The application of enzyme engineering in the degradation of plastics

Towards sustainable recycling of PET by improved enzymes

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History of plastics: moldable materials

ca. 1600 — 1856 — 1907 — 1950s

Natural plastics: Rubber

Parkesine: First man-made plastic

Altered natural cellulose

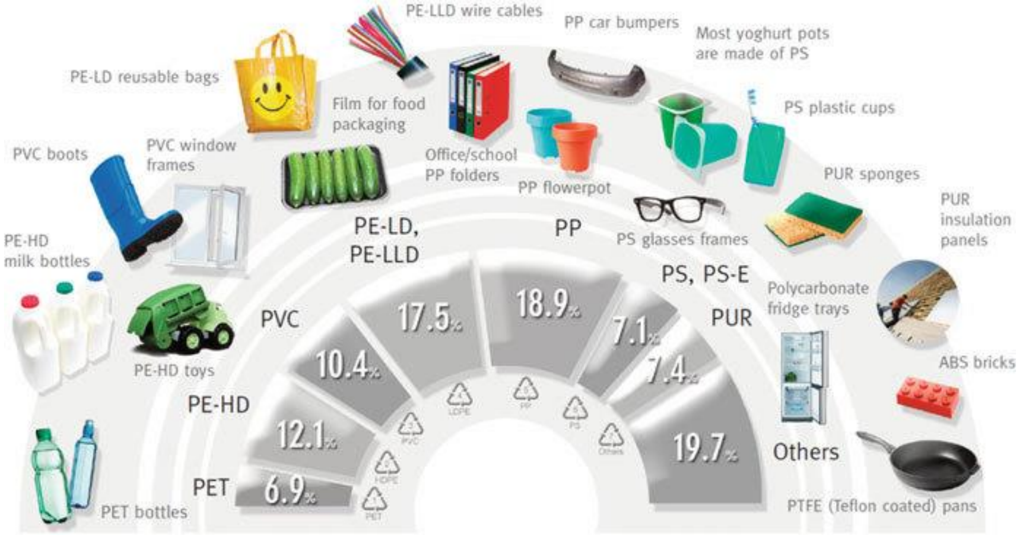
Bakelite: First synthetic plastic

Modern synthetic plastics:

PVC, PET, PP…

Plastics: versatile and durable

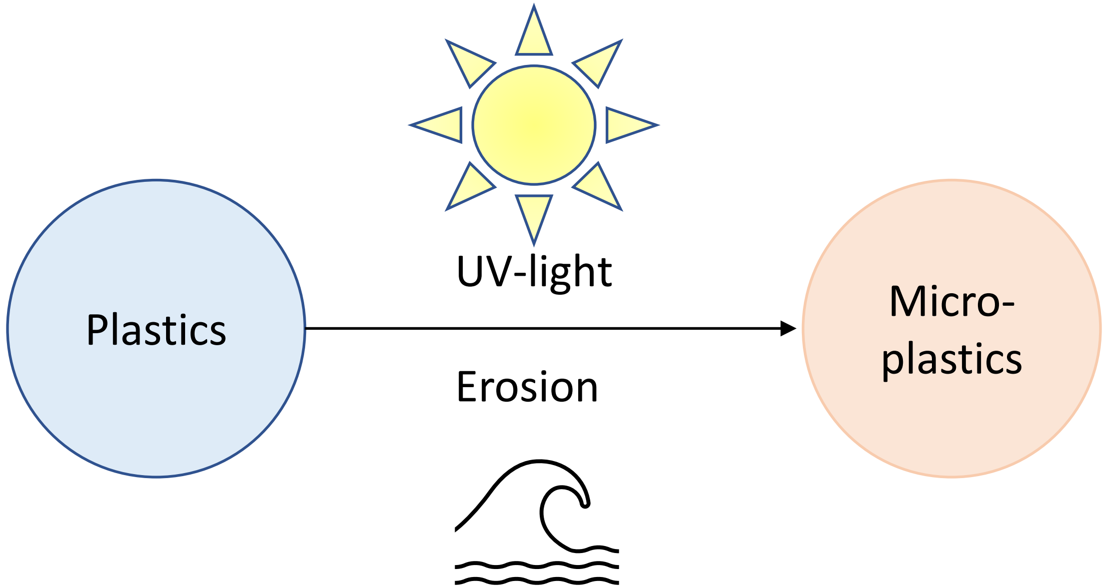
- 359 million tons of plastics are produced every year
- Plastic materials are versatile and very durable



Lackner, 2017

Millions of metric tons of plastic accumulates in the environment each year

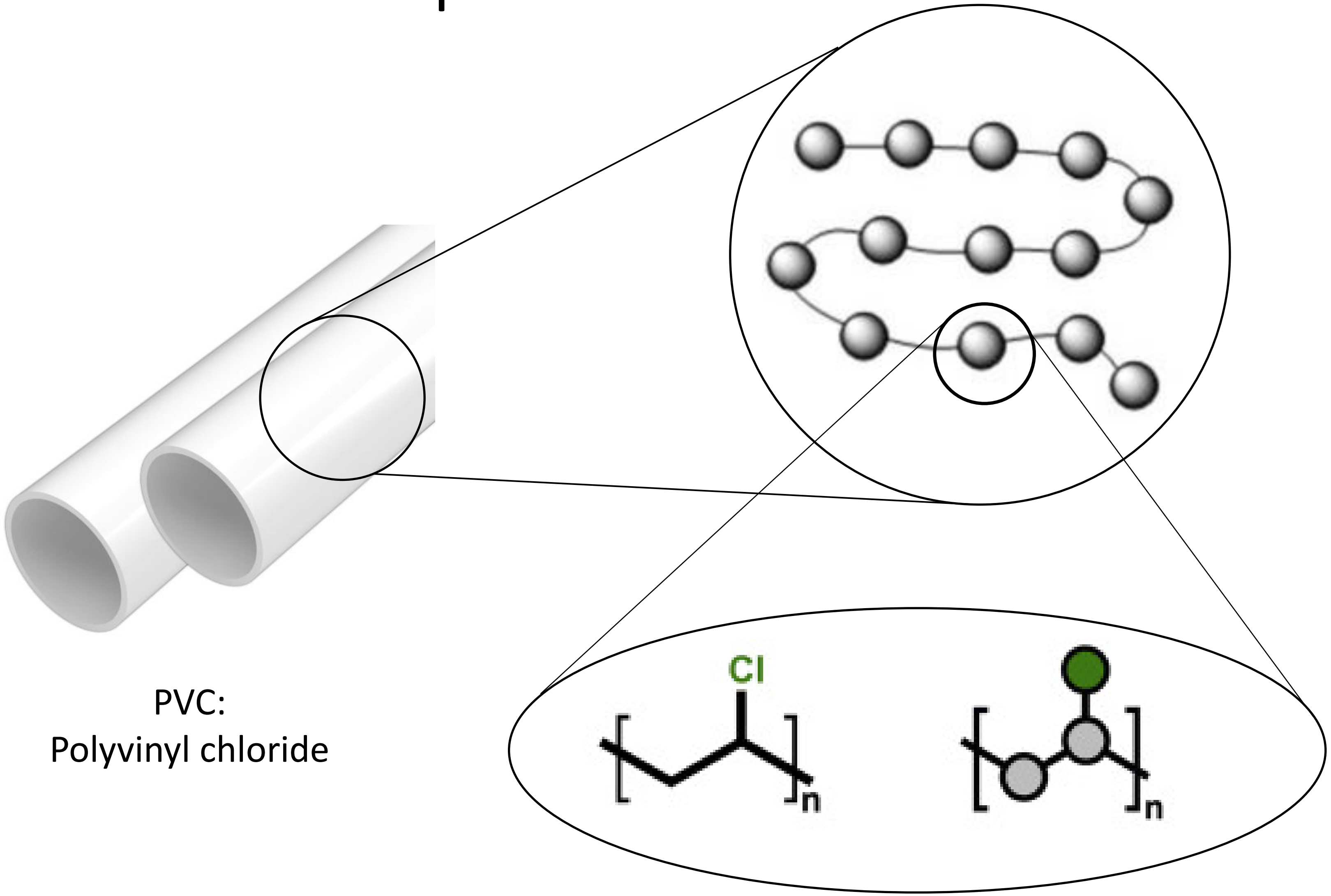
An additional threat: Microplastics



- Smaller particles are easily ingested by animals and fish
- Humans ingest microplastics via animal consumption

Lavender Law et al, 2014

What are plastics?



