



DER FORSCHUNG | DER LEHRE | DER BILDUNG

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**FuturEnzyme**

Technologies of the FUTURe for low-cost ENZYMES for environment-friendly products

# Mining the microbial diversity for esterases, lipases and plastic degrading enzymes

CLIB Event

Fantastic enzymes:

Where and how to find them



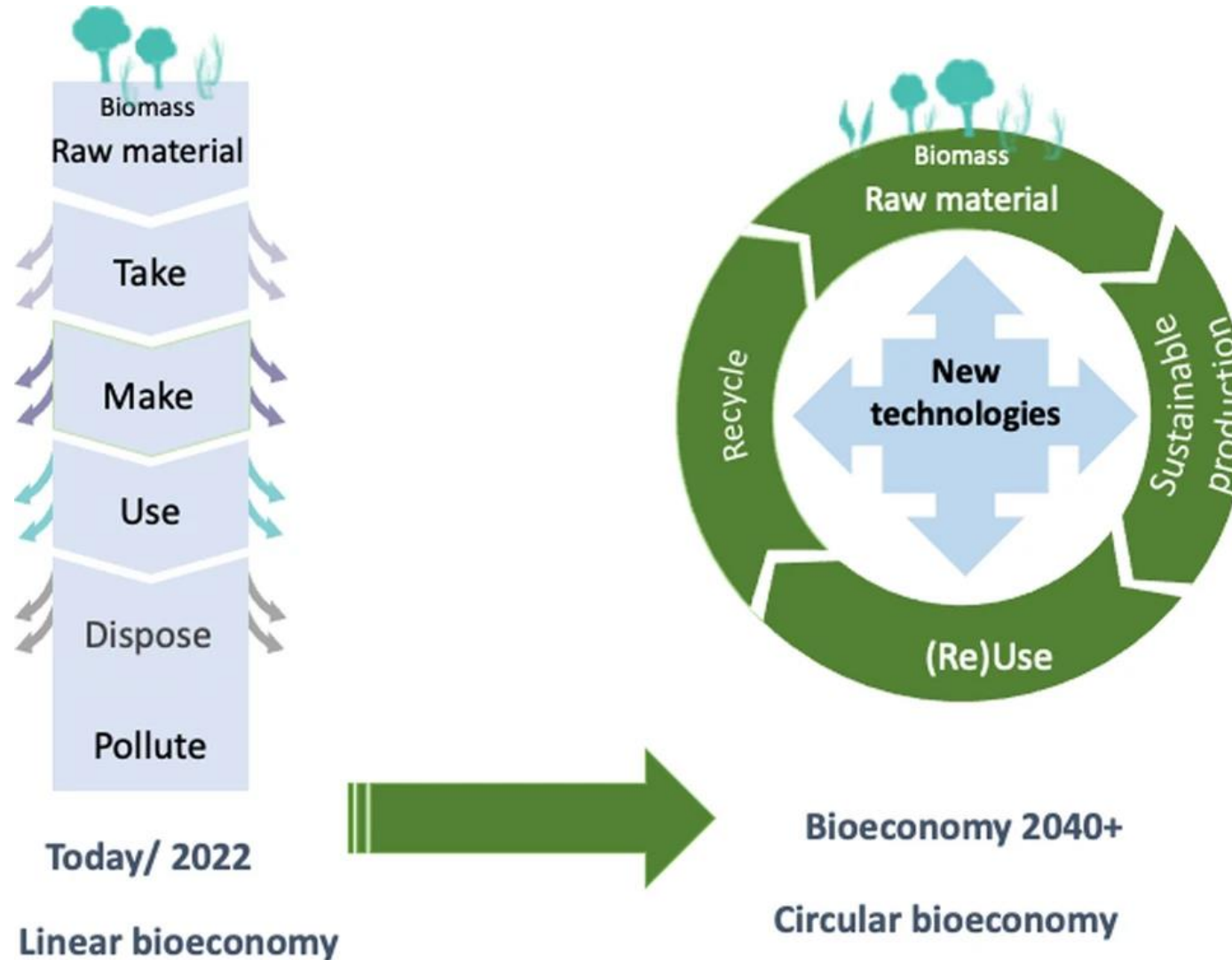
Project funded by the European Union's Horizon 2020  
Research and Innovation Programme under grant agreement No [101000327]

6th of September 2022



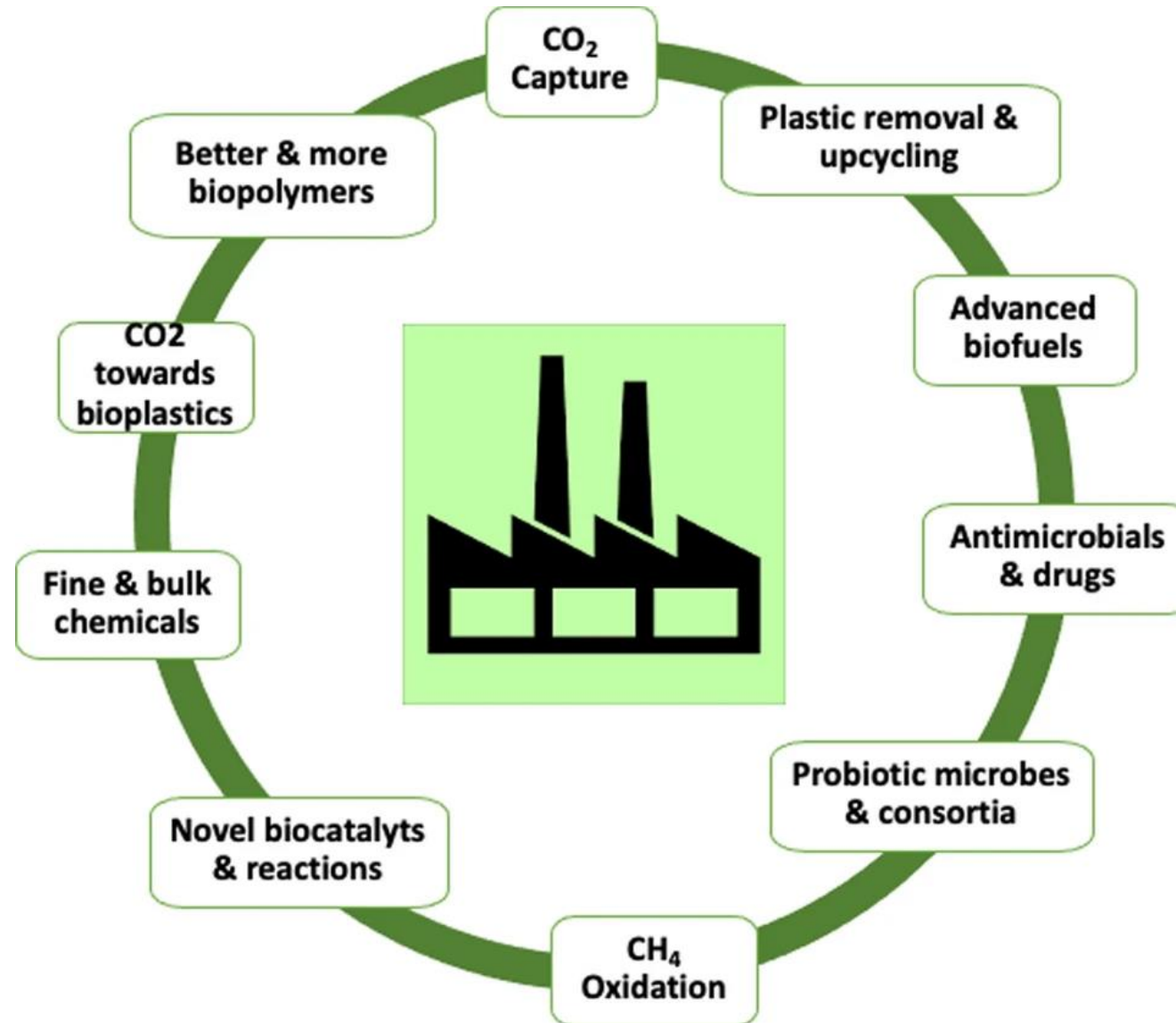


# Shifting towards a circular bioeconomy





# Key developments for a sustainable economy





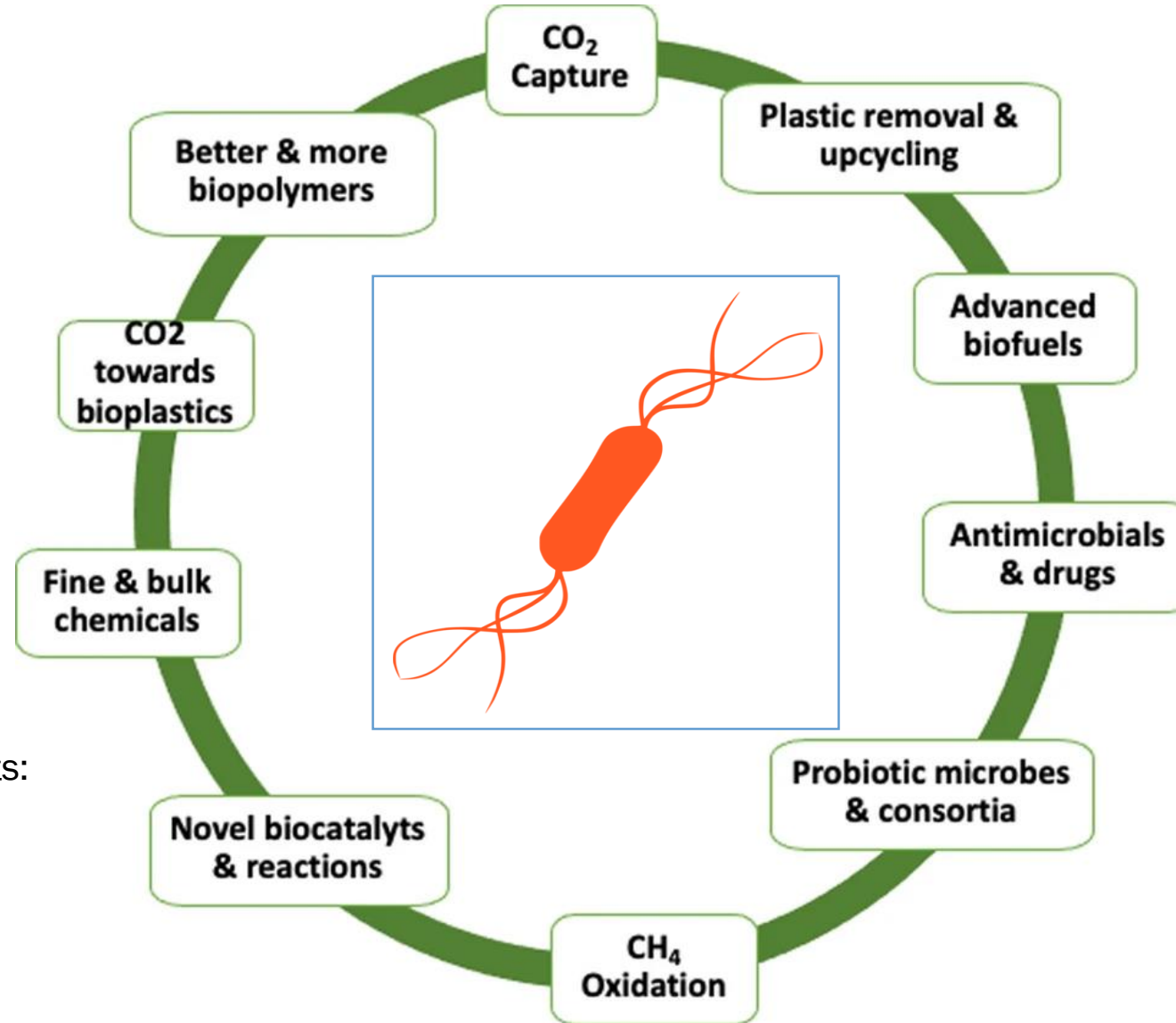
# Key developments for a sustainable economy

