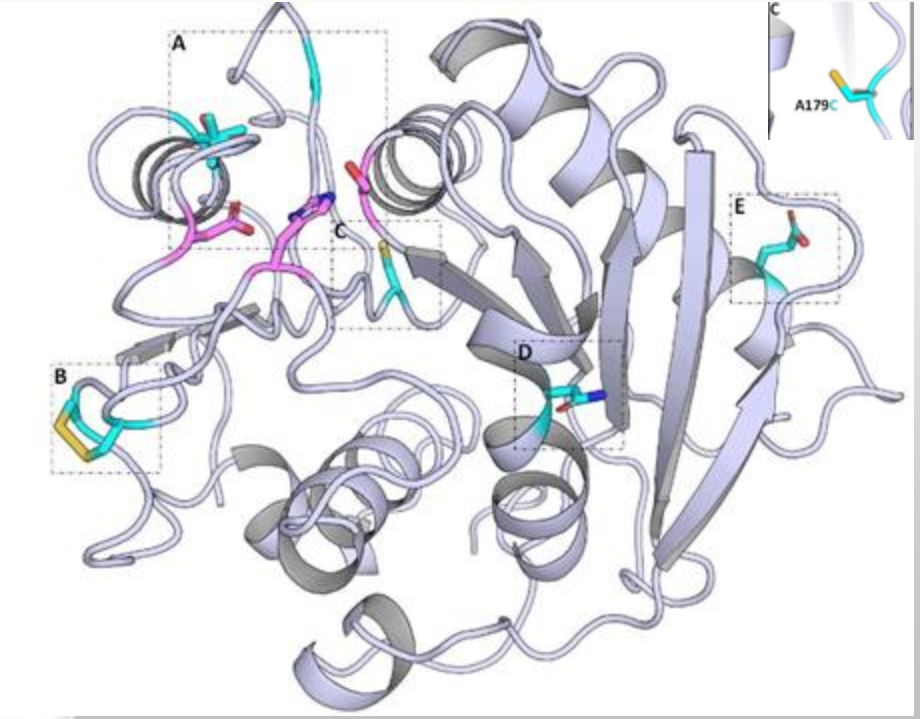
↓

Introducing disulfide bridge using N233C and S282C

↓

Triple mutant with increased melting temperature and activity at 50°C

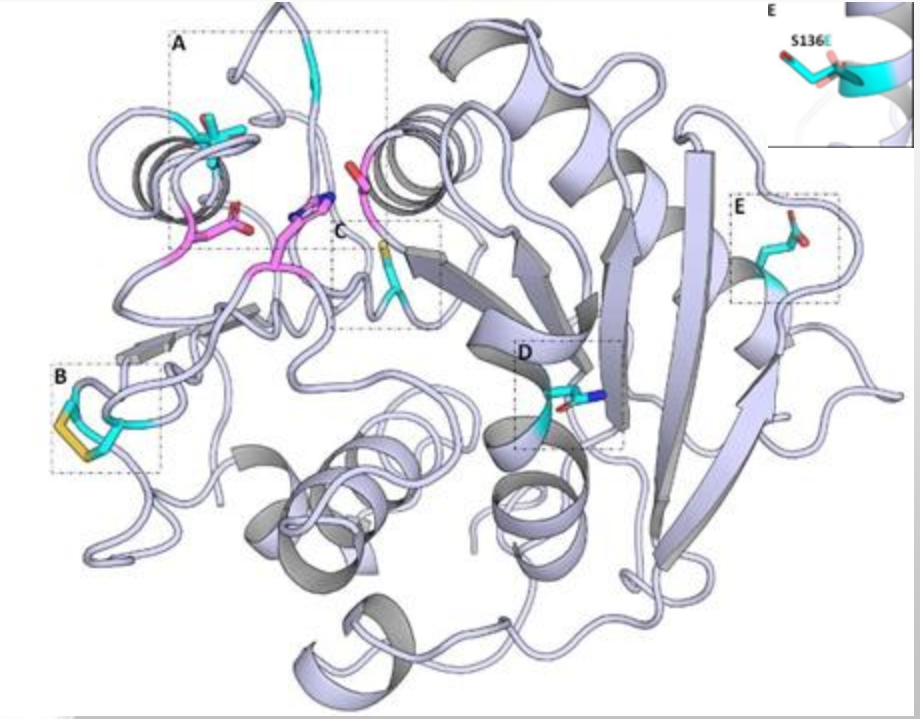
PETase^{ACCCET}: Generation 3



To further improve thermostability:

- A179C → The variant fills a hydrophobic cavity in the core of the enzyme
- S136E → Increase hydrolytic activity
- S214T → The mutation breaks the hydrogen bond with P184 located in the loop, increasing flexibility and enzyme activity

PETase^{ACCCET}: Generation 3



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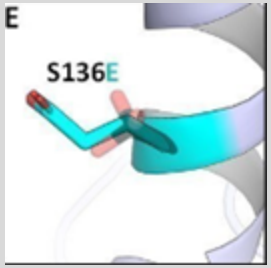

	Mutant number	Mutations	Purification yield (mg/L)	Activity at 45 °C (μM) TPA	Tm (°C)
	Scaffold	D186A + N233C + S282C (new scaffold) → PETase^{ACC}		427.0	64.7 ±0.11
9	321	PETase^{ACC} + S136E (E)	3.8	567	65.6 ±0.19