PVC:
Polyvinyl chloride

Carbon polymer chains of varying lengths

- Cross-links can be added to increase strength
- Additives such as antioxidants, flame retardants add to the qualities of the material

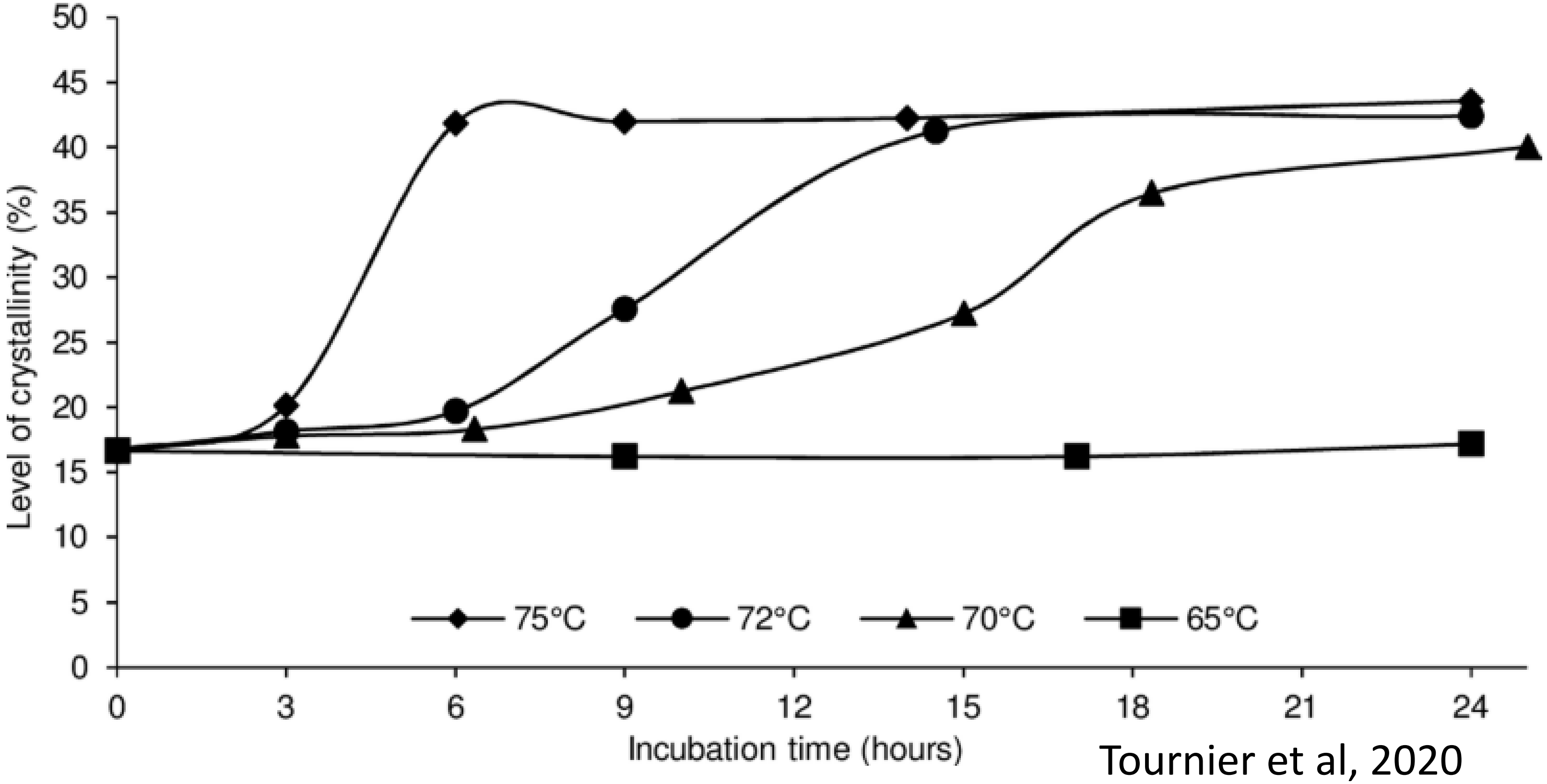
- The degree of ordered regions is reflected in the degree of crystallinity