Global biocatalysts market  
2020: 117 kilotons

Dominant type of biocatalysts:  
**Hydrolases**

<https://www.expertmarketresearch.com/>

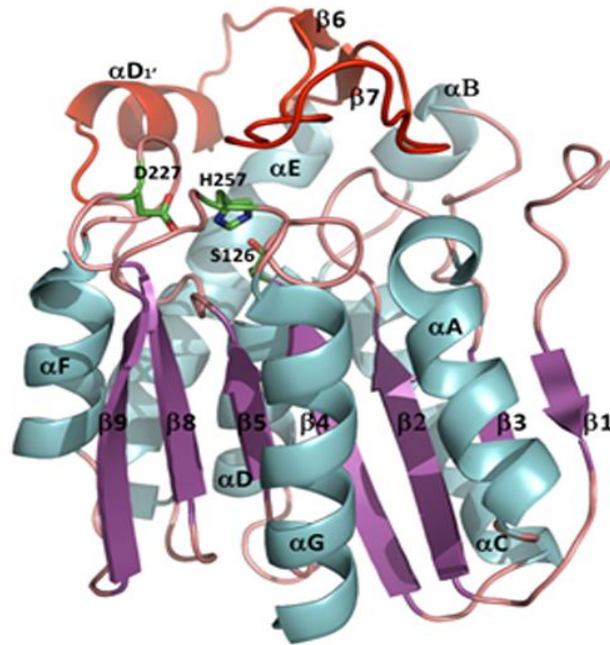
Microbes harbour the key to a  
circular economy







# Lipases and carboxylesterases in biotechnology



## Food

Pasta and bread dough, oils and fats, drinks

## Washing detergents

## Biodiesel

## Textile-, paper production

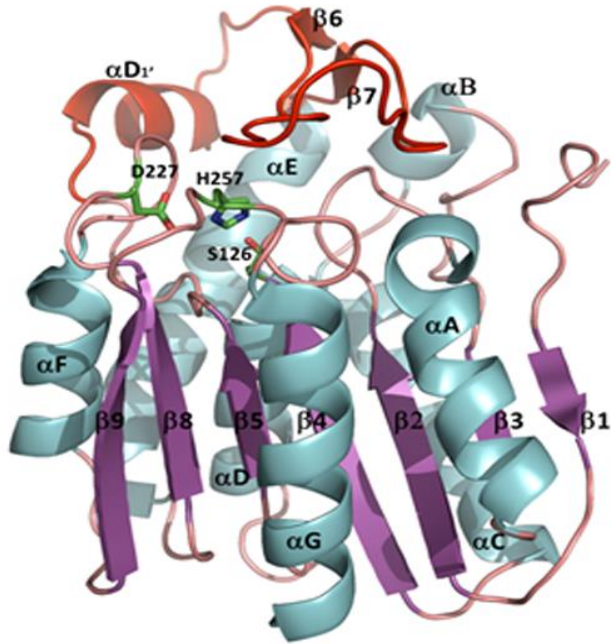
## Organic Syntheses

Polyesters,  
Pharmaceuticals, basic chemicals

Cosmetics



# Lipases and carboxylesterases in microbes



## Versatile biocatalysts

- Highly specific or promiscuous
- Secreted enzymes can be highly stable

## Metabolism

Lipid degradation  
Lipid modification  
Membrane adaptation...

## Growth promotion

Carbon source  
Degradation of toxins  
Quorum Quenching  
Colonization of niches  
Symbiosis...

## Virulence

Degradation of the plant cell wall  
Production of surface-active substances  
Degradation of host lipids...



# Metagenomics

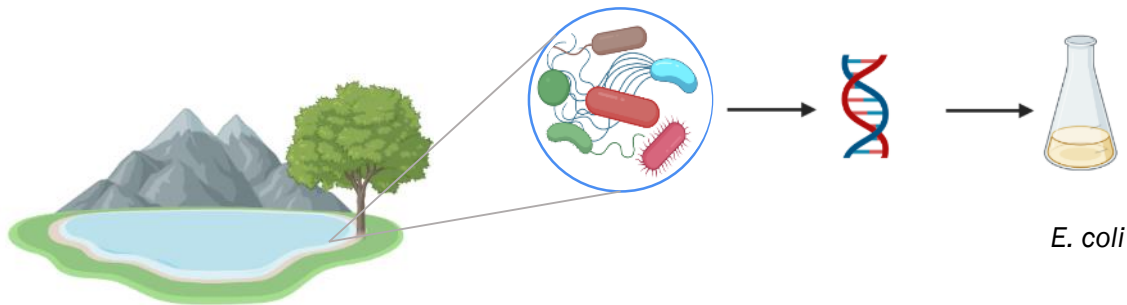
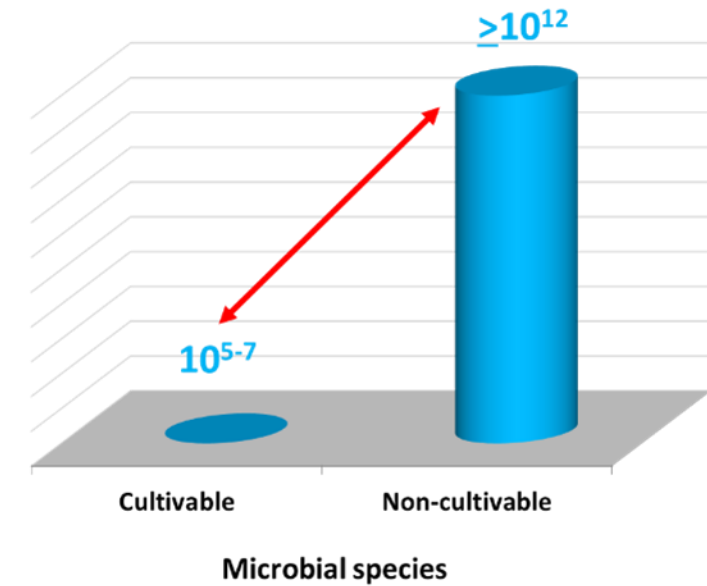
Bacteria/Archaea:  $10^{30}$  cells on earth

$10^{7-12}$  microbial species

$10^{10-15}$  bacterial/archaeal enzymes

Billions of different reactions

Billions of potential biomolecules/compounds for different industrial applications

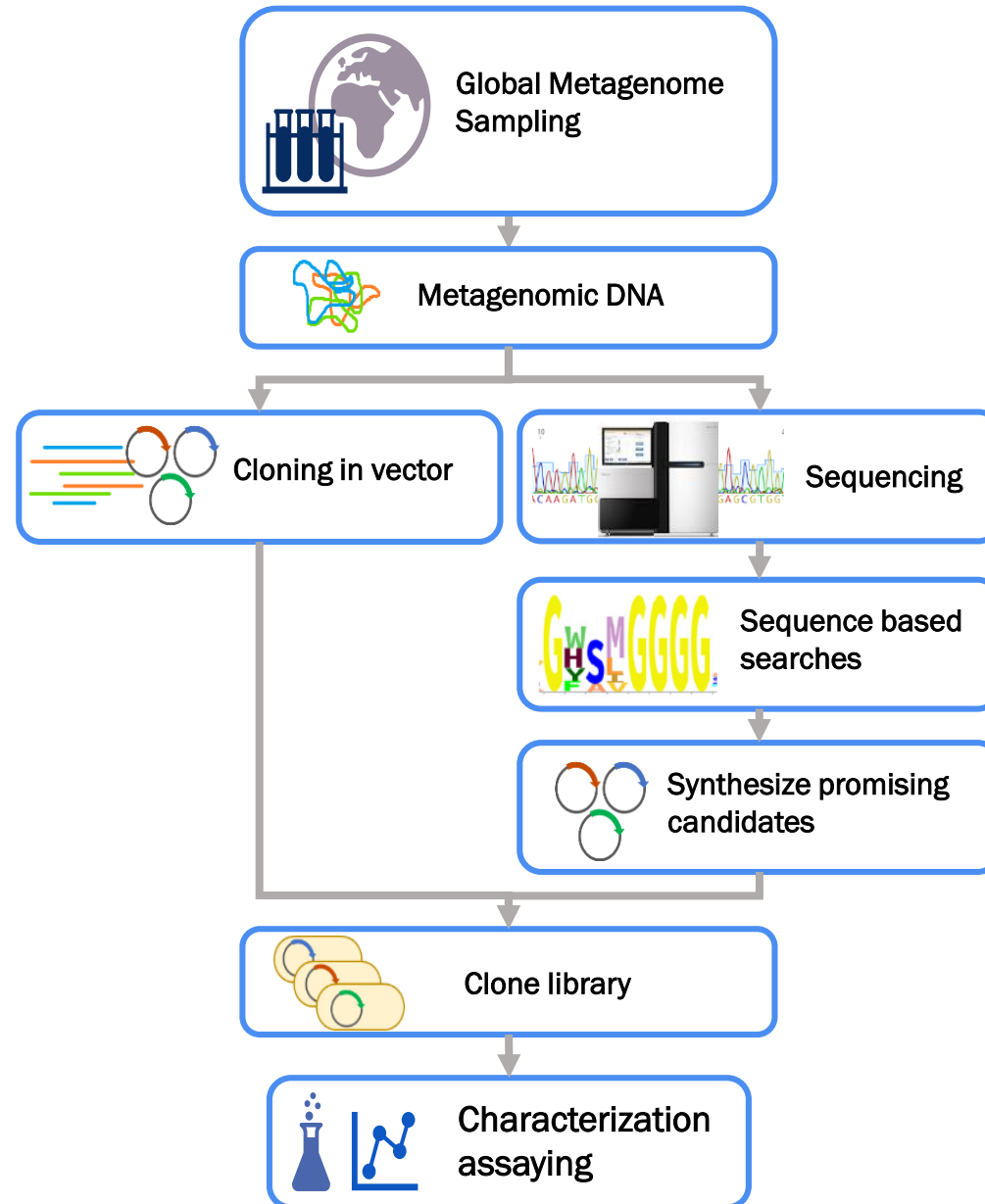


Metagenome: The entirety of mixed microbial communities genomes

Direct DNA extraction from environmental samples without prior cultivation



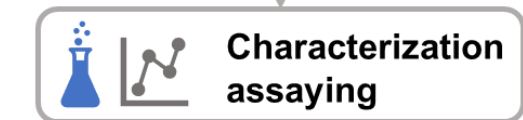
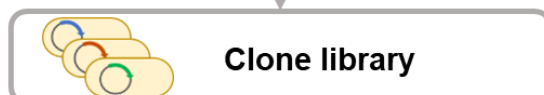
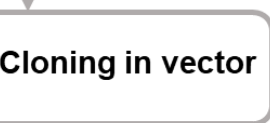
# Metagenomics



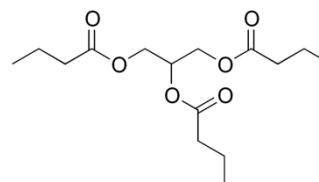




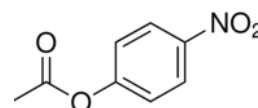
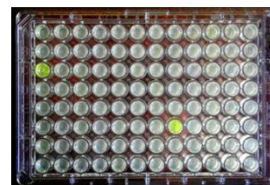
# Lipase toolbox @UHAM



Tributylin (TBT)



*para*-Nitrophenol (pNP)



Metagenome library	No. of clones
Elephant faeces	20,064
Teufelsbrück, river Elbe sediment	20,256
Glückstadt, river Elbe sediment	23,520
Algae photobioreactor biofilm	14,976
Esterase-lipase-box (collection of metagenome-derived enzymes)	81
Total = 78,897	