It shows a 1.3-fold increase in hydrolytic activity

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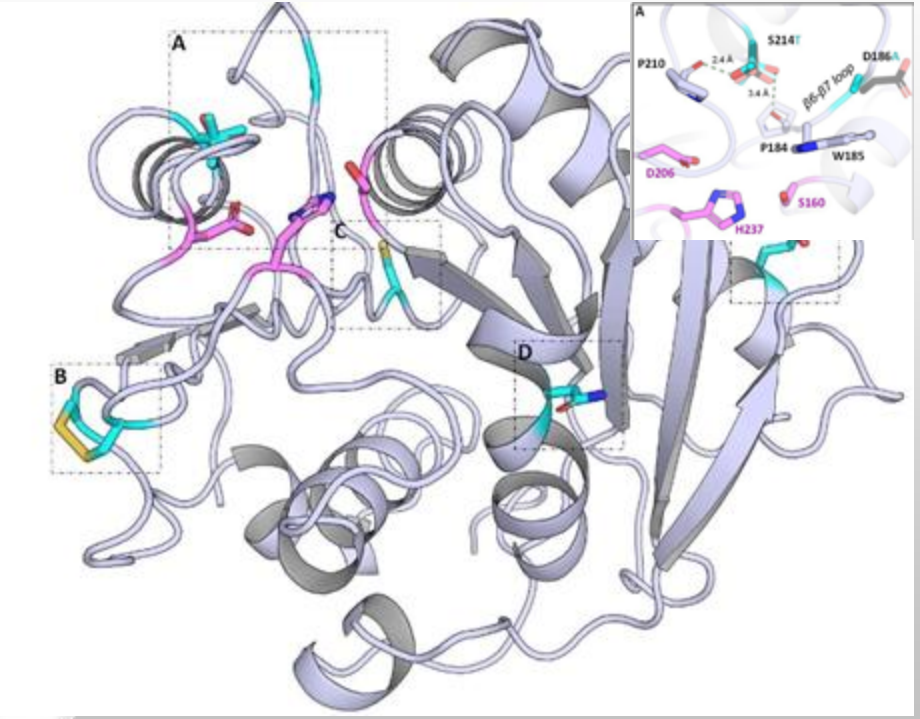
PETase^{ACCCET}: Generation 3



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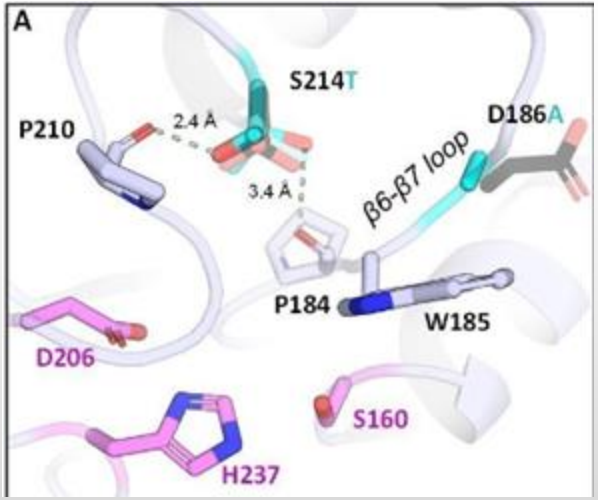

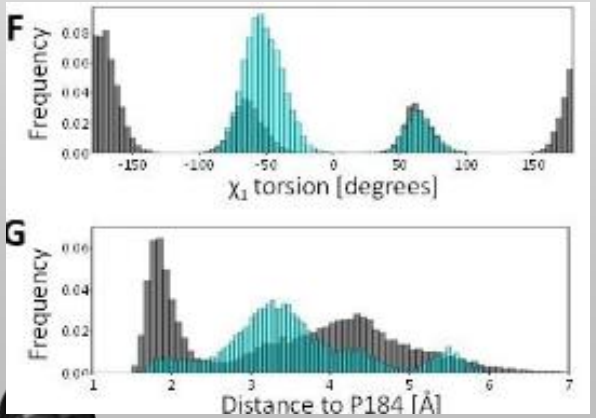

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PETase^{ACCCET}: Generation 3

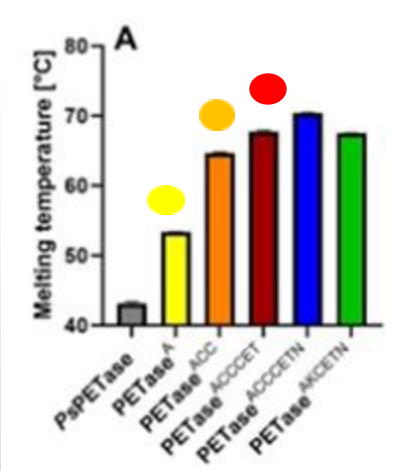
	Mutant number	Mutations	Purification yield (mg/L)	Activity at 45 °C (μM) TPA	Tm (°C)
	Scaffold	D186A + N233C + S282C (new scaffold) → PETase^{ACC}		427.0	64.7 ±0.11
5	304	PETase^{ACC} + S214T (T)	5	480.2	66.8 ±0.11

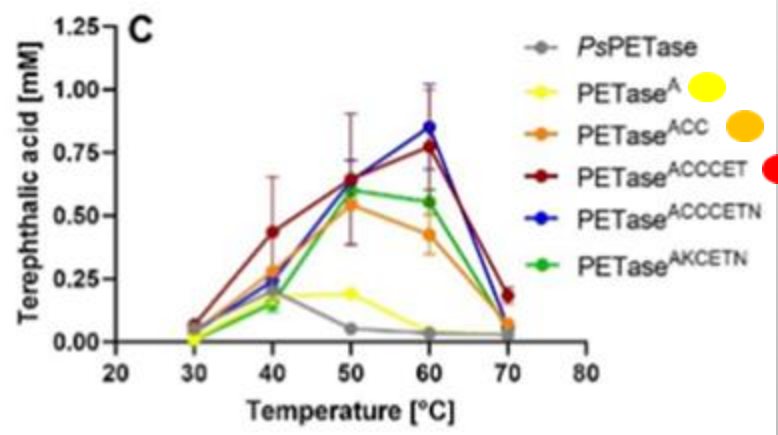
The variant increases the enzymatic activity by 12%

To further improve thermostability:

- A179C → The variant fills a hydrophobic cavity in the core of the enzyme
- S136E → Increase hydrolytic activity
- S214T → The mutation breaks the hydrogen bond with P184 located in the loop, increasing flexibility and enzyme activity