Vashchuck et al, 2018

- The degree of ordered regions is reflected in the degree of crystallinity

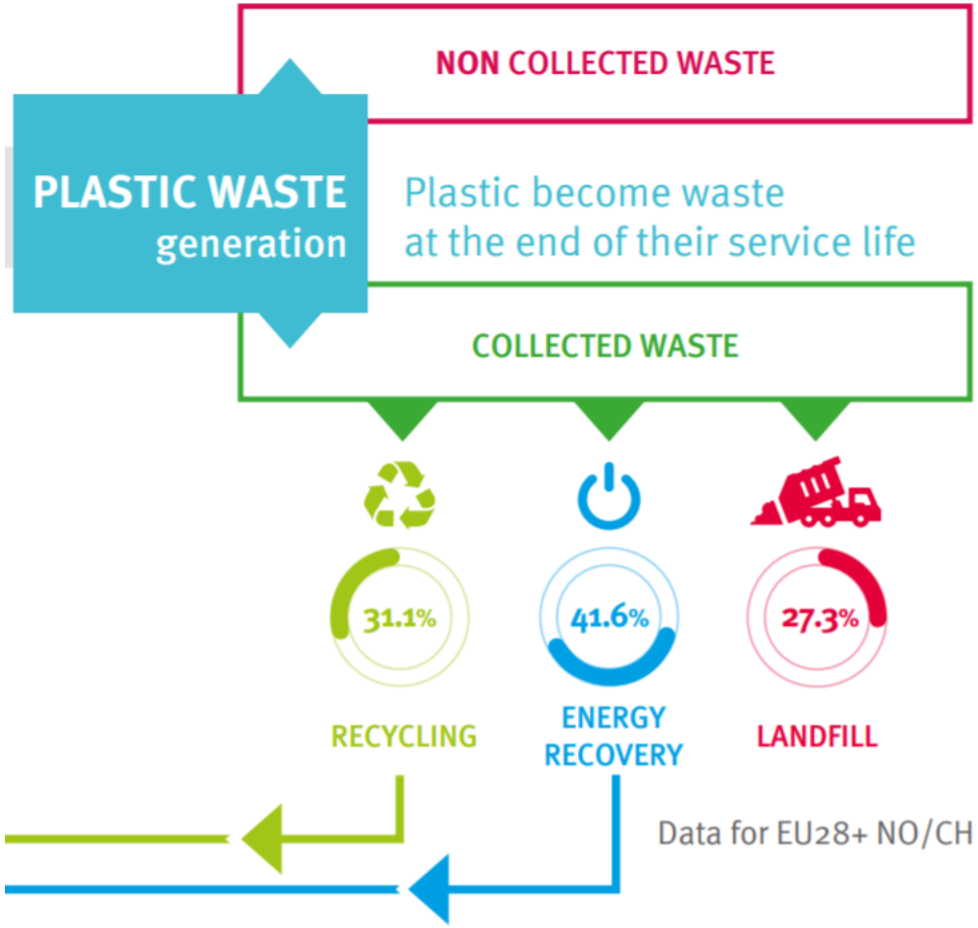
- Induced by heat, stress…

Crystallinity of PET



Tournier et al, 2020

Current recycling of plastics is not efficient enough



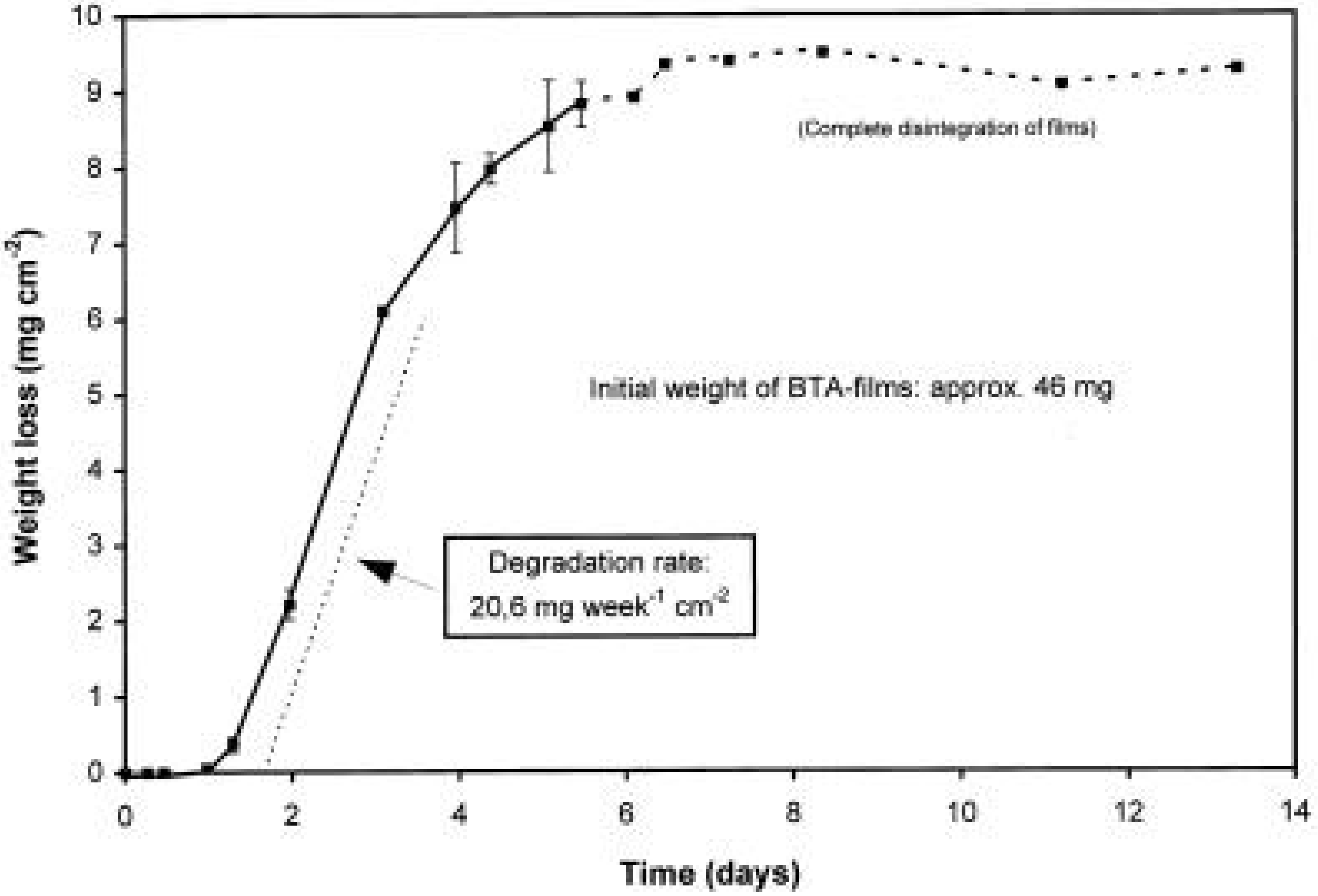
PlasticsEurope, 2018

- Currently, there is still an increase in landfill waste
- Mechanical and chemical recycling methods produce **toxic byproducts**

- Polyethylene terephthalate (PET), mainly used in packaging, is one of the main contributors to waste

Biological degradation of PET by micro-organisms

- *Thermomonospora fusca* was found to be able to degrade PET
- Hydrolases were responsible for this conversion



Kleeberg et al, 1998

- Many other enzymes have been found to degrade PET in the last 20 years

Enzyme (if specified)	Source	Substrate
TfH	*Thermobifida fusca*	Melt pressed PET from beverage bottle (10 % crystallinity)
HiC	*Thermomyces insolens*	*lc*PET (7 % crystallinity), *bo*PET (35 % crystallinity)
Thh_Est	*Thermobifida halotolerans*	PET film (properties not reported)
LC-Cutinase	Metagenome from leaf branch compost	Amorphous PET film
	Nocardia species	PET transparency sheets
Tcur1278 Tcur0390	*Thermomonospora curvata*	Nanoparticles of *lc*PET film
Cut190 (S226P/R228S)	*Saccharomonospora viridis*	*lc*PET (6–7 % crystallinity) PET film for packaging (PET-S) (8.3 % crystallinity)
	Comamonas testosterone F6.	Micro-size PET particles (23 % crystallinity)
	Uncultured bacterium	Amorphous PET foil
	Streptomyces species	Powdered PET from beverage bottle
PETase	*Ideonella sakaiensis* 201-F6	*lc*PET (1.9 % crystallinity) and high crystallinity *hc*PET (commercial bottle)

Papadoulou et al, 2019

Challenges in enzymatic PET degradation

Enzyme	Organism of origin	Estimated Tm by DSF (°C) ± s.d.	Specific activity ($mg_{TAeq}.h^{-1}.mg_{enzyme}^{-1}$) ± s.d. 65°C
BTA-hydrolase 1	*Thermobifida fusca*	70.8 ± 0.0	**2.76 ± 0.70**
BTA-hydrolase 2	*Thermobifida fusca*	67.2 ± 0.3	**2.10 ± 0.41**
FsC	*Fusarium solani pisi*	56.2 ± 0.2	n. d.
Is-PETase	*Ideonella sakaiensis 201-F6*	46.4 ± 0.2	n. d.
LCC	Uncultured bacterium	84.7 ± 0.2	**93.19 ± 0.29**

Tournier et al, 2020

- Low thermal stability if the enzyme
- Slow conversion rate
- Aspecificity in substrates
- Cost of production