# Plastics: A global challenge & no effective solutions

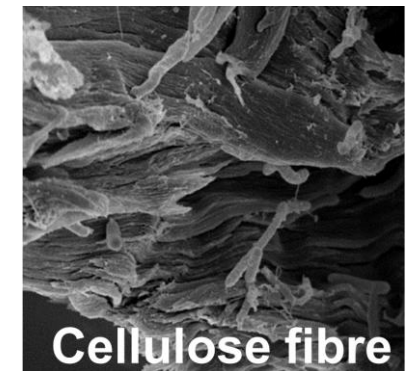
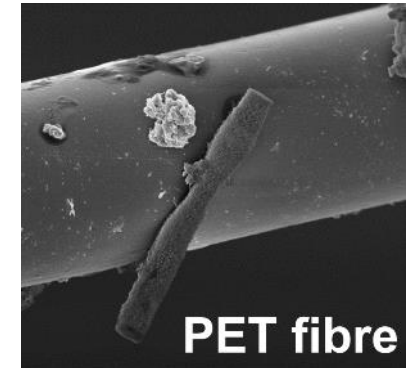
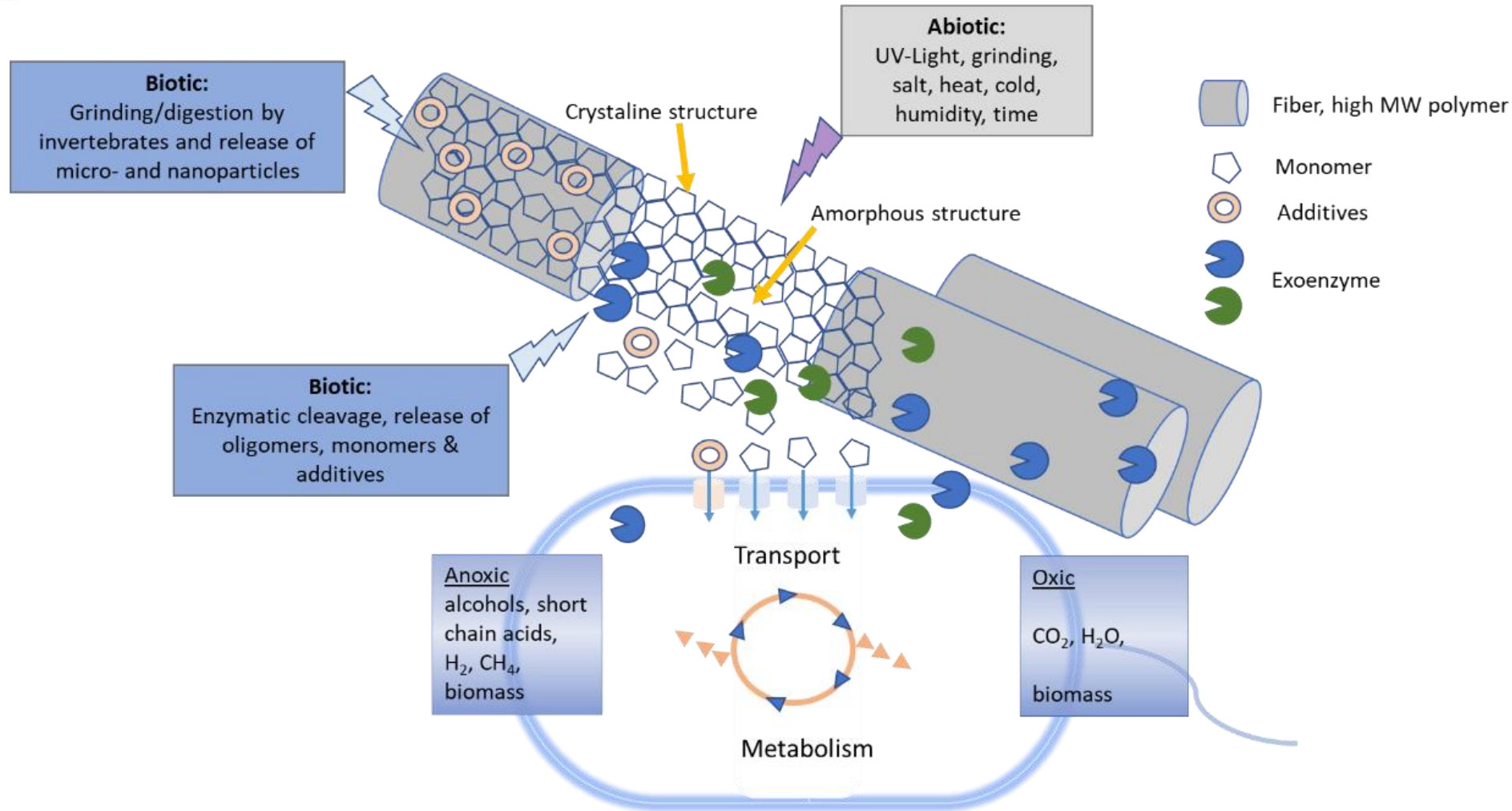


- 380-450 Mio tons produced annually
- 50% single use products
- Recycling rate less than 10 %
- Much of it ends up in terrestrial & aquatic environments
- Microplastics most likely pose the far more serious problem
- We eat more than 20 kg of plastic in our life

**Microbial enzymes as promising approach to plastic waste reduction**



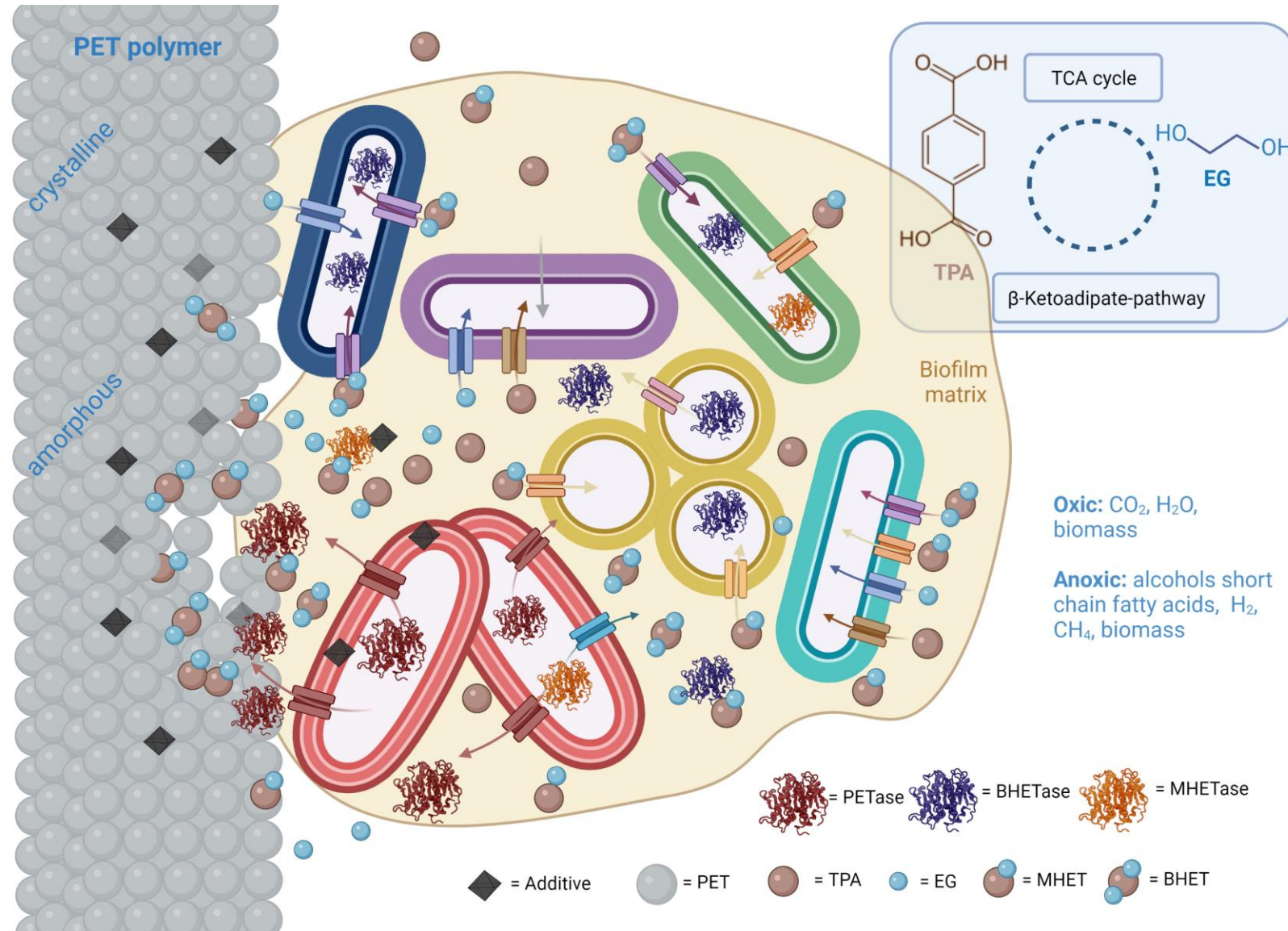
# A model for microbial plastic degradation





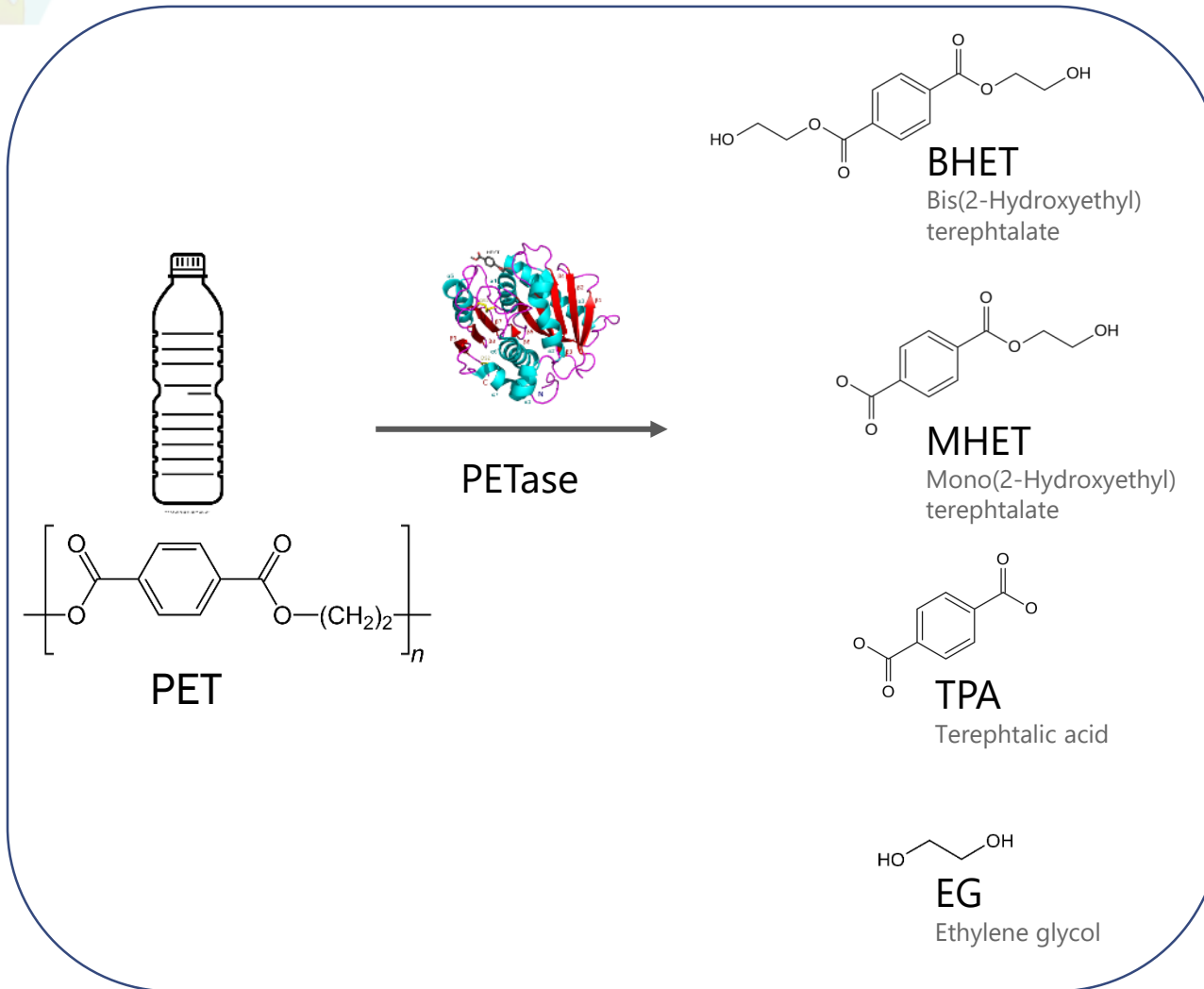


# Microbial degradation – a community job?





# PET hydrolases



## Best performing enzymes on PET

- **LCC** - Leaf compost cutinase - Actinobacteria  
(Sulaiman, 2012)
- **IsPETase** – *Ideonella sakaiensis* -Proteobacteria  
(Yoshida 2016)