PET*ase*^{ACCCET}: Generation 3





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- A179C → The variant fills a hydrophobic cavity in the core of the enzyme
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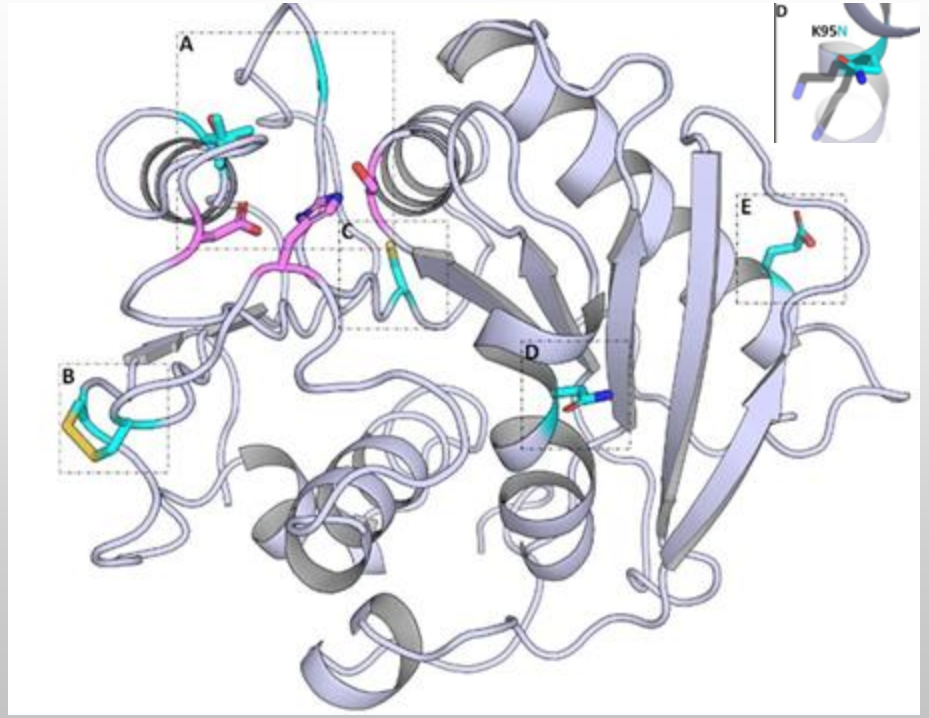
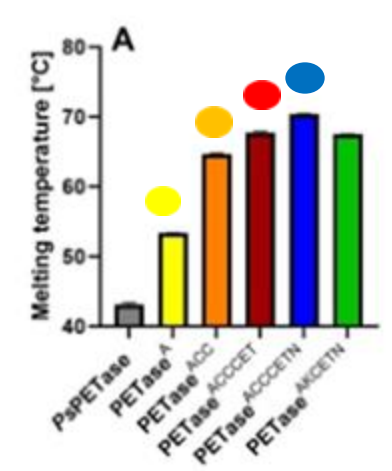
The effect of mutations is additive