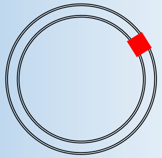
→ Enzyme engineering

Enzyme engineering

Rational

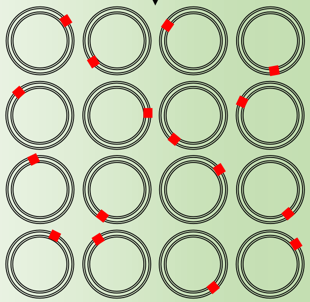
Structure-based design

Site-specific mutagenesis

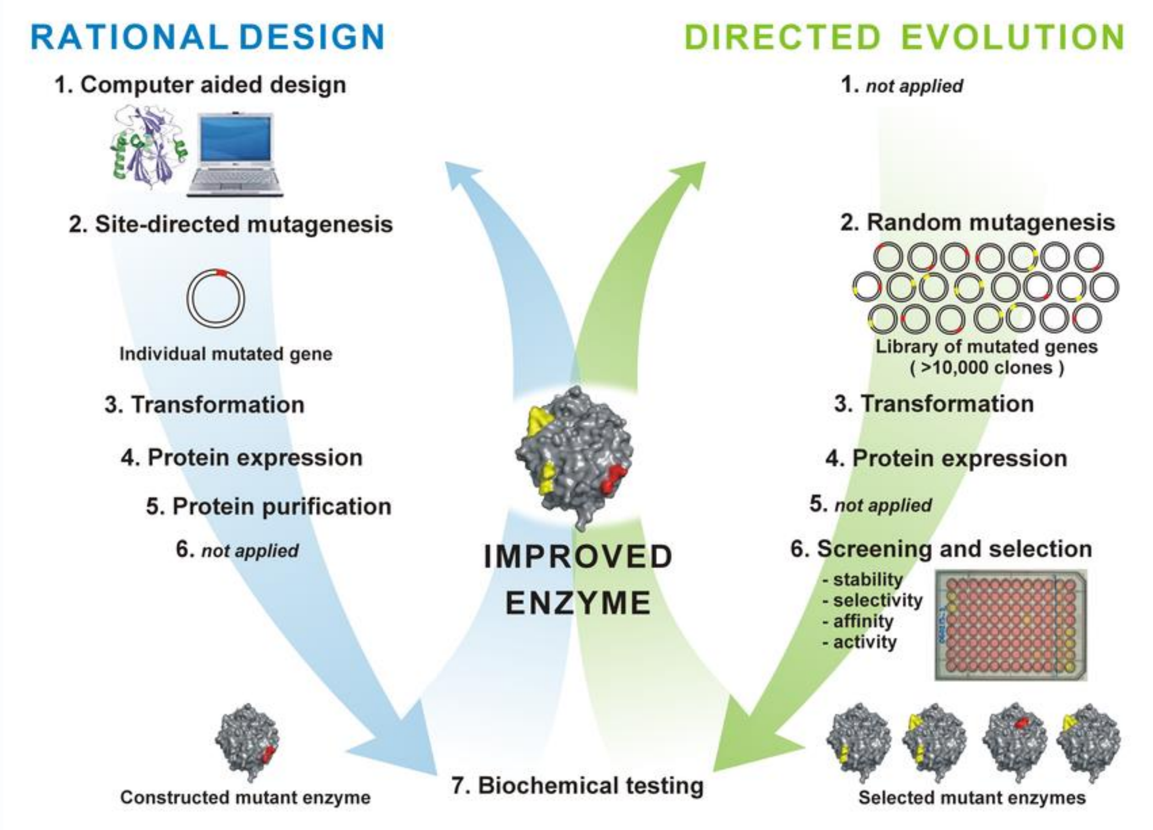


Random

Random mutagenesis



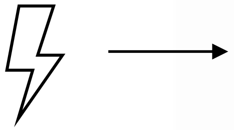
Screening and selection

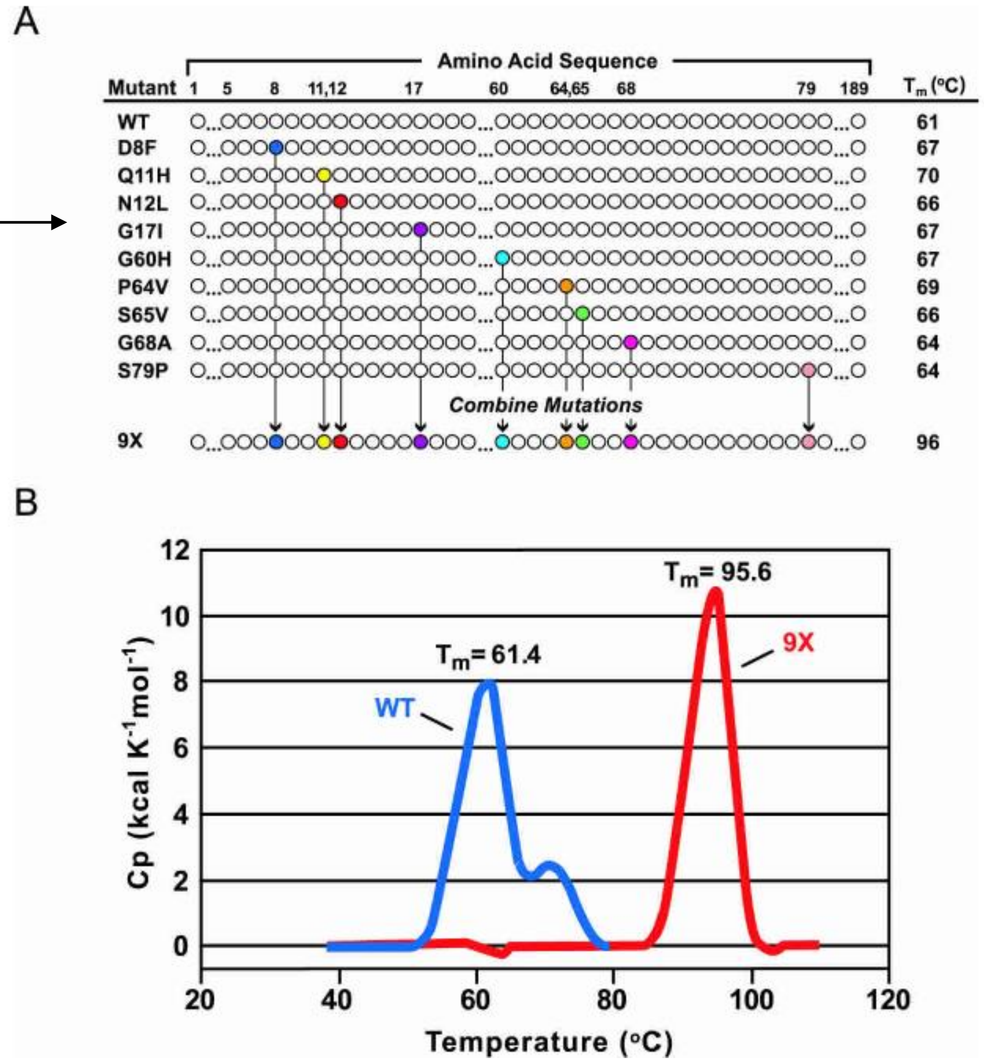


Dvorak, 2007

Protein expression & validation

Example of random design: Thermostability improvement of a xylanase



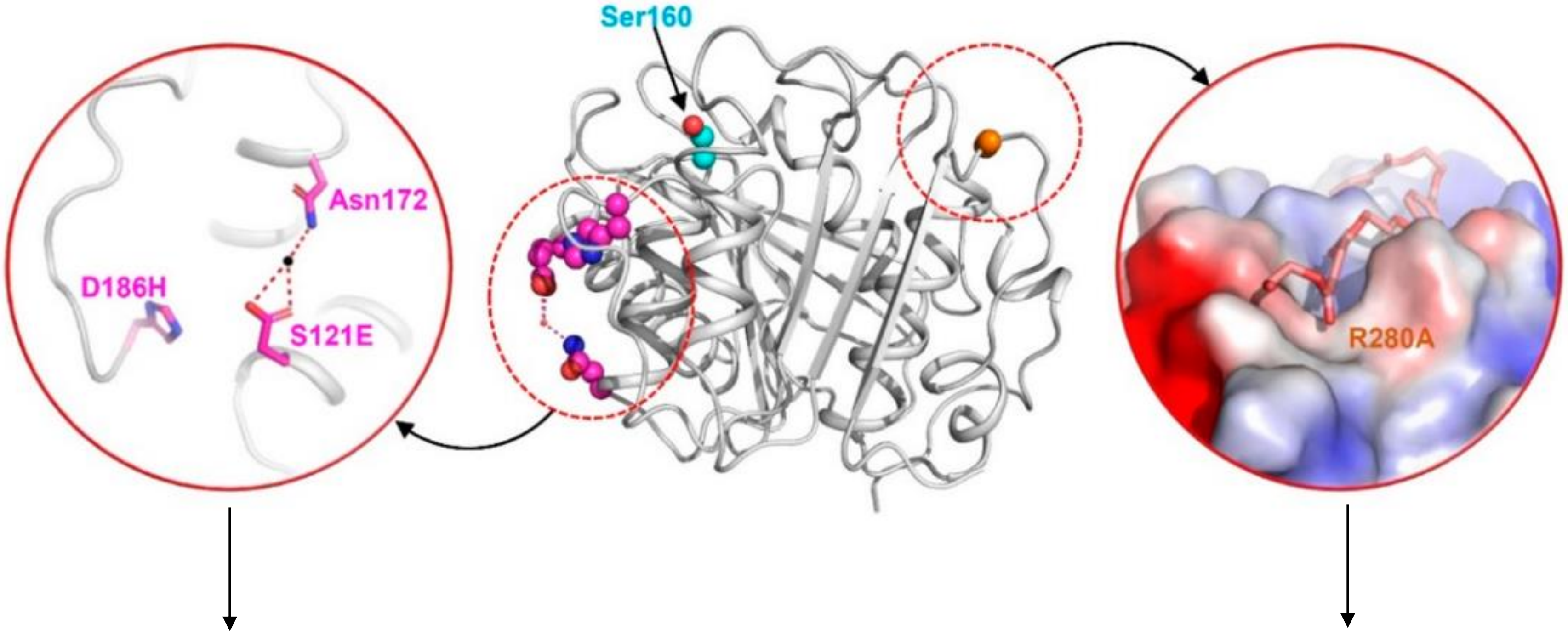


- Xylanases can hydrolyse the biopolymer xylan, a principal component in hemicellulose
- Xylanases are used in food industry, animal feed production and other industries