## Application in bioindustries

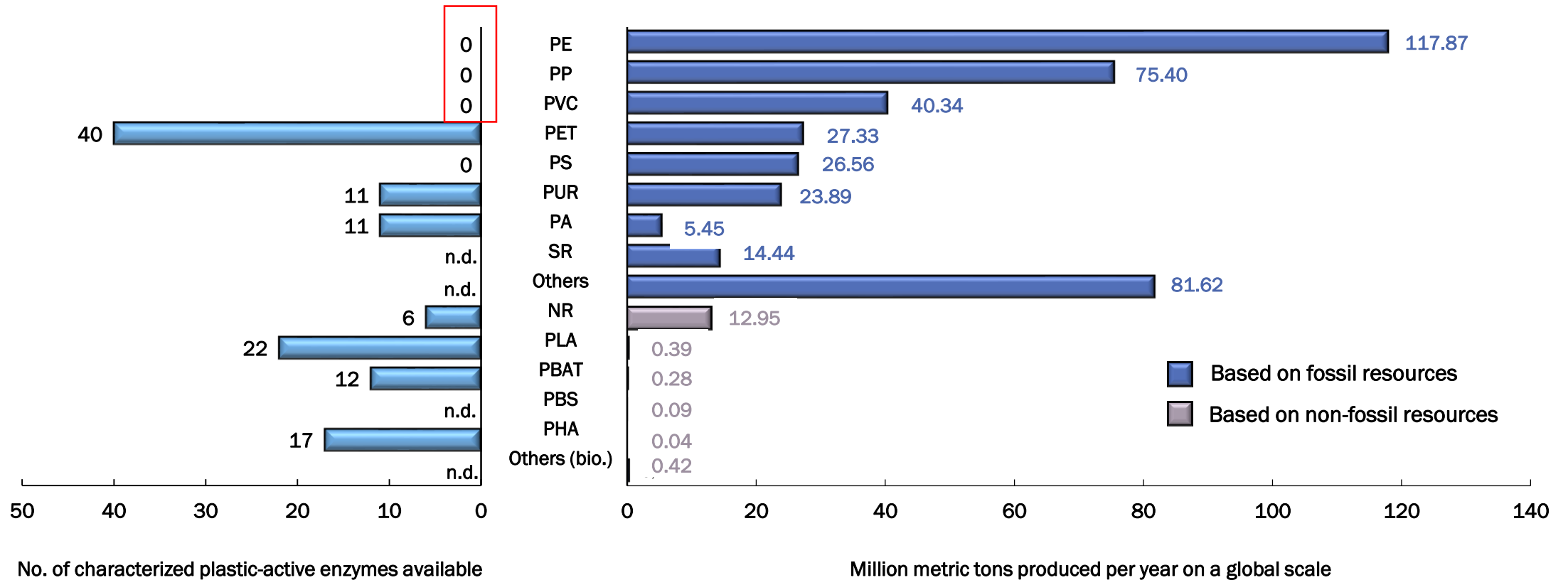
- Reduction of waste streams
- Re-valorization
- Reduction of microplastics in waste water





# Enzymatic plastic depolymerization – Where are we?

Known active enzymes mostly for PET & ester-based PUR!





# PAZy - The Plastics-Active Enzymes Database

<https://pazy.eu/>

PAZY

Log In

## PAZy - The Plastics-Active Enzymes Database

Plastics are widely used in our economy and each year, at least 350-400 million tons are being produced at a global level<sup>1,2</sup>. Due to poor recycling and low circular use, tens of millions of tons accumulate annually in marine and terrestrial environments. While it has become obvious that micro and macroplastics contaminate our environments recent research has identified few bacteria and fungi actively degrading plastics by enzymatic reactions. In general these are promiscuous enzymes (hydrolases) acting on low crystalline and mostly low density polymers of PET, ester-based PUR and oligomers of PA. Notably today, no enzymes have been characterized on a biochemical level for polymeric and crystalline PE, ether-based PUR, PS, PVC, PP. While many publications report on plastic degradation often, no convincing biochemical data have been published. Therefore the PAZy database lists **exclusively biochemically** characterized plastic-active enzymes. Predicted and putative enzymes that were not characterized on a biochemical, functional or structural level are not included in the PAZy database. The entries are manually curated.

**Cite:** Buchholz, P.C.F., Feuerriegel, G., Zhang, H., Perez-Garcia, P., Nover, L.-L., Chow, J., Streit, W.R. and Pleiss, J. (2022). Plastics degradation by hydrolytic enzymes: The Plastics-Active Enzymes Database - PAZy. <https://doi.org/10.1002/prot.26325> (download) [link for reference manager](#)

PAZy collects activity, gene and protein data for verified enzymes acting on the following synthetic polymers:

Fossil fuel-based polymers	Biochemically characterized wt enzymes
<a href="#">Polyethylene terephthalate (PET)</a>	40
<a href="#">Polyurethane</a>	11
<a href="#">Polyethylene (PE)</a>	0
<a href="#">Polyamide (PA)</a>	11, only enzymes acting on oligomers are known
<a href="#">Polystyrene (PS)</a>	0
<a href="#">Polyvinylchloride (PVC)</a>	0
<a href="#">Polypropylene (PP)</a>	0
<a href="#">Other types of polymers</a>	0

Polymers from mainly renewable resources	Biochemically characterized wt enzymes
<a href="#">Polylactic acid (PLA)</a>	22
<a href="#">Polyhydroxyalkanoates (PHA)</a>	16
<a href="#">Polybutylene adipate terephthalate (PBAT)</a>	12
<a href="#">Natural rubber (NR)</a>	6

Total number of enzymes: 110

PAZY

Log In

## Polyethylene terephthalate (PET)

The diagram illustrates the degradation of Polyethylene terephthalate (PET) into its constituent monomers. PET is shown as a repeating unit of terephthalic acid and ethylene glycol. The reaction proceeds through intermediates: BHET (bis(2-hydroxyethyl) terephthalate), MHET (mono(2-hydroxyethyl) terephthalate), TPA (terephthalic acid), and EG (ethylene glycol).

Polyethylene terephthalate ([CHEBI:131701](#)) is a synthetic polymer of repeating units of terephthalic acid ([CHEBI:30043](#)) and ethylene glycol ([CHEBI:30742](#)). PET is mainly used for the production of PET bottles, foil and various fibers in textile industry. PET is one of the few polymers, for which a number of microbial enzymes are known. They belong to the cutinases ([EC 3.1.1.74](#)), lipases ([EC 3.1.1.3](#)) and carboxylesterases ([EC 3.1.1.1](#) / [EC 3.1.1.101](#) / [EC 3.1.1.2](#)) and can act on amorphous but not crystalline PET. These enzymes break the ester bond in the polymer to either produce [bis \(2-hydroxyethyl\) terephthalate](#) (BHET), [mono \(2-hydroxyethyl\) terephthalate](#) (MHET) or [terephthalic acid](#) (TPA) and [ethylene glycol](#) (EG). The enzymes act as *exo*-enzymes. MHET can subsequently be cleaved with a specific MHETase ([EC 3.1.1.102](#)) and the TPA monomers degraded via cleavage of the aromatic ring structure using known aryl pathways ([Wei et al., 2017](#); [Danso et al., 2019](#); [Taniguchi et al., 2019](#)). PET-active enzymes are often designated 'PETases'. Notably, the majority of all PET-active enzymes are promiscuous enzymes.

Currently known and **biochemically characterized** active PET hydrolases are listed below and grouped according to their phylogenetic affiliation. Predicted and not characterized enzymes are not included. For multiple PDB entries only one lead structure is included.

In case you are aware of PET-active biochemically characterized enzymes that are not listed below, please contact the PAZy team.

**Please cite the database by referring to:** P.C.F. Buchholz, H.L. Zhang, P. Perez-Garcia, L.-L. Nover, J. Chow, W. R. Streit, J. Pleiss (2022). Plastics degradation by hydrolytic enzymes: the Plastics-Active Enzymes Database - PAZy. <https://doi.org/10.1002/prot.26325>

Microbial host/enzyme/gene	EC number	Reference	GenBank/ UniProt/MGnify	PDB entry	NCBI BLAST
<b>Proteobacteria</b>					
<i>Ideonella sakaiensis</i> 201-F6, ISPETase, <i>ISF6_4831</i> ( <a href="#">↪DuraPETase</a> ) ( <a href="#">↪ThermoPETase</a> ) ( <a href="#">↪Fast-PETase</a> )	<a href="#">EC 3.1.1.101</a>	<a href="#">Yoshida, 2016</a> ; <a href="#">Han 2017</a> ; <a href="#">Cui, 2021</a> ; <a href="#">Son, 2019</a> ; <a href="#">Lu, 2022</a>	<a href="#">A0A0K8P6T7</a>	<a href="#">5XFY</a> , <a href="#">and others</a>	<a href="#">WP_054022242.1</a>
<i>Oleispira antarctica</i> RB-8, PET5, <i>LipA</i> ( <a href="#">=Oacut</a> )	<a href="#">EC 3.1.1.1</a>	<a href="#">Danso, 2016</a> , <a href="#">Blasquez-Sanchez, 2021</a>	<a href="#">R4YKL9_OLEAN</a>		<a href="#">MBQ0729274.1</a>
<i>Vibrio gazogenes</i> , PET6, <i>BSQ33_03270</i>	<a href="#">EC 3.1.1.1</a>	<a href="#">Danso, 2018</a>	<a href="#">A0A1Z2SIQ1_VIBGA</a>	<a href="#">7Z6B</a>	<a href="#">ASA57064.1</a>
<i>Polyangium brachysporum</i> , PET12, <i>AAW51_2473</i>	<a href="#">EC 3.1.1.1</a>	<a href="#">Danso, 2018</a>	<a href="#">A0A0G3BI90_9BURK</a>		<a href="#">AKJ29164.1</a>
<i>Pseudomonas pseudoalcaligenes</i> DSM 50188, PpCutA	<a href="#">EC 3.1.1.74</a>	<a href="#">Ingils, 2011</a> ; <a href="#">Haervall, 2017</a> ,	<a href="#">KU695574</a>		<a href="#">ANP21911.1</a>
<i>Pseudomonas pelagia</i> DSM 25163, PpelaLip	<a href="#">EC 3.1.1.74</a>	<a href="#">Haervall, 2017</a>	<a href="#">KU695573</a>		<a href="#">ANP21910.1</a>
<i>Pseudomonas aestusnigri</i> VGX014, PE-H, <i>B7O88_11480</i>	<a href="#">EC 3.1.1.1</a>	<a href="#">Bollinger, 2020</a>	<a href="#">A0A1H6AD45</a>	<a href="#">6SBN</a> , <a href="#">and others</a>	<a href="#">WP_088276085.1</a>
<i>Pseudomonas mendocina</i> ATCC 53552, PmC	<a href="#">EC 3.1.1.3</a>	<a href="#">Ronkvist, 2009</a>	<a href="#">N20M5AZM016</a>	<a href="#">2FX5</a>	<a href="#">2FX5_A</a>
<i>Moraxella</i> sp.TA144, <i>lip1</i> , Mors1	<a href="#">EC 3.1.1.3</a>	<a href="#">Blasquez-Sanchez, 2021</a>	<a href="#">P19833</a>		<a href="#">P19833.1</a>
<i>Pseudomonas pseudoalcaligenes</i> , PpEst ( <i>tesA</i> )	<a href="#">EC 3.1.1.2</a>	<a href="#">Haervall, 2017</a> , <a href="#">Wallace, 2016</a>	<a href="#">W6R2Y2</a>		<a href="#">WP_003460012.1</a>
<i>Pseudomonas</i> sp., esterase MG8	<a href="#">EC 3.1.1.1</a>	<a href="#">Eiamthong, 2022</a>	<a href="#">MGYP000532440779</a>		<a href="#">MGYP000532440779</a>
<i>Rhizobacter eumimophilus</i> NS21, <i>RaPETase</i>	<a href="#">EC 3.1.1.1</a>	<a href="#">Sagong, 2021</a>	<a href="#">A4W93_05950</a>	<a href="#">7DZT</a> and others	<a href="#">A4W93_05950</a>