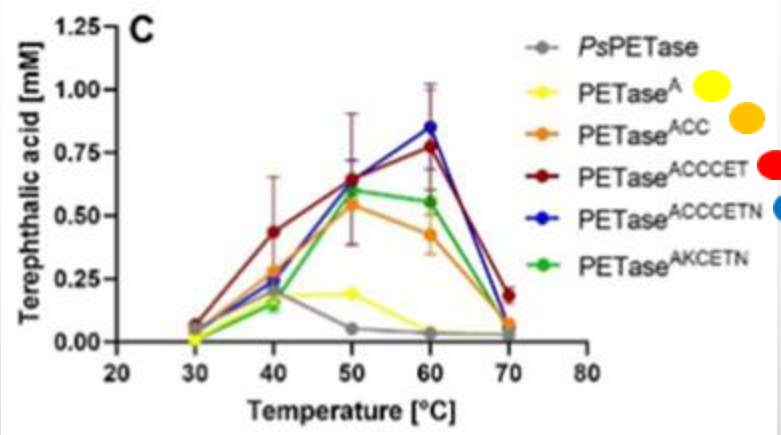
PETase^{ACCETN}: Generation 4



- Ancestral Sequence Recostruction
- **K95N mutation**

PETase^{ACCETN}: Generation 4



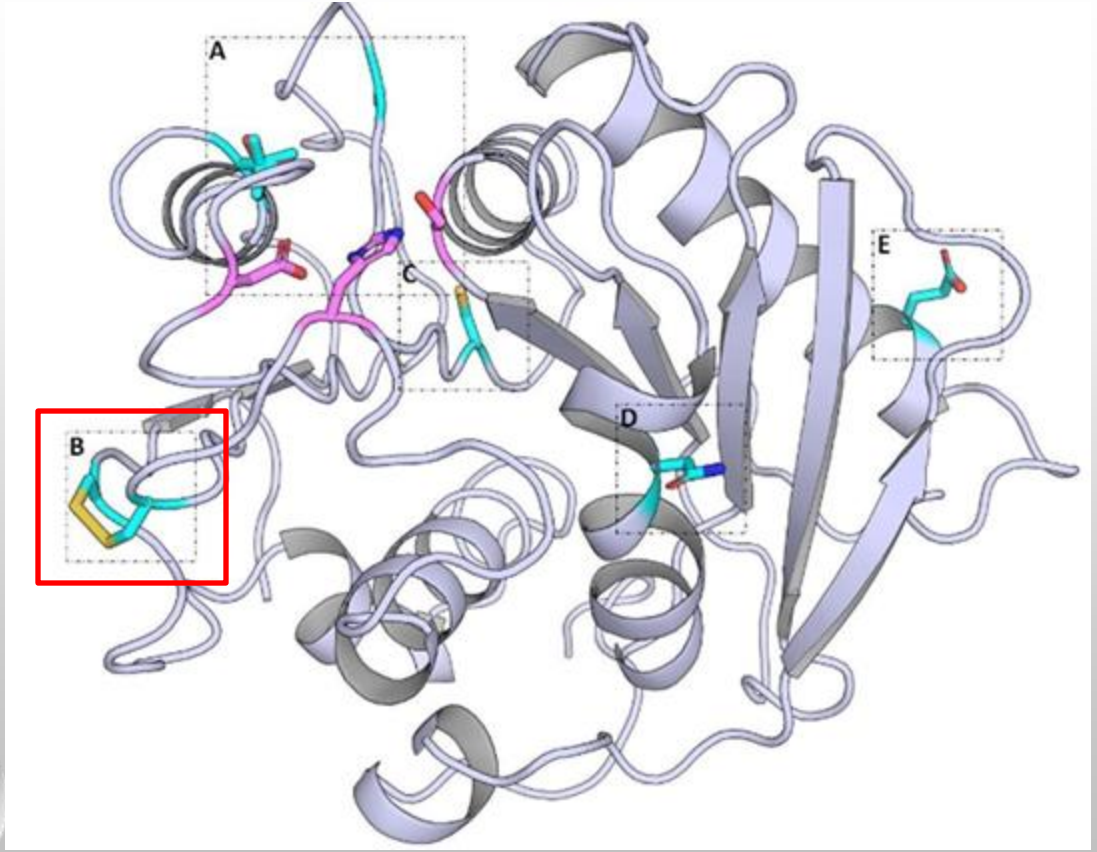


- Ancestral Sequence Recostruction
- **K95N mutation**

	Mutant number	Mutations	Purification yield (mg/L)	Activity at 50°C for 1h (μM) TPA	Tm (°C)
	Scaffold	PETase^{ACCCET}		447.8	~68.7 ±0.14
6	**615**	**PET^{ACCCET} + K95N**	13.8	**499.4**	**70.4 ±0.04**

Thermal stability at 70.4°C and 12% increase in terms of enzymatic activity

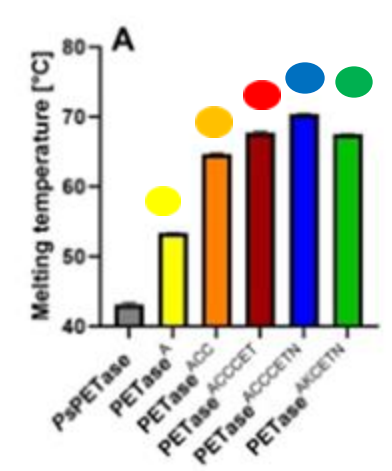
PETase^{AKCETN}: Generation 5

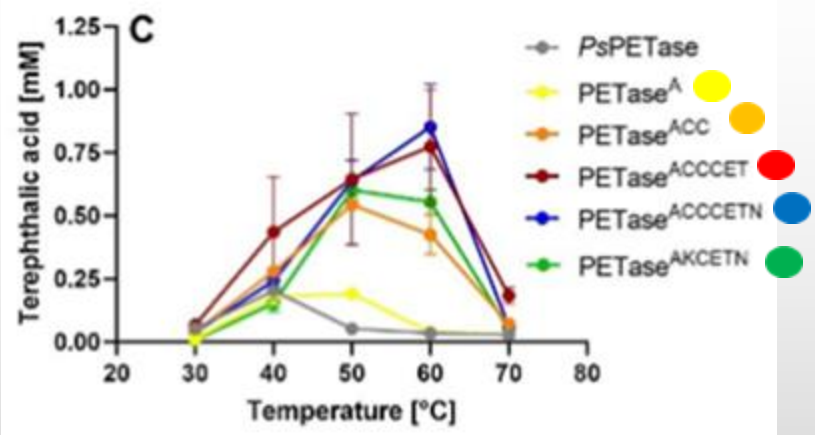


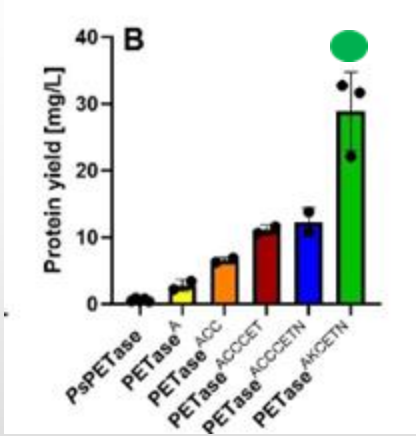
- Machine Learning
- **N233K mutation**

This mutation breaks the engineered disulfide bridge between N233C and S282C

PETase^{AKCETN}: Generation 5







- Machine Learning
- **N233K mutation**

→

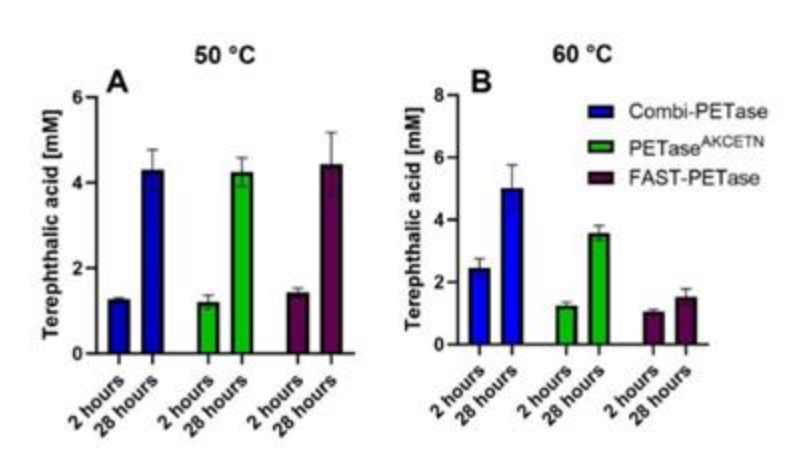
- Modest Tm decrease to 67.5°C compared to Combi-PETase
- reduction of 10°C of the optimum temperature back to 50°C
- However, the yield of the PETaseAKCETN variant is much higher