Palackal et al, 2004

Example of rational design of a PETase from *Ideonella sakaiensis*

- A PET-degrading enzyme of *Ideonella sakaiensis* lacked **thermal stability**
- By structural analysis, residues were selected to increase thermal stability

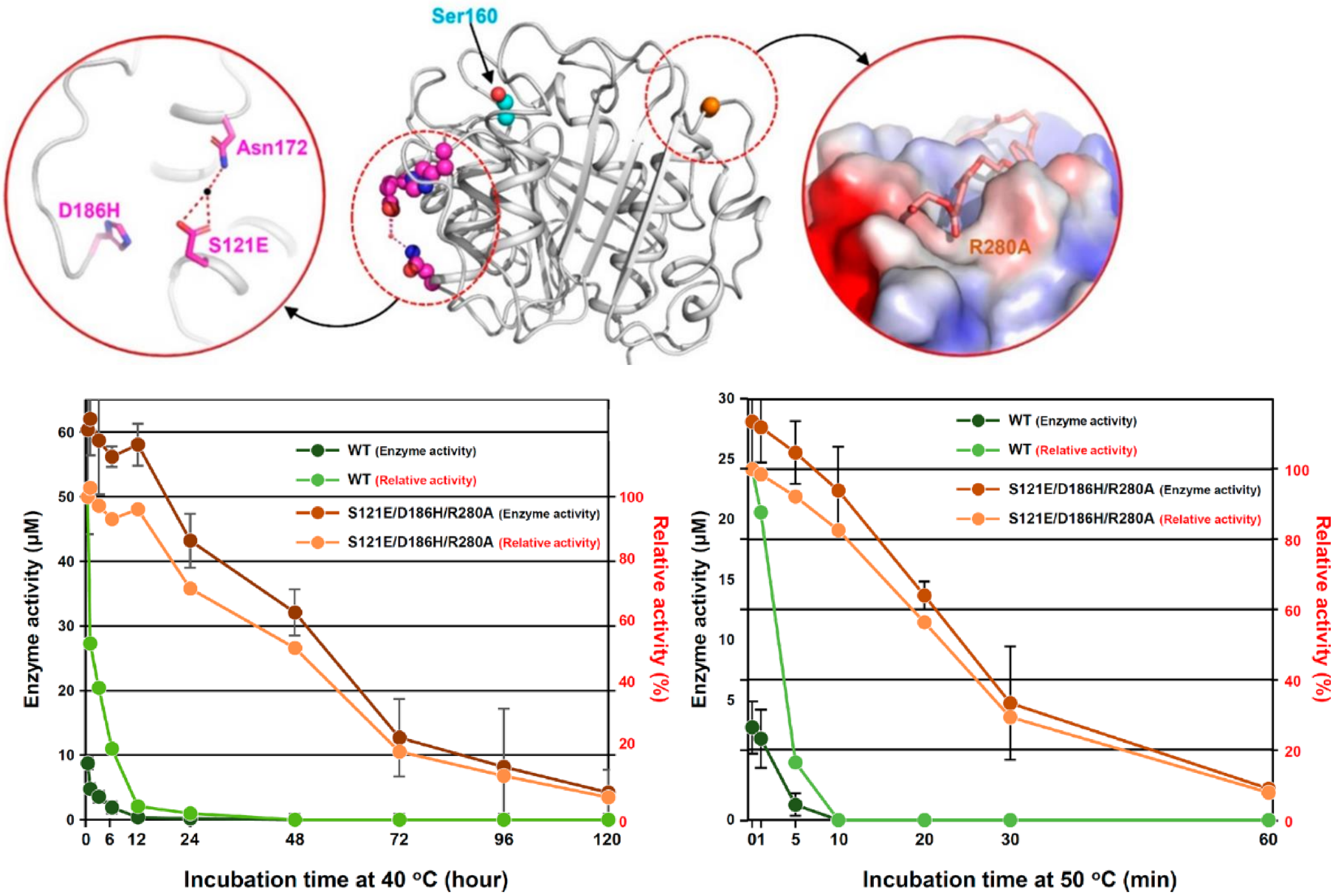


- Comparison with a **homologue**
- Replacement with **compatible residues**
- Hydrogen bonding

- Identify **substrate binding subsites**
- Replace hypothesized sterically hindering **arginine with alanine**

Son et al, 2019

Increase in thermal stability



Combining rational and random engineering in a PETase

Leaf-branch compost cutinase (LCC) was subjected to rational and random engineering

Enzyme	Organism of origin	Estimated Tm by DSF (°C) ± s.d.	Specific activity ($mg_{TAeq}.h^{-1}.mg_{enzyme}^{-1}$) ± s.d. 65°C
BTA-hydrolase 1	*Thermobifida fusca*	70.8 ± 0.0	**2.76 ± 0.70**
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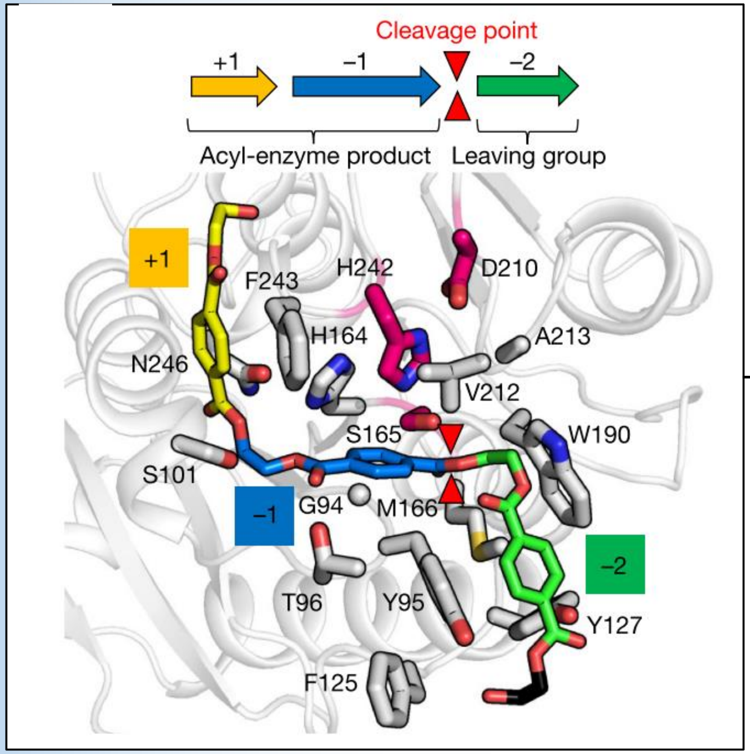
Tournier et al, 2020

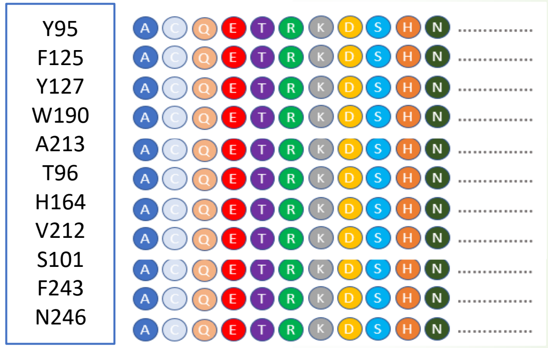
Substrate of LCC is amorphous (non-crystalline) PET, or Gf-PET