In collaboration with AG Jürgen Pleiss



**Universität Stuttgart**  
Institut für Biochemie und Technische Biochemie



**Universität Hamburg**

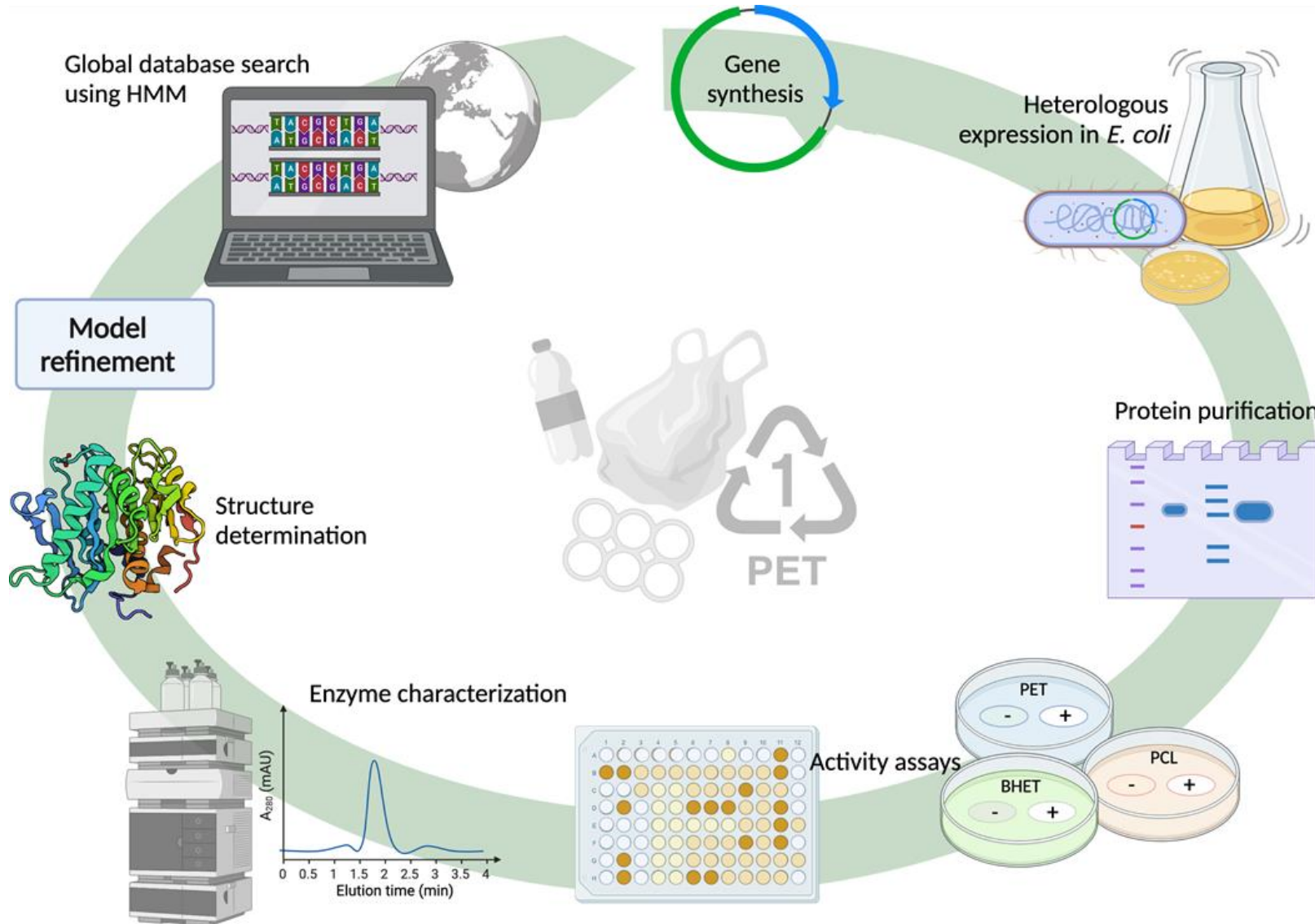
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Buchholz, P.C.F., Feuerriegel, G., Zhang, H., Perez-Garcia, P., Nover, L.-L., Chow, J., Streit, W.R. and Pleiss, J. (2022) *Proteins* 90 7





# How to find new PET degrading enzymes







# Finding plastic eating bacteria: Metagenomics



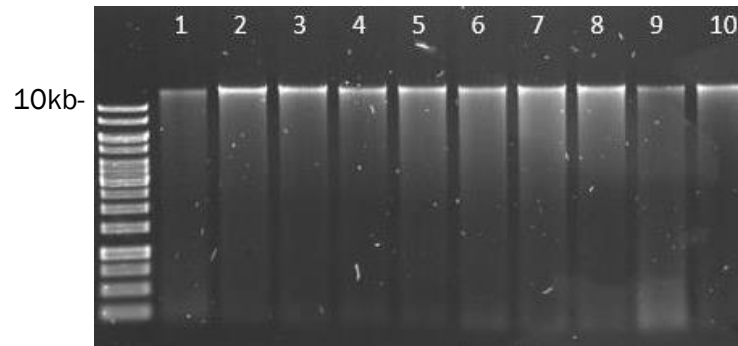
Global Metagenome Sampling



Metagenome DNA

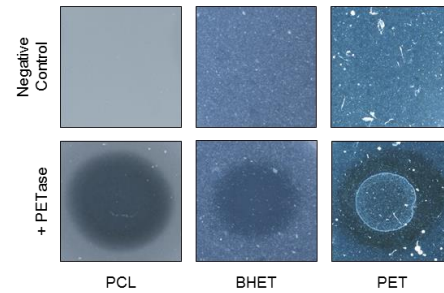
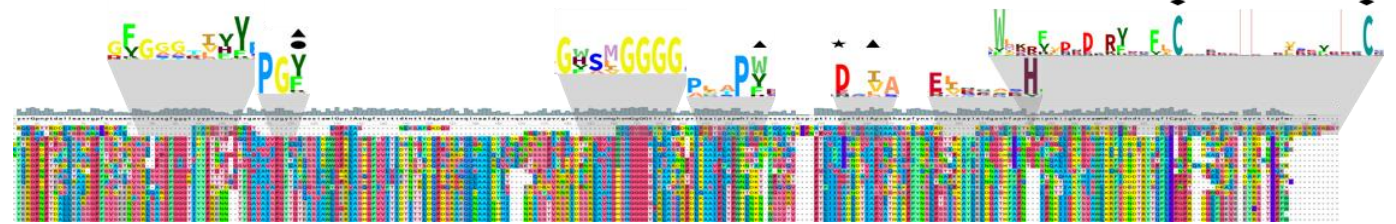
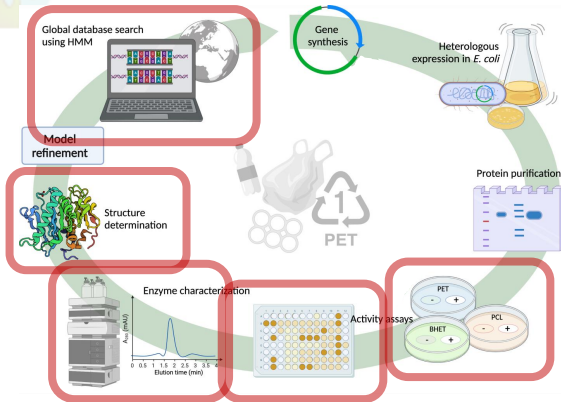


Environmental DNA samples

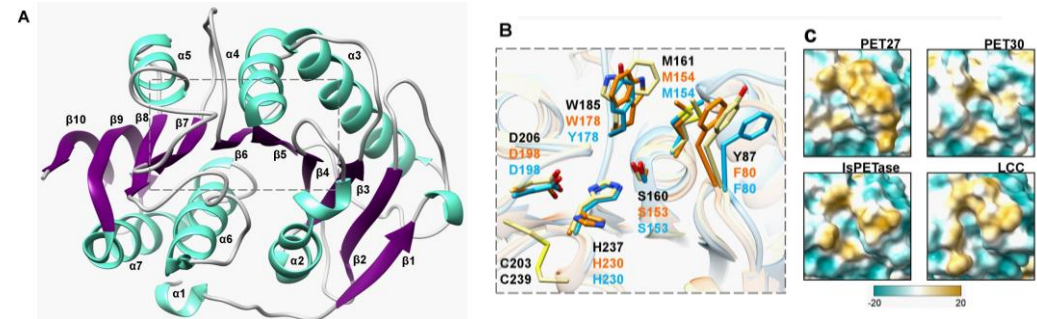
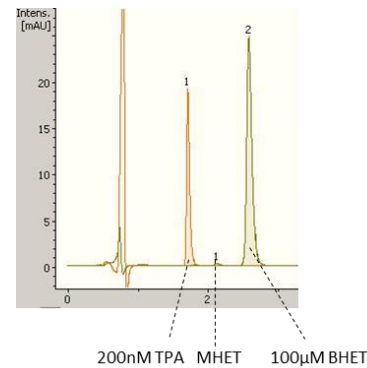
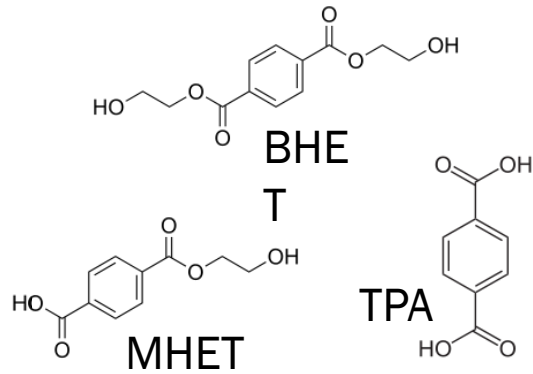
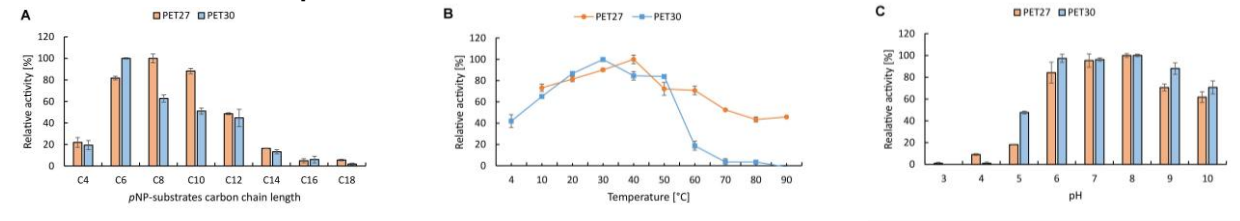




# How to find new PET-degrading enzymes



## Optimization of reaction conditions:

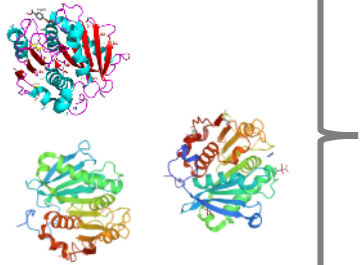






# Finding plastic eating bacteria: HMM screening

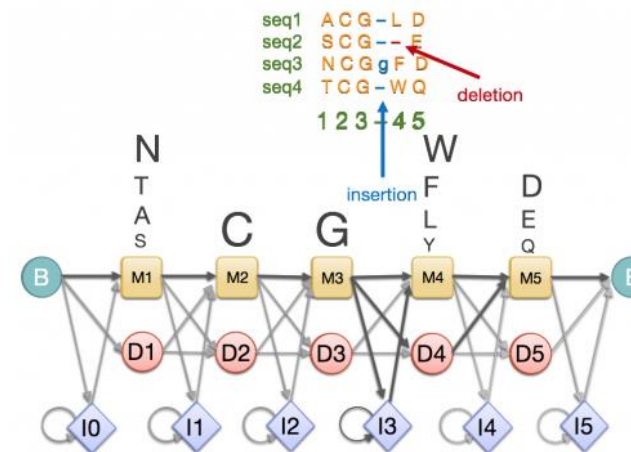
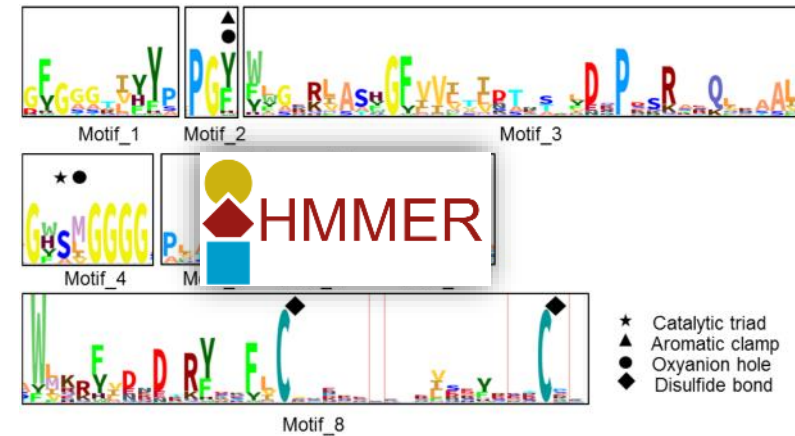
Known enzymes  
With desired  
function