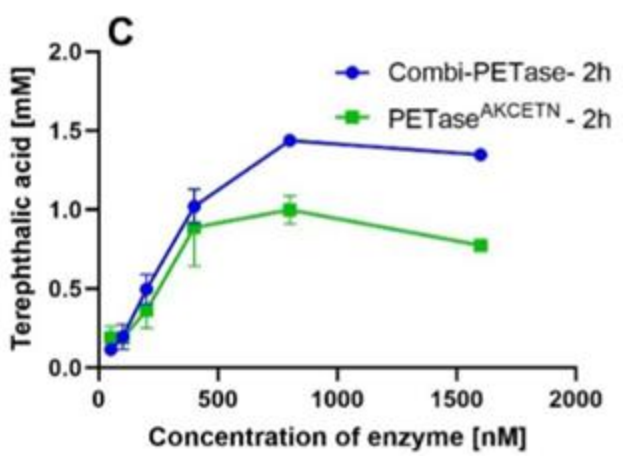
SUITABILITY FOR INDUSTRIAL APPLICATIONS

Table 1. Summary of thermostability, activity, and soluble yield for selected engineered PETase variants. The melting temperature was determined by differential scanning fluorimetry (DSF). PET degradation activity was measured as concentration of released TPA products after incubating hc-PET substrate with 500 nM enzyme concentration at the enzyme's optimal temperature for one hour and shaking at 1000 rpm.

Variant/Approach	Mutations	Activity [TPA mM/1 h]	Tm [°C]	Protein Yield [mg/L]
*Ps*PETase	None	0.20 ± 0.04	43.2 ± 0.2	0.5
PETase^{A} RD (rational design)	D186A	0.19 ± 0.02	53.3 ± 0.1	2.9
PETase^{ACC} RD	D186A/N233C/S282C	0.54 ± 0.01	64.7 ± 0.1	6.6
PETase^{ACCCET} RD	ACC/A179C/S136E/ S214T	0.77 ± 0.2	68.6 ± 0.2	11.1
PETase^{ACCCETN} (Combi-PETase) RD + ASR	ACCCET/K95N	0.85 ± 0.2	70.4 ± 0.04	12.3
PETase^{AKCETN} RD + ASR + AI	D186A/N233K/A179C/S136E/S214T/K95N	0.56 ± 0.05	67.5 ± 0.1	29

SUITABILITY FOR INDUSTRIAL APPLICATIONS





Long-term reaction experiments at high temperatures and high enzyme concentrations:

- at 60°C Combi-PETase performs better than PETaseAKCETN, which in turn is better than FAST-PETase
- 4-fold increase in the optimal enzyme concentration for the reaction

SUITABILITY FOR INDUSTRIAL APPLICATIONS

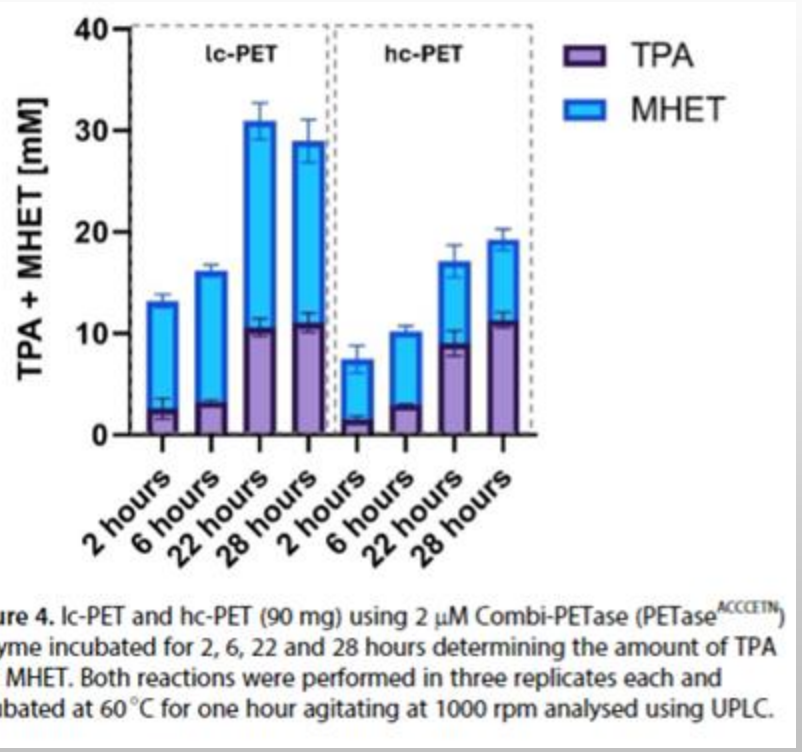
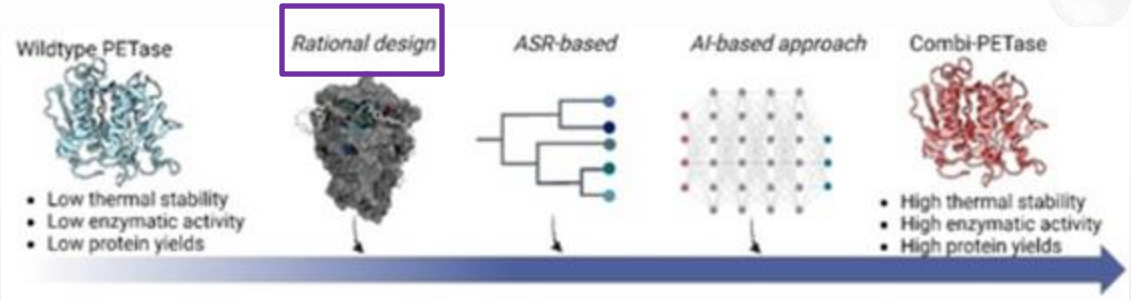
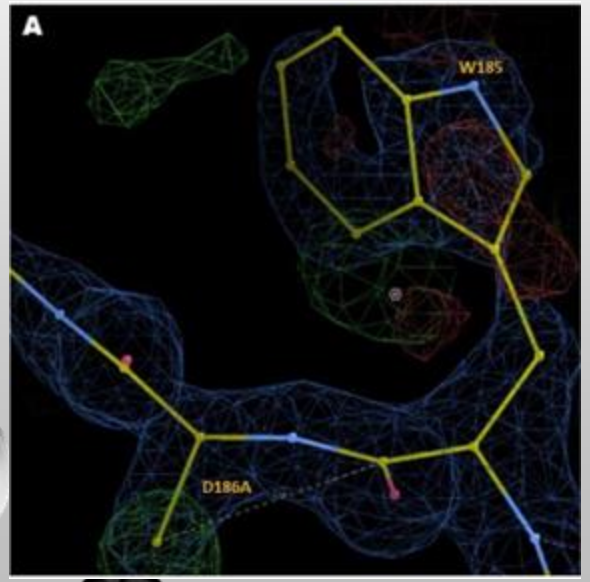