Activity improvement of LCC by semi-rational design

LCC



Substrate binds in elongated, hydrophobic groove



Site specific saturation mutagenesis of first shell contact residues

Protein expression & screening

>100% of wt activity

F243I
F243W

> T_m

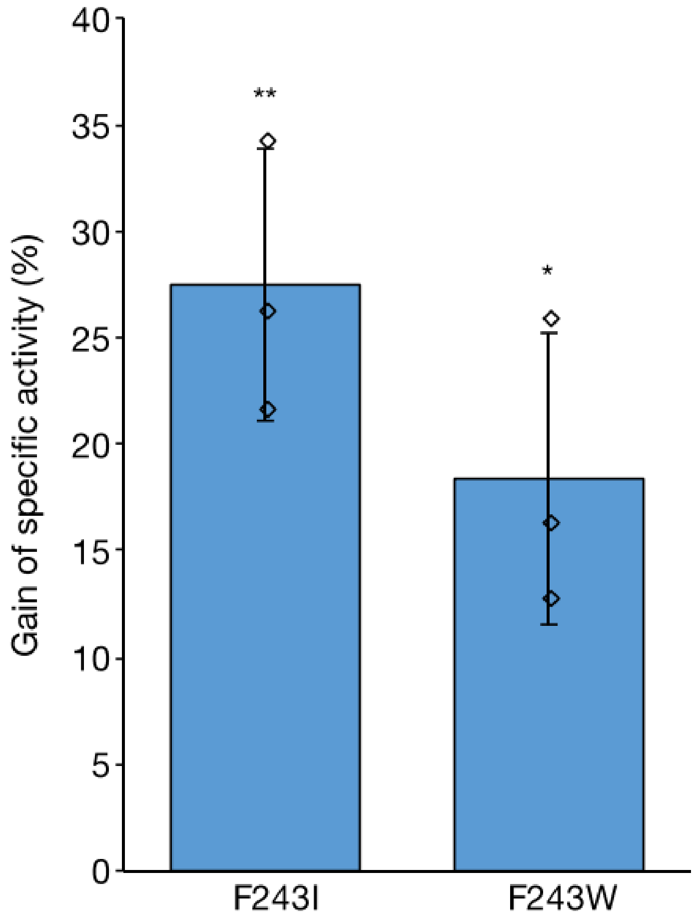
T96M
Y127G
N246D
N246M

Activity improvement of LCC by semi-rational design

Protein expression
& screening

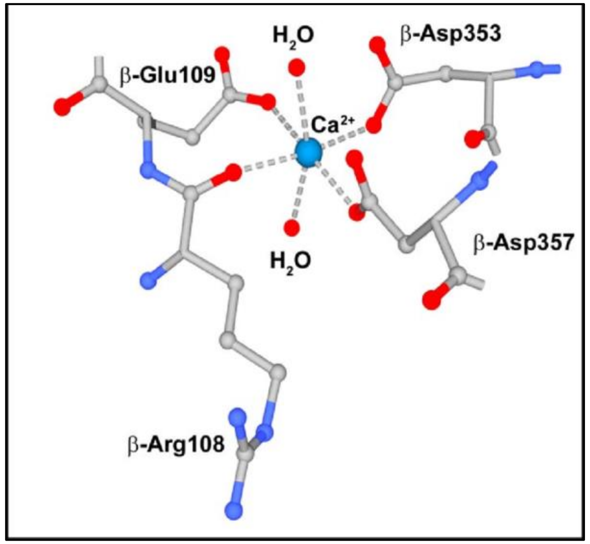
>100% of wt
activity

F243I
F243W



Thermal stability improvement of LCC by rational design

- Divalent metal-binding sites in homologous PET-cutinase structures increase stability
- Addition of Ca^{2+}-ions to LCC led to an increase in thermal stability

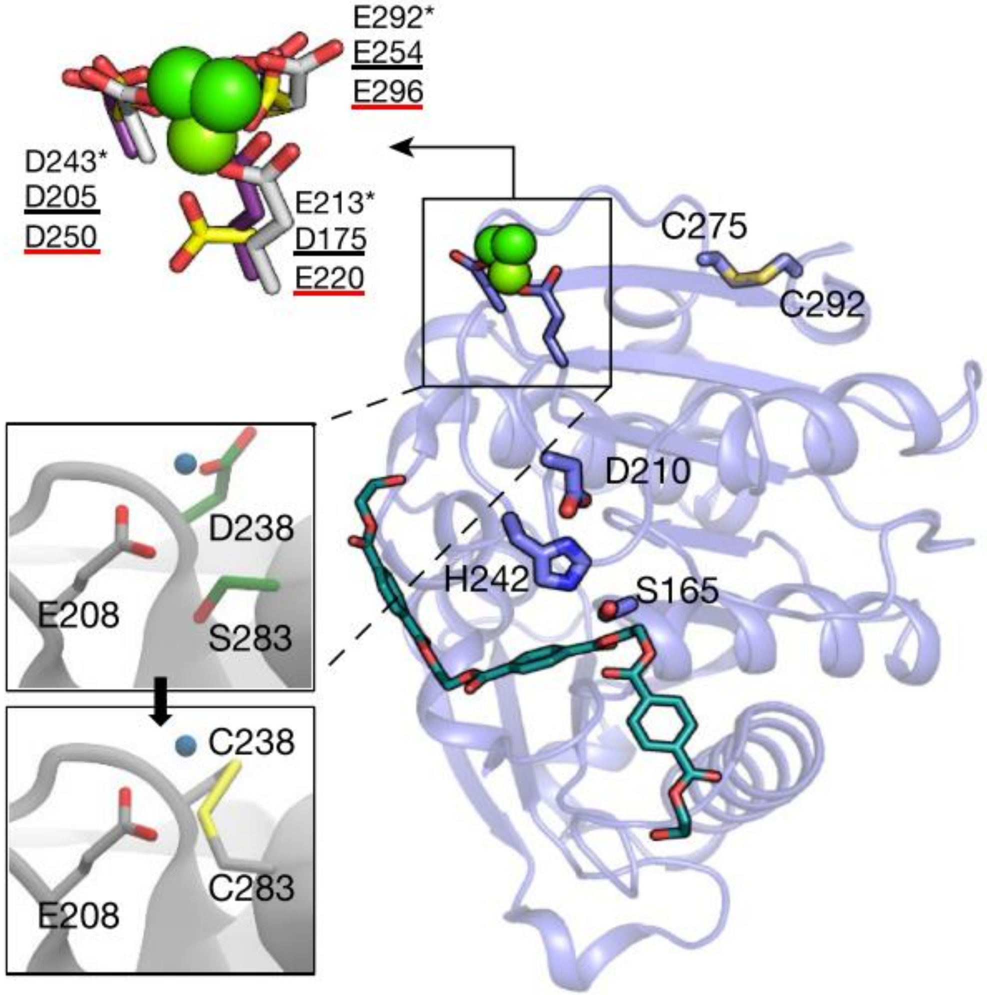


Divalent metal-binding sites:
Three residues bind to metal-ions

Thermal stability improvement of LCC by addition of a disulfide bridge

- Mutation of two equivalent residues from homologous structures to cysteines
- These mutations create a stabilizing disulfide bridge