Multiple sequence  
alignment

<i>Idionella sakaiensis</i>	L	Q	S	S	S	Q	M	A	R	Q	V	A	S	L	N	G	T	S	S	S	P	T	Y	G	V	T	
<i>Thermobifida alba</i>	L	Q	S	S	A	E	L	N	A	A	N	H	M	I	-	-	N	R	A	S	T	R	R	S	R	S	
<i>Thermobifida fusca_Cut1</i>	L	Q	S	S	A	E	L	N	A	A	N	H	M	I	-	-	N	R	A	S	T	R	R	S	R	S	
<i>Thermobifida cellulolytica</i>	L	Q	S	S	A	E	L	N	A	A	N	H	M	I	-	-	N	R	A	S	T	R	R	S	R	S	
<i>Thermobifida halotolerans</i>	L	Q	S	S	A	E	L	N	A	A	N	H	M	I	-	-	N	R	A	S	T	R	R	S	R	S	
<i>Saccharomonospora viridis</i>	L	Q	S	S	A	E	L	N	A	A	N	H	M	I	-	-	N	R	A	S	T	R	R	S	R	S	
<i>LCC</i>	L	Q	S	S	A	E	L	N	A	A	N	H	M	I	-	-	N	R	A	S	T	R	R	S	R	S	
<i>Thermobifida fusca_Cut2</i>	L	Q	S	S	A	E	L	N	A	A	N	H	M	I	-	-	N	R	A	S	T	R	R	S	R	S	
<i>Thermomonospora curvata</i>	L	Q	S	S	A	E	L	N	A	A	N	H	M	I	-	-	N	R	A	S	T	R	R	S	R	S	

Hidden Markov Model construction and  
search



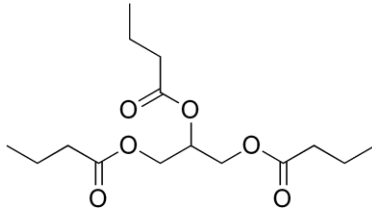


# PET esterase screening substrates



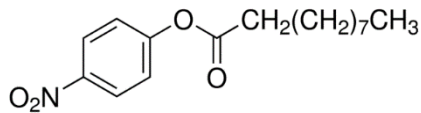
## TBT

Tributyryl



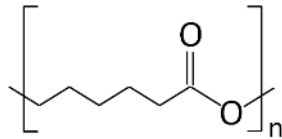
## pNP-esters

Para Nitrophenol – esters



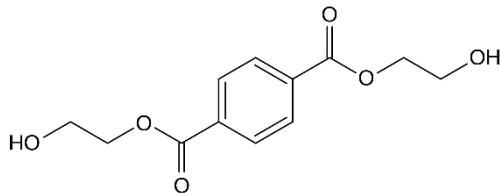
## PCL

Polycaprolactone



## BHET

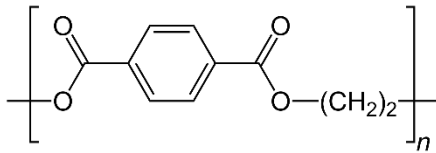
Bis(2-Hydroxyethyl) terephthalate



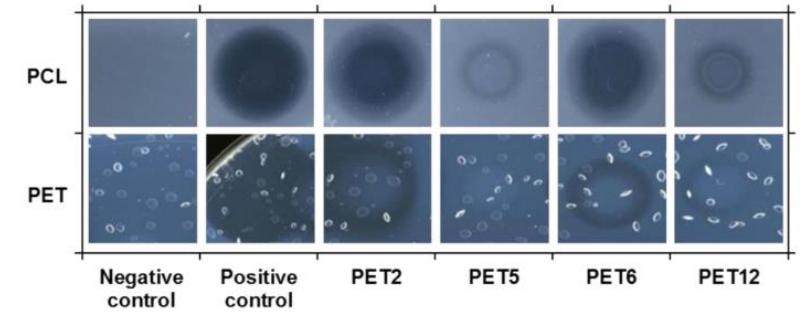
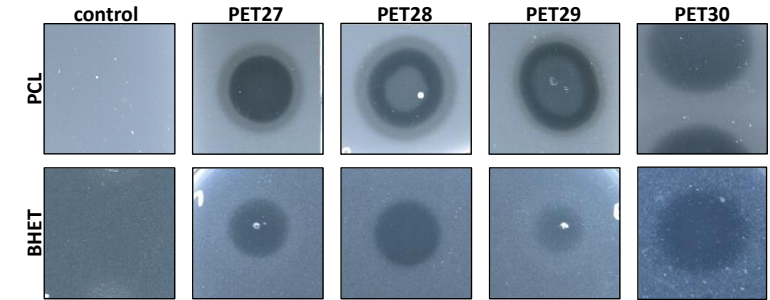
## PET Film

## PET Powder

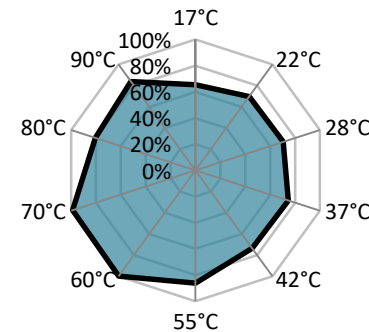
Polyethylene terephthalate



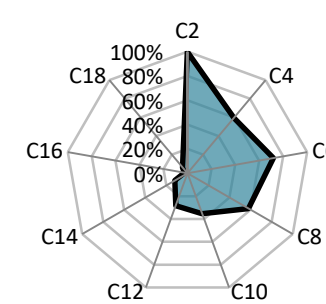
Agar clearing assays



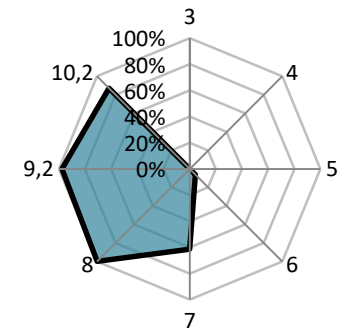
pNP-ester degradation



Temperature



Substrate chain length



pH

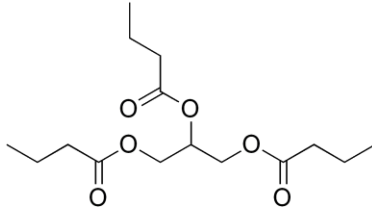




# PET esterase screening substrates

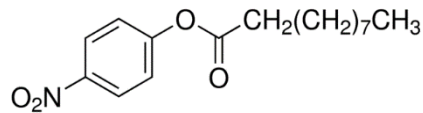
**TBT**

Tributyryl



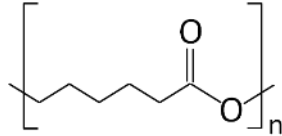
**pNP-esters**

Para Nitrophenol –  
esters



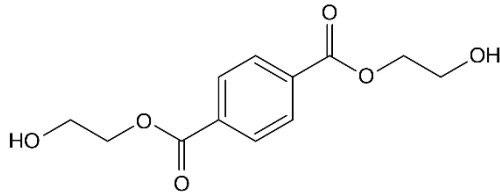
**PCL**

Polycaprolactone



**BHET**

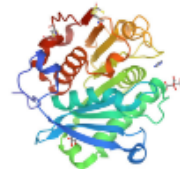
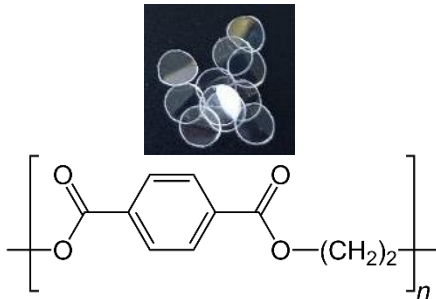
Bis(2-Hydroxyethyl)  
terephthalate



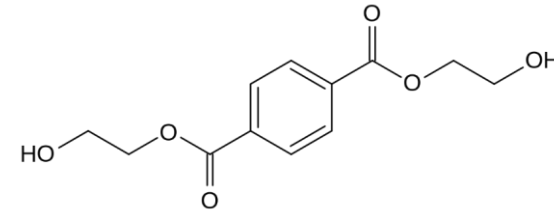
**PET Film**

**PET Powder**

Polyethylene  
terephthalate

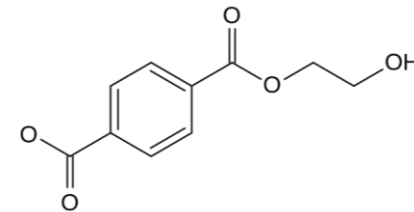


Degradation



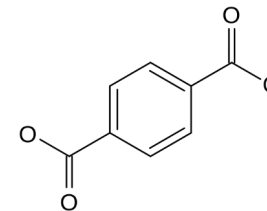
**BHET**

Bis(2-Hydroxyethyl)  
terephthalate



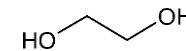
**MHET**

Mono(2-Hydroxyethyl)  
terephthalate



**TPA**

Terephthalic acid



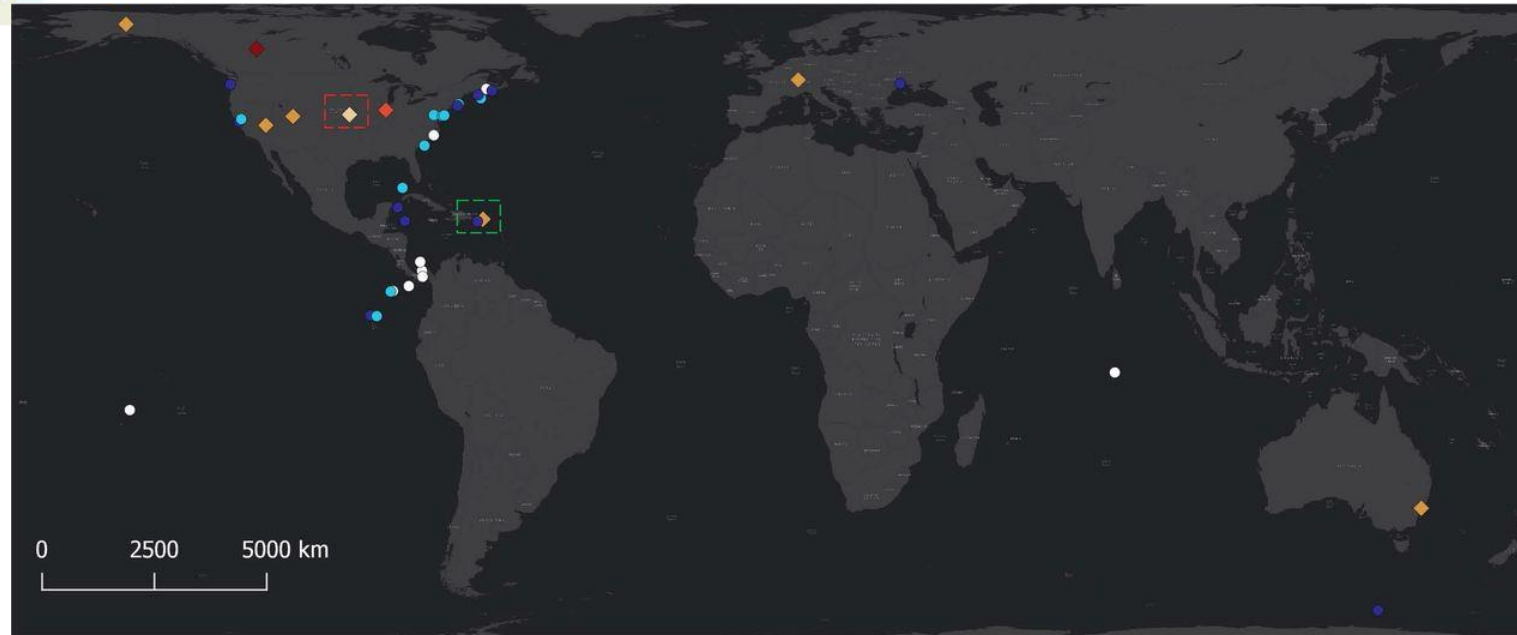
**EG**

Ethylene glycol

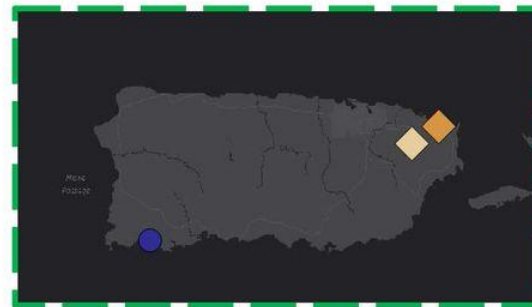
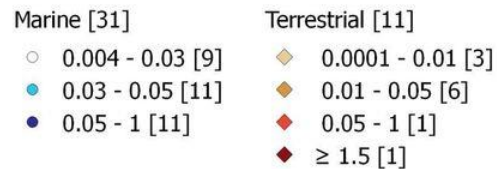