Figure 4. lc-PET and hc-PET (90 mg) using 2 μM Combi-PETase (PETase^{ACCCETN}) enzyme incubated for 2, 6, 22 and 28 hours determining the amount of TPA and MHET. Both reactions were performed in three replicates each and incubated at 60 °C for one hour agitating at 1000 rpm analysed using UPLC.

- Test of the residual activity of the enzyme against high-cristalline PET substrate;
- **The new variant retains 65% of the activity, which is very high compared to other PETase enzymes.**

SUMMARY

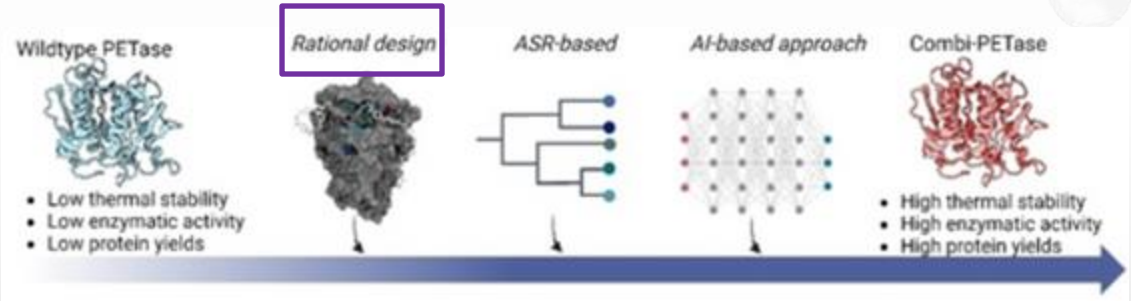


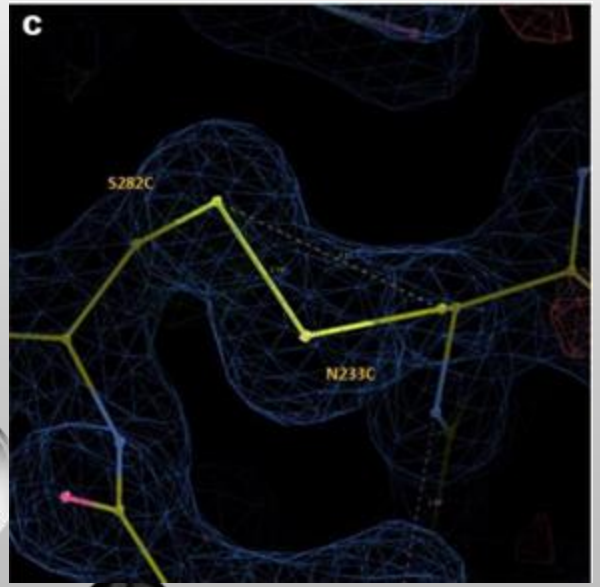


First Generation:

- Electron density around the ‘wobbly’ W185 residue, with the D186A mutation;

SUMMARY

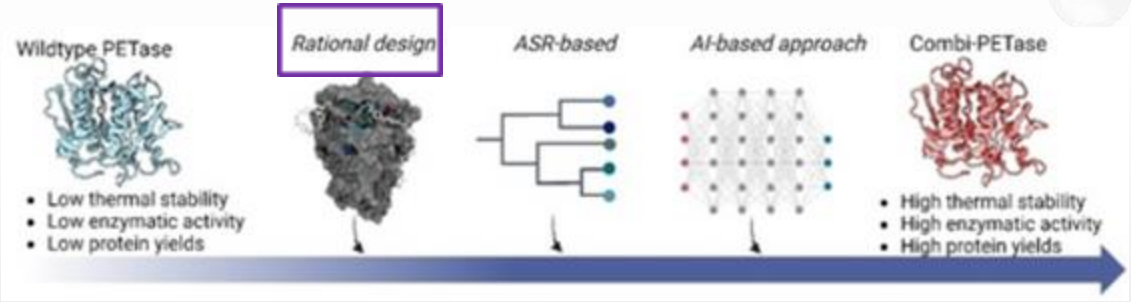


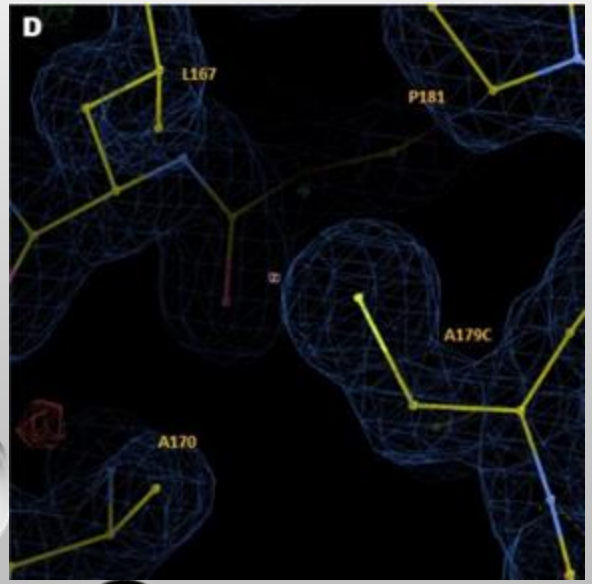


Second Generation:

- Electron density at the N233C and S282C mutations;