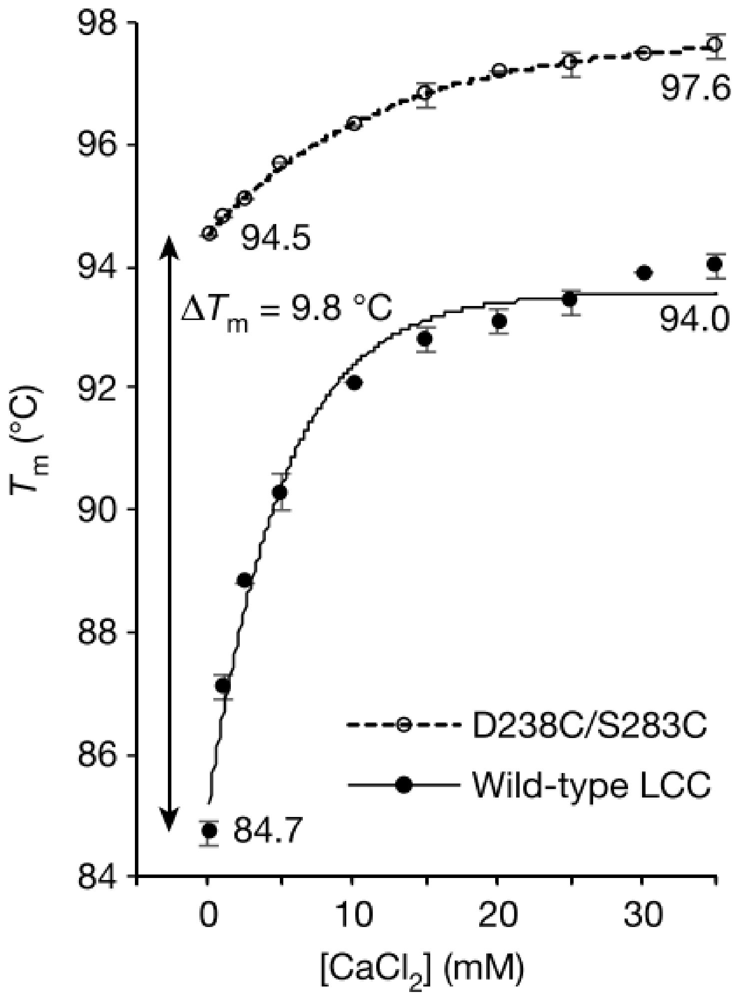
D238C/S283C



Thermal stability improvement of LCC by addition of a disulfide bridge

- Mutation of two equivalent residues from homologous structures to cysteines
- These mutations create a stabilizing disulfide bridge

D238C/S283C



Combining all improvements

Thermal stability improvement

D238C/S283C

F243I
F243W

Activity improvement

	ICC	WCC
	F243I D238C/S283C	F243W D238C/S283C
Activity	122%	98%
T_m	+6.1°C	+10.1°C