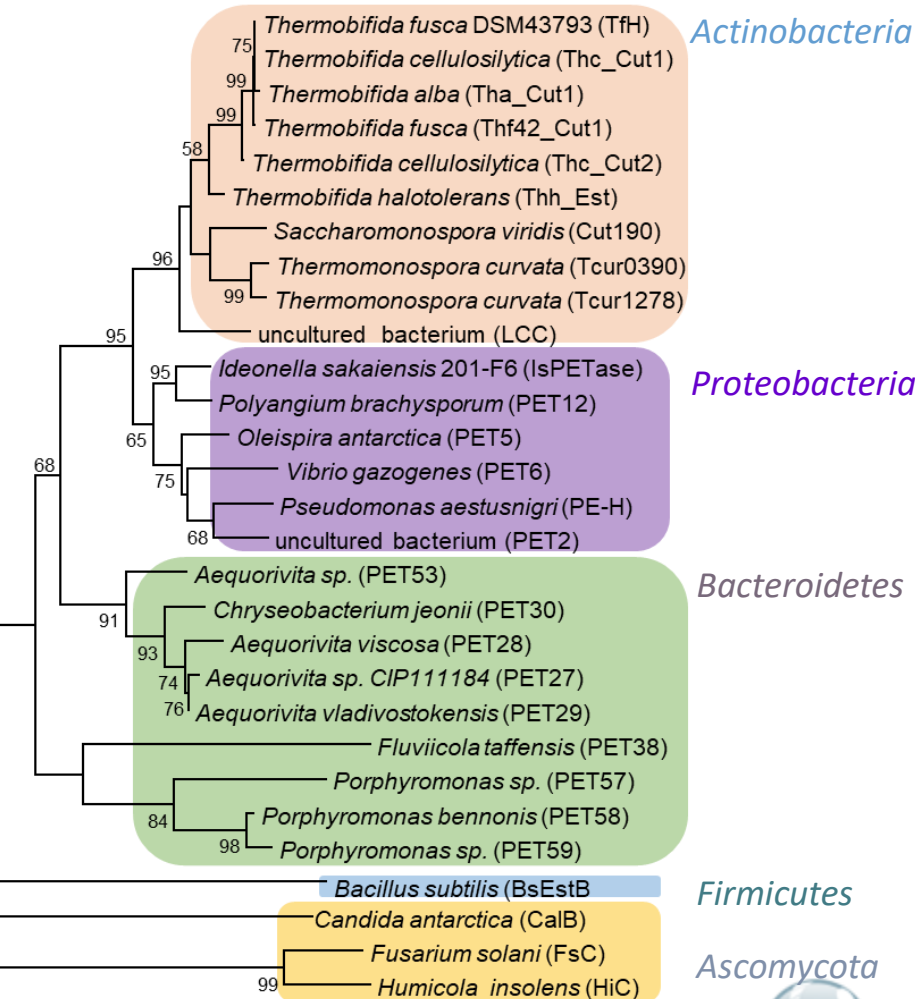
# PET degrading enzymes are hard to find



Frequency of potential  
PET hydrolases in hits/Mb



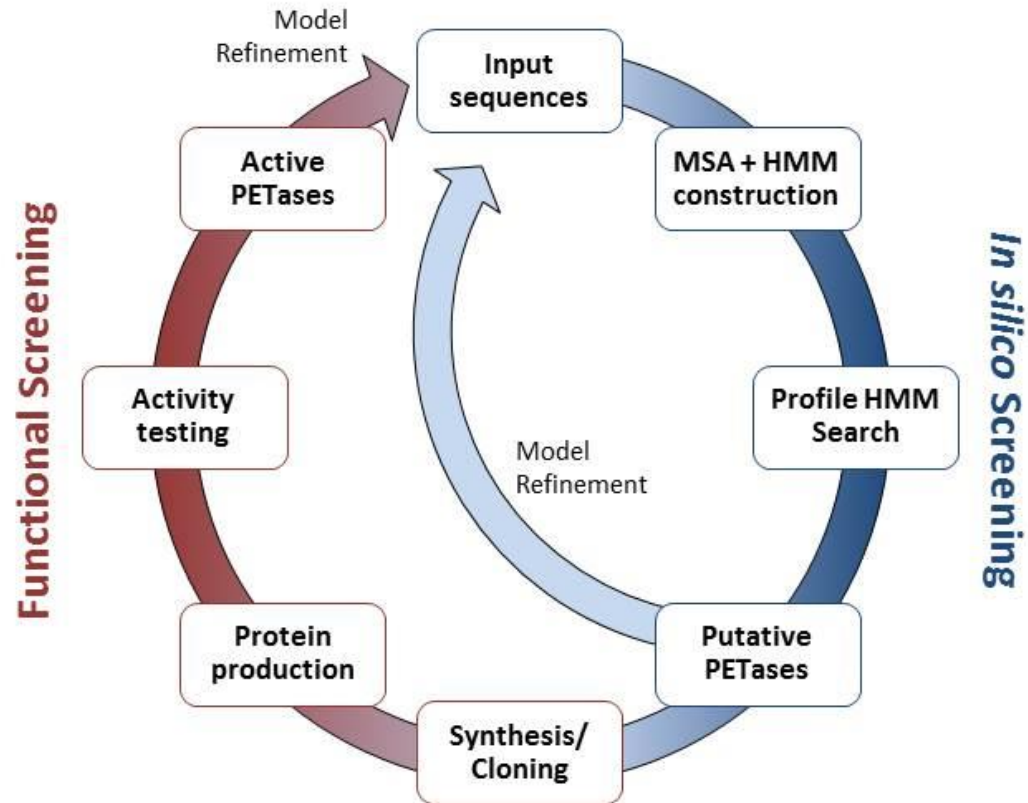
## 29 PETases in 5 phylogenetic clusters



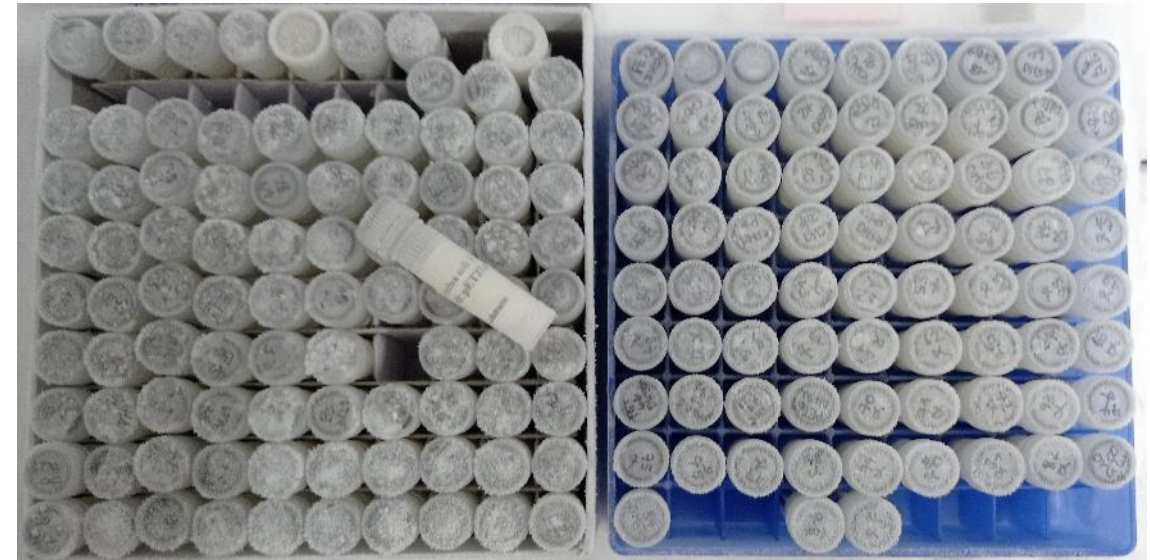




# Finding plastic eating bacteria: An ongoing search...

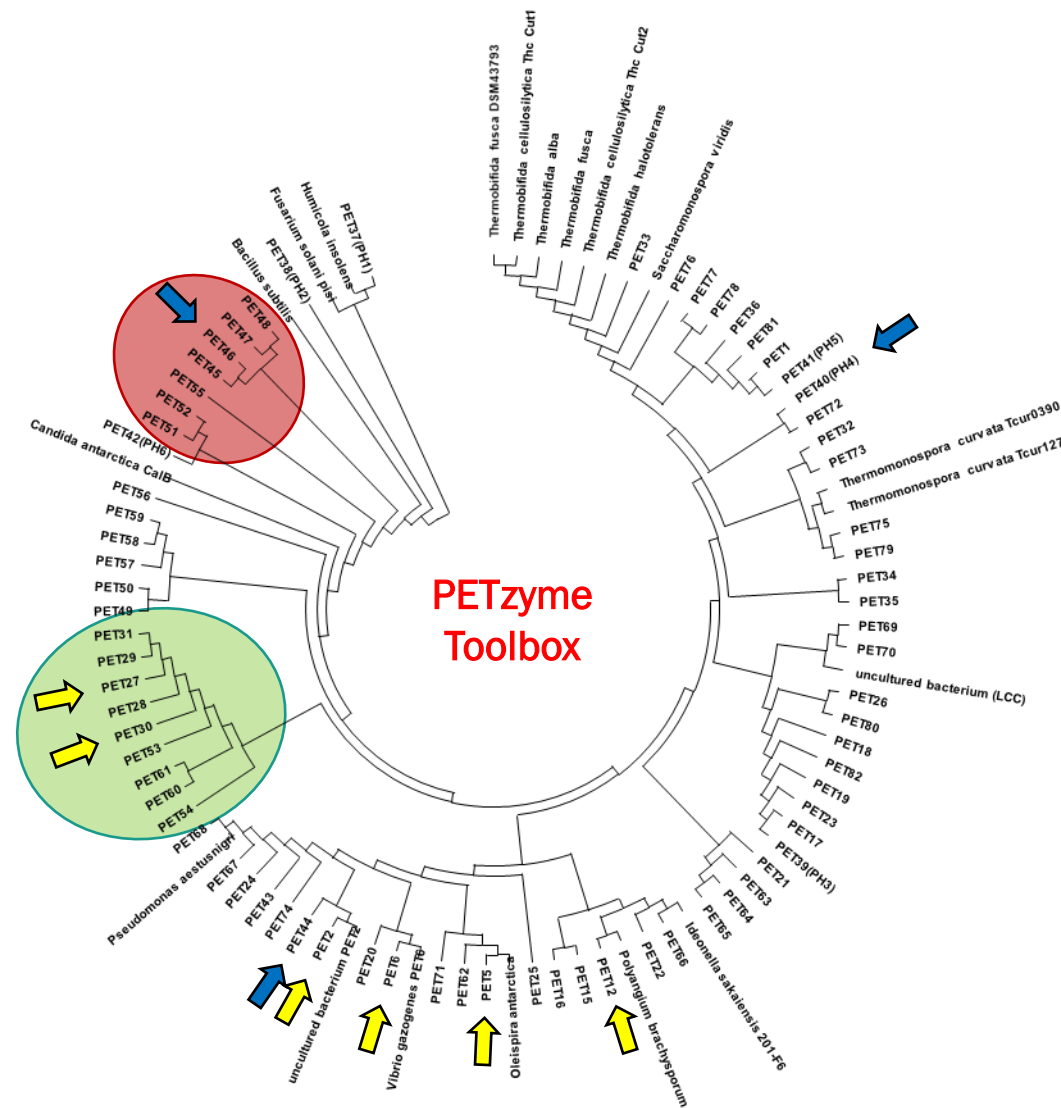
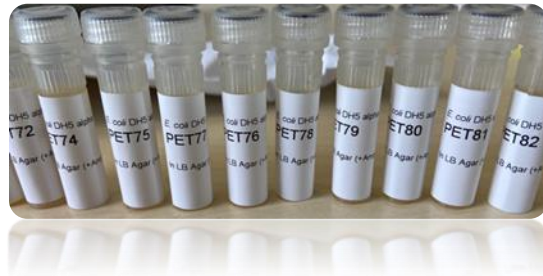
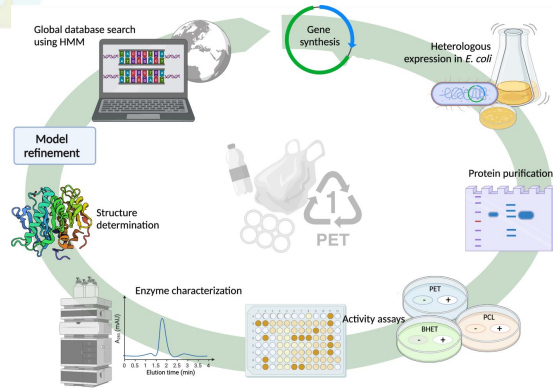