SUMMARY

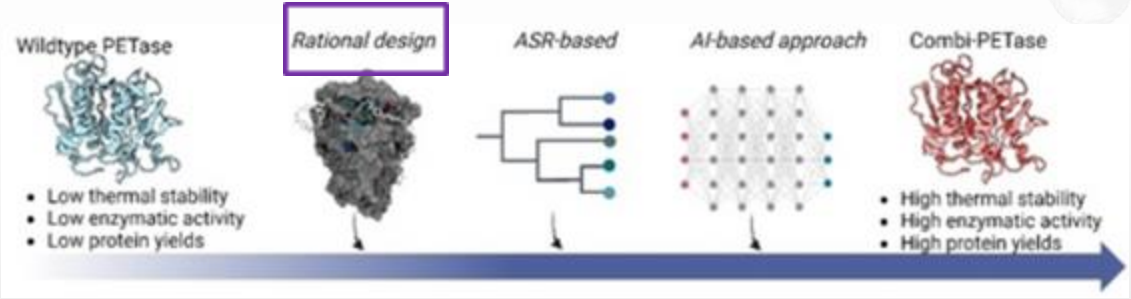


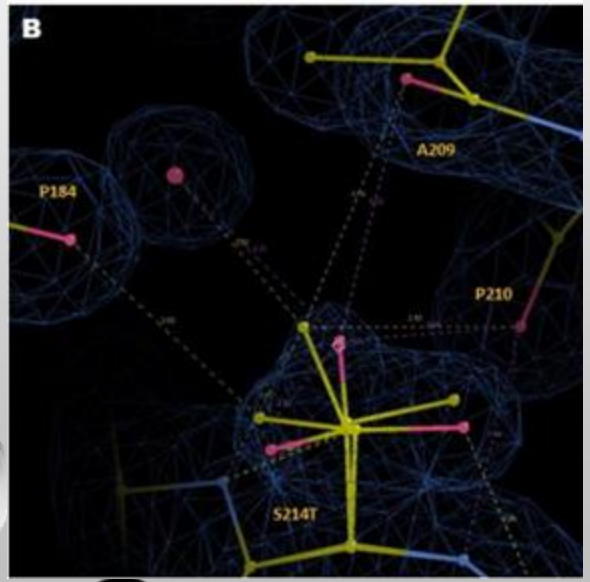


Third Generation:

- The mutation fills the hydrophobyc cavity in the core of the enzyme;

SUMMARY

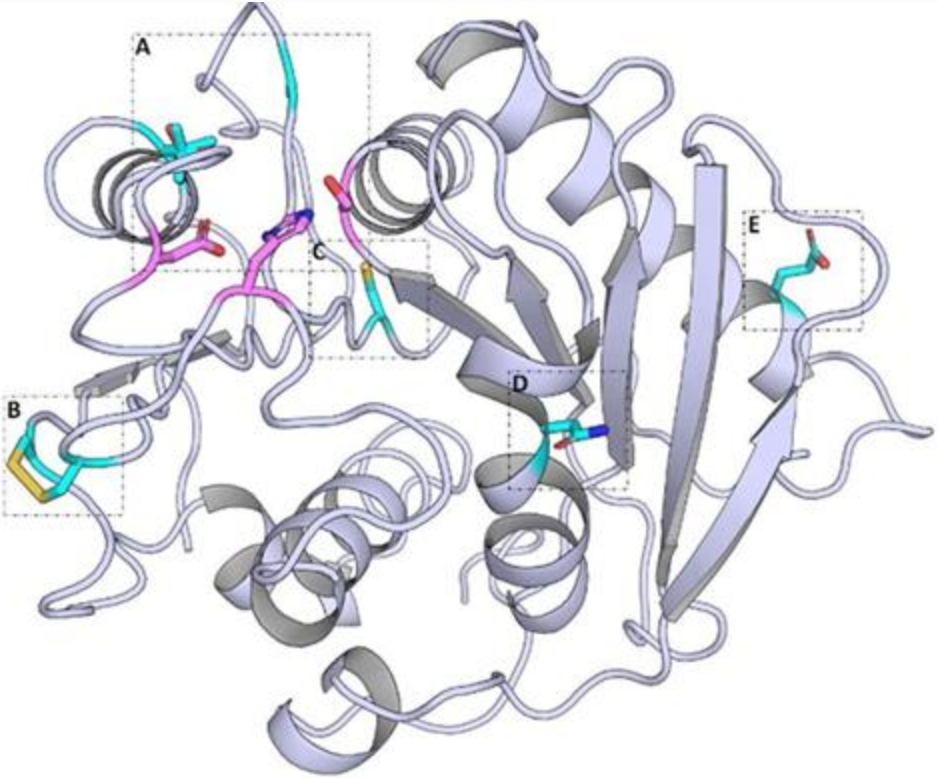




Third Generation:

- The mutation S214T breaks the interaction with P184;