Thermal stability improvement

Y127G
N246M

ICCG

ICCM

WCCG

WCCM

4 engineered LCC enzymes

Assessment of 4 engineered enzymes in bioreactor conditions

ICCG

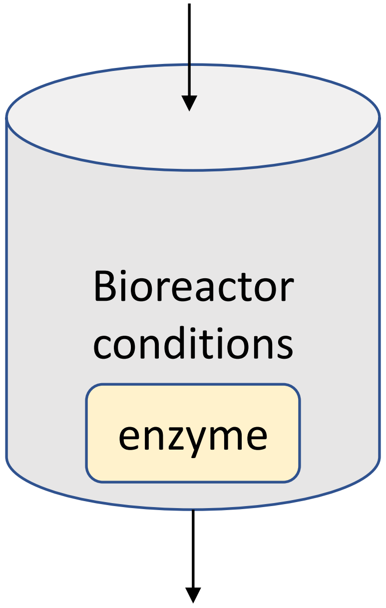
ICCM

WCCG

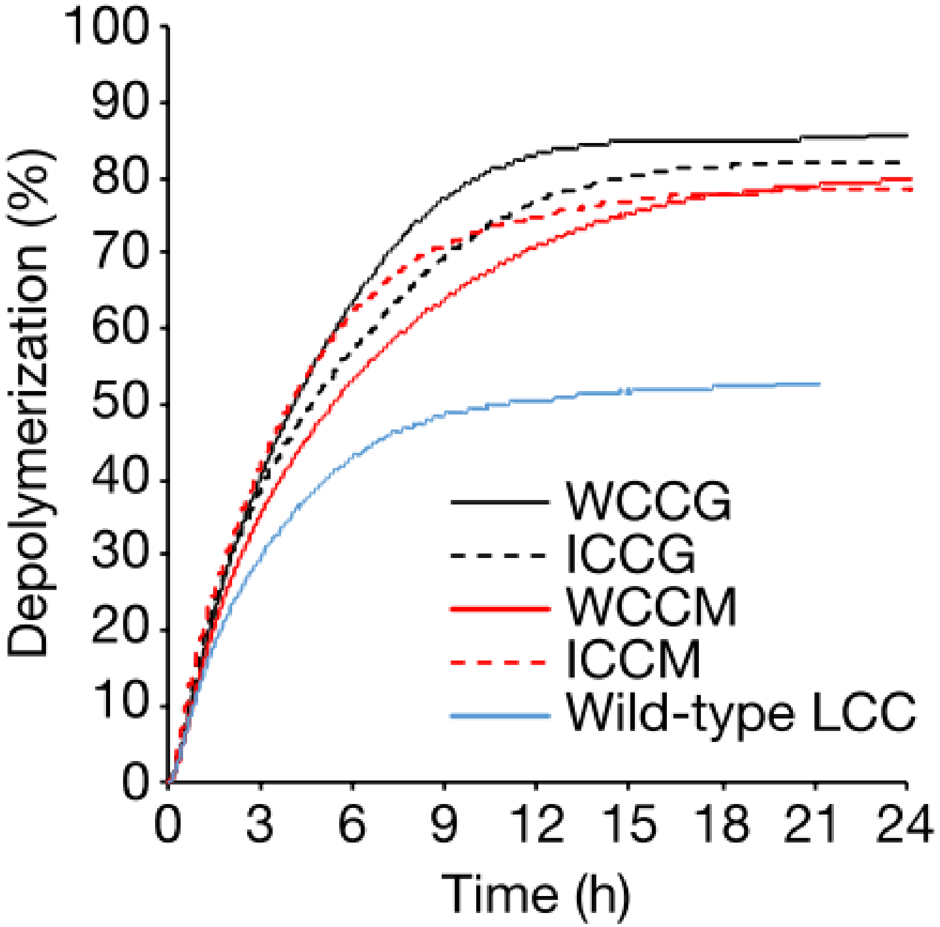
WCCM

PcW-PET:
post-consumer coloured-flake PET waste

extrusion and micronization

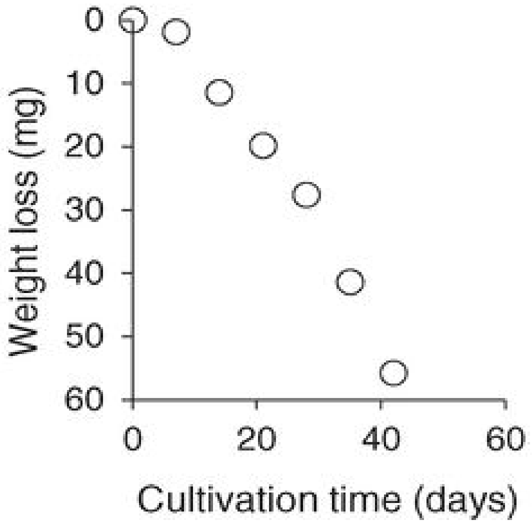


Ethylene glycol
+
Terephthalic acid

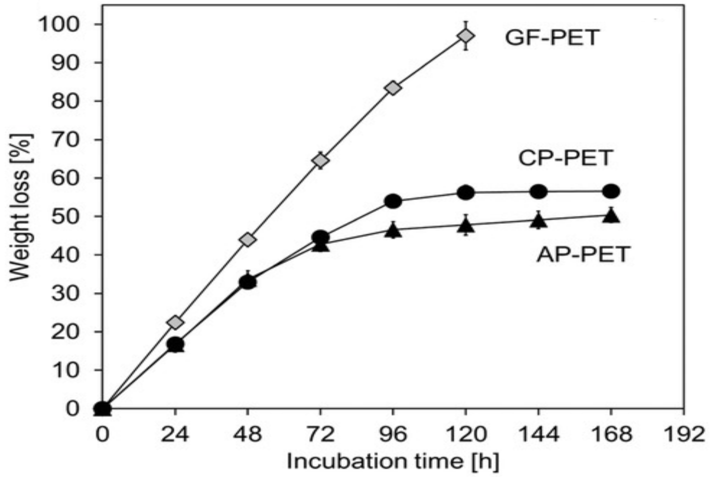


72 °C

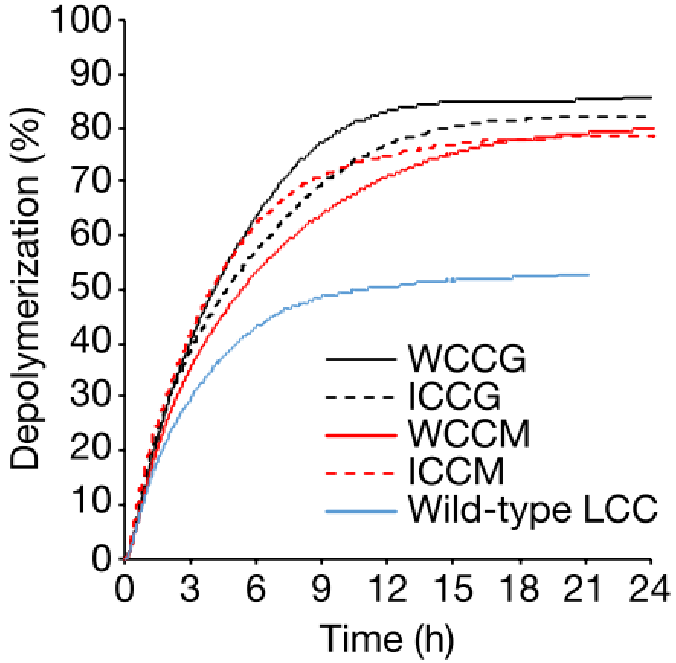
Engineered LCC outperforms other PETases to date



I. sakaiensis – Yoshida et al, 2016

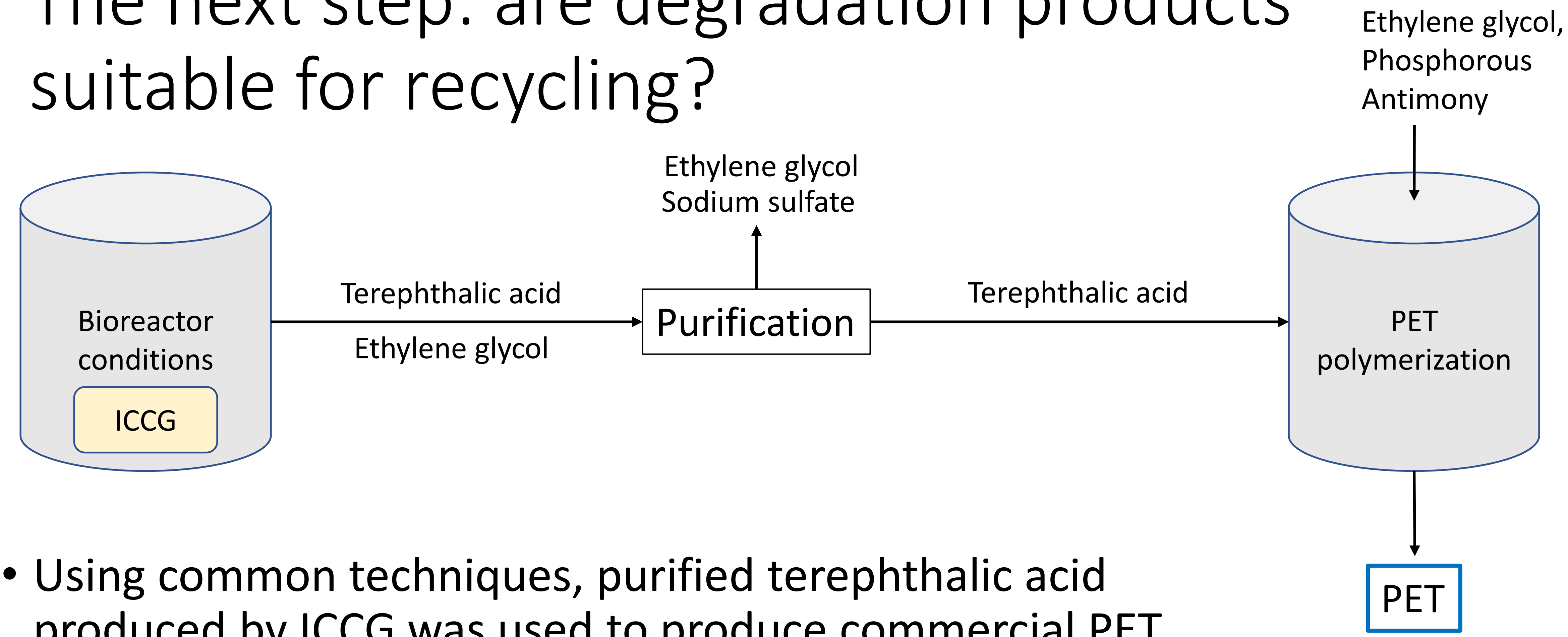


Tfcut2 – Wei et al, 2019



- Previously reported cultures and engineered PET-degrading enzymes require days to partly convert amorphous PET (Gf-PET)
- The engineered LCC variants convert **82-85% of PcW-PET in 15-20 h**

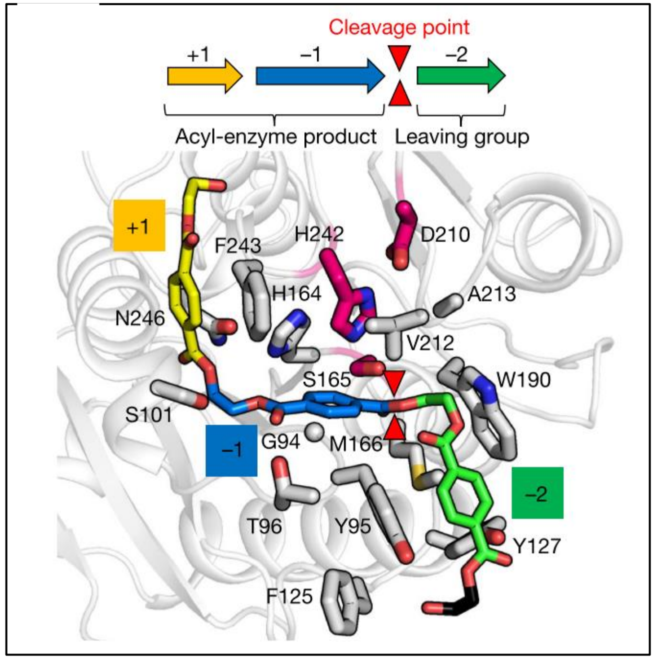
The next step: are degradation products suitable for recycling?

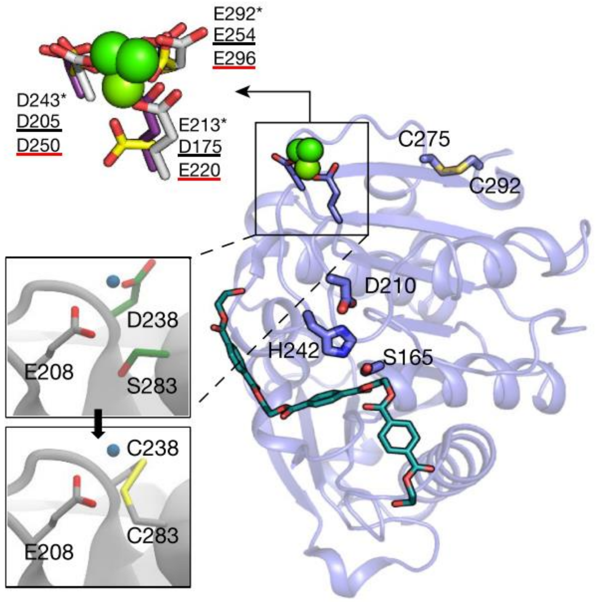


- Using common techniques, purified terephthalic acid produced by ICCG was used to produce commercial PET.
- Recycled PET-bottles had similar properties as newly synthesized PET

Conclusion

- Combining rational and random protein engineering resulted in an improved version of PET-degrading enzyme LCC
- Applying protein engineering techniques can help chemical processes to become sustainable





Questions?