Until now: Synthesized a collection of > 80 different potential and verified PETase genes





# PETzyme: A toolbox of microbial PET-degrading enzymes



Published PETases: 40

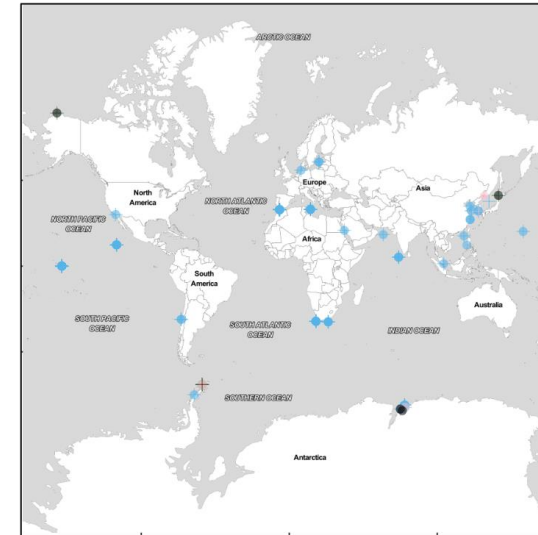
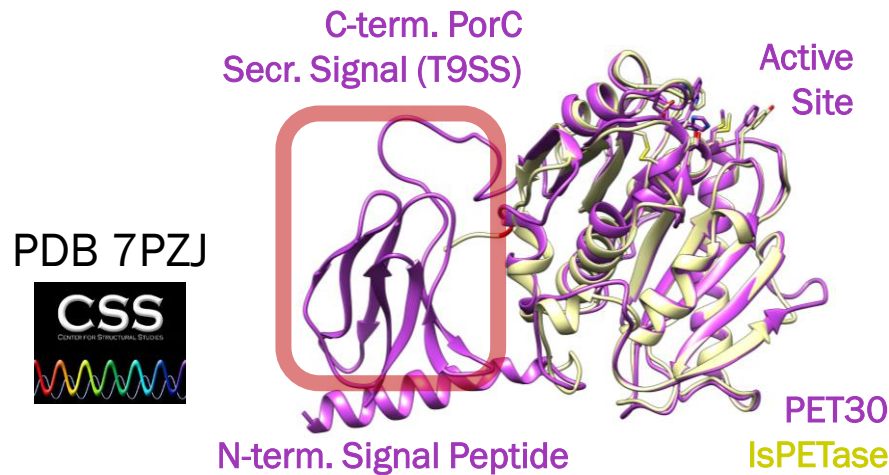
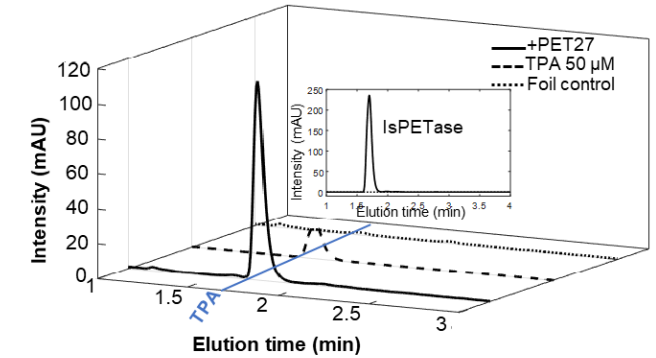
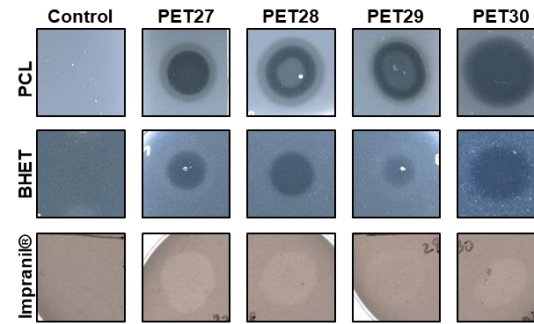
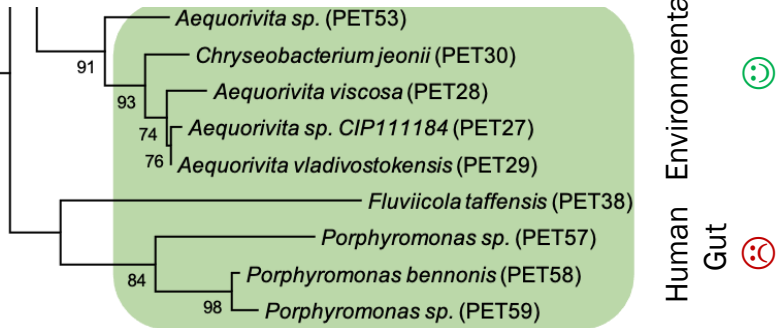
PETzyme toolbox: 80+

→ Published  
→ Soon to be submitted

PET2, 5, 6, 12: Danso, D., Schmeisser, C., Chow, J., Zimmermann, W., Wei, R., ...and Streit, W.R. (2018) Appl Environ Microbiol 84  
 PET27-31: Zhang, H., Perez-Garcia, P., Dierkes, R.F., Applegate, V., ...Pleiss, J., ...Smits, S.H.J., Chow, J., and Streit, W.R. (2022) Front Microbiol 12: 803896  
 PET6: Weigert, S., Perez-Garcia, P.,... Chow, J., Streit, W.R. and Höcker, B. (2022) Proteins, under review



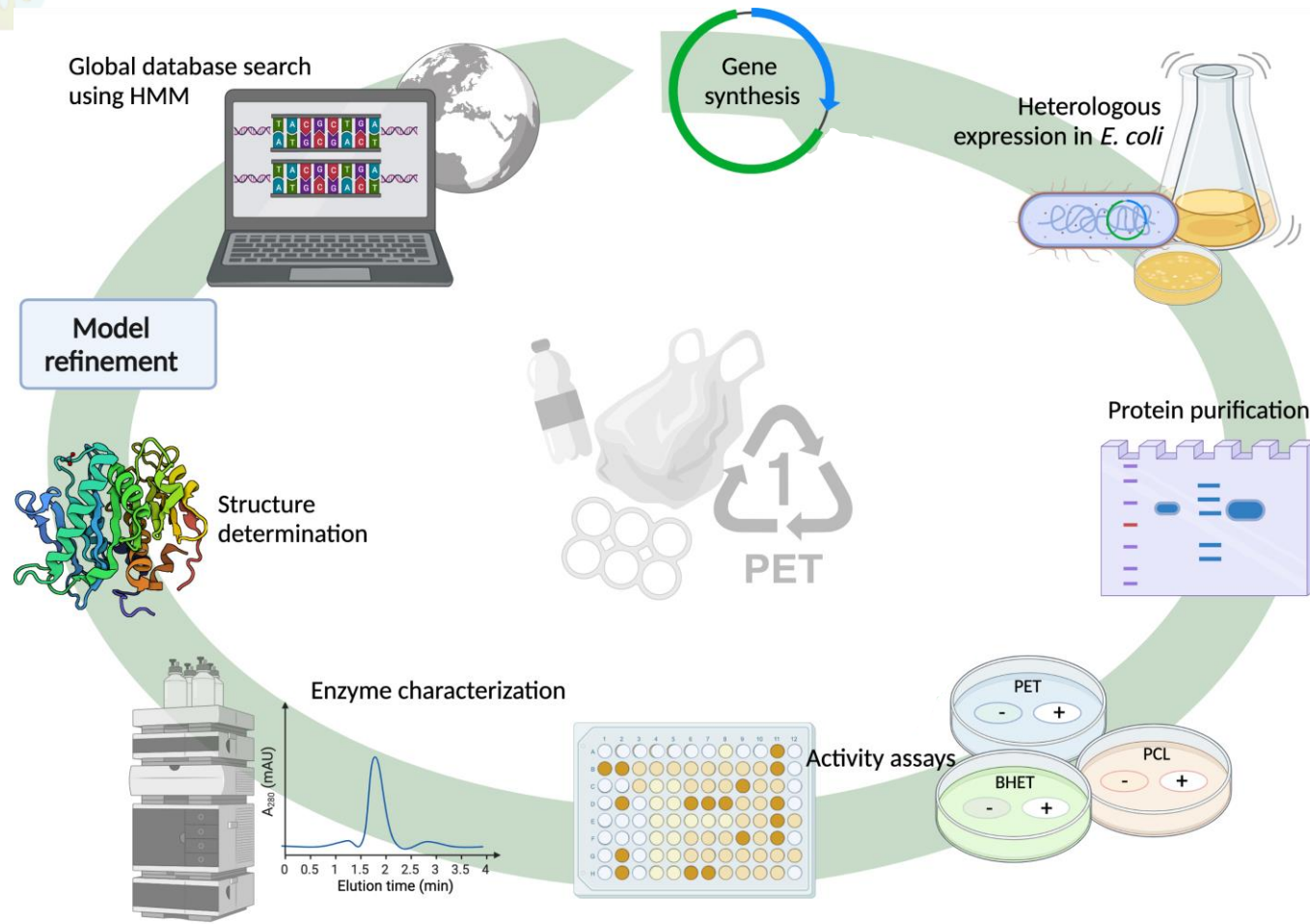
# The first bacteroidetal PET-degrading enzymes



Bacteroidetal PETases are spread worldwide



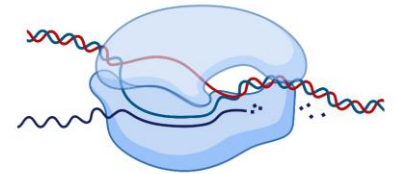
# *In vitro* transcription and translation of PETases



PCR of candidate genes



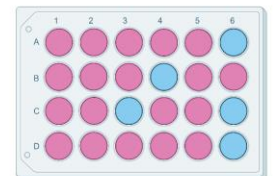
*In vitro* transcription



*In vitro* translation

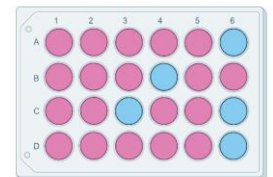
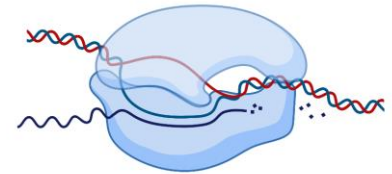