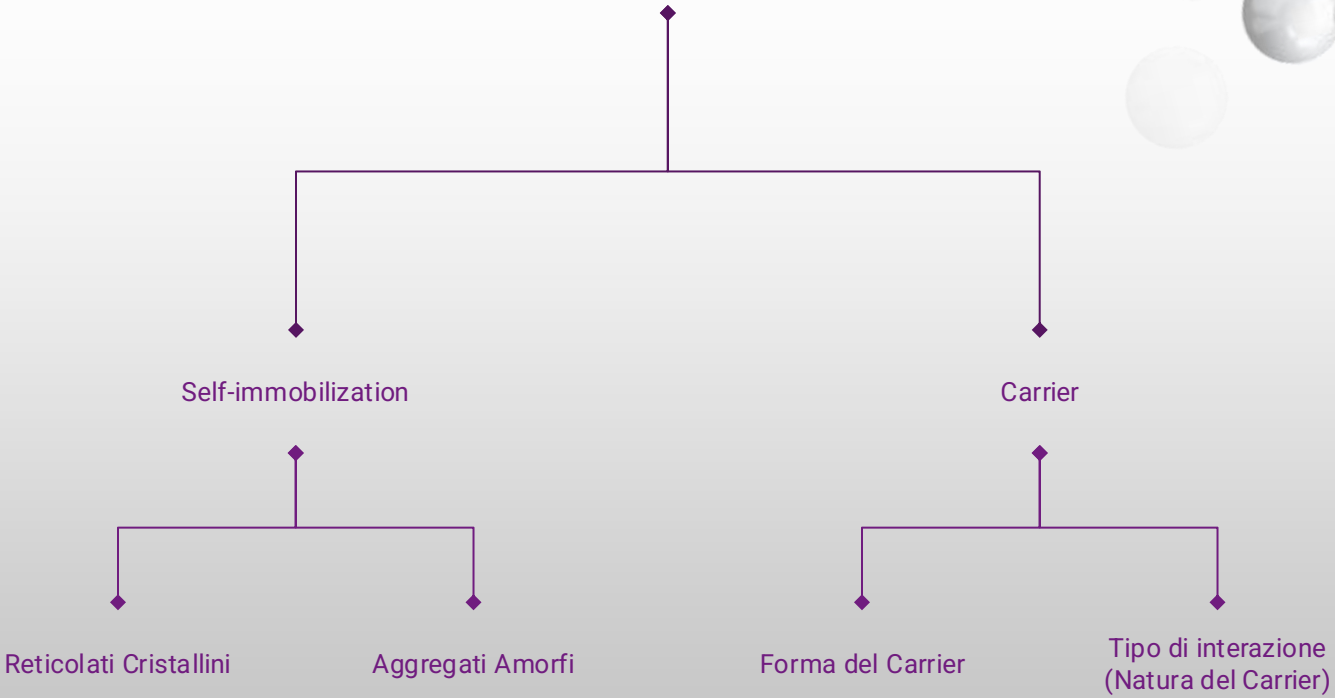
THE NEW Combi-PETase

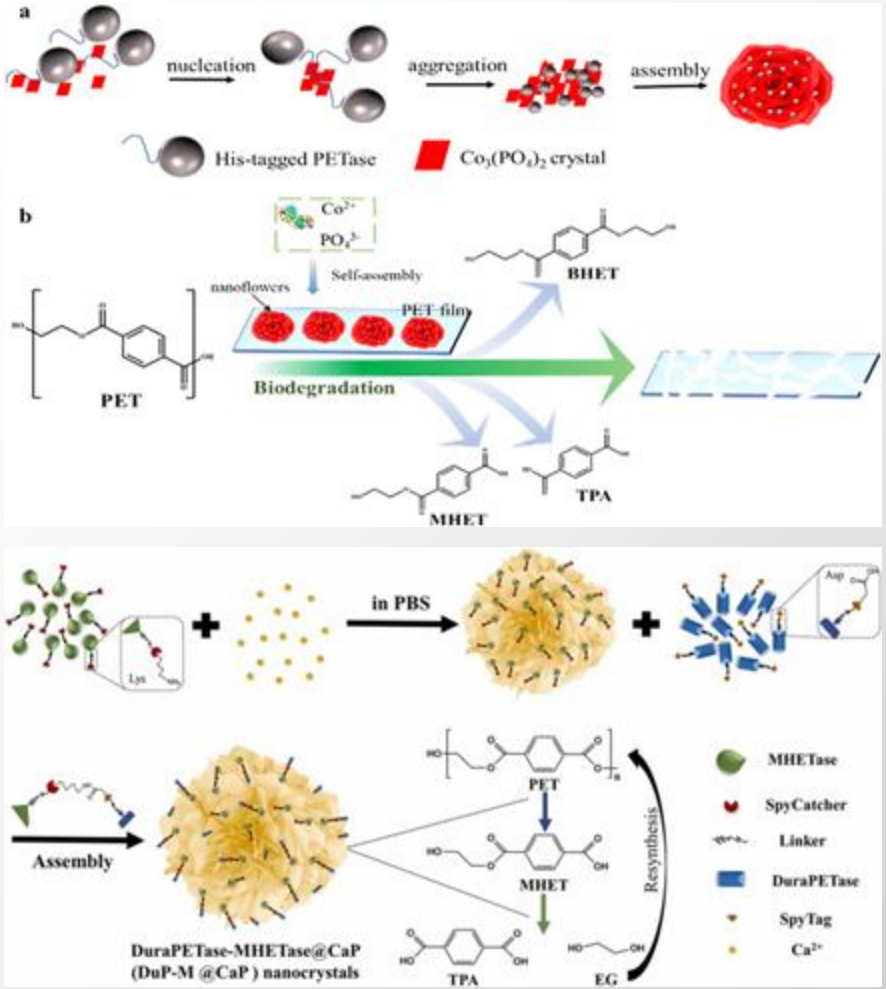


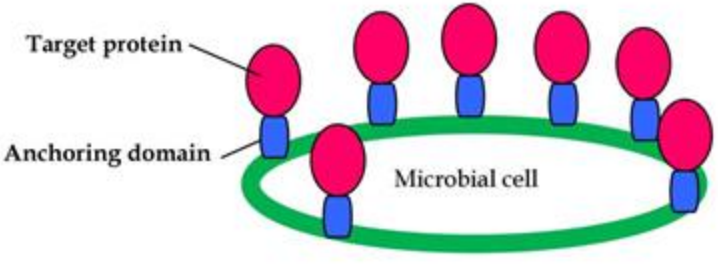
They have engineered the Combi-PETase variant to perform better in terms of:

- Protein yields
- Thermo stability
- Activity
- Tolerance to high enzyme concentration
- Longer reaction times

IMMOBILIZZAZIONE ENZIMA




a
nucleation
aggregation
assembly
His-tagged PETase
$Co_3(PO_4)_2$ crystal
b
Co^{2+}
PO_4^{3-}
Self-assembly
nanoflowers
PET film
BHET
PET
Biodegradation
MHET
TPA
in PBS
Lys
Asp
Assembly
PET
MHET
TPA
EG
Resynthesis
DuraPETase-MHETase@CaP
(DuP-M @CaP) nanocrystals
MHETase
SpyCatcher
Linker
DuraPETase
SpyTag
Ca^{2+}


Target protein
Anchoring domain
Microbial cell

Thanks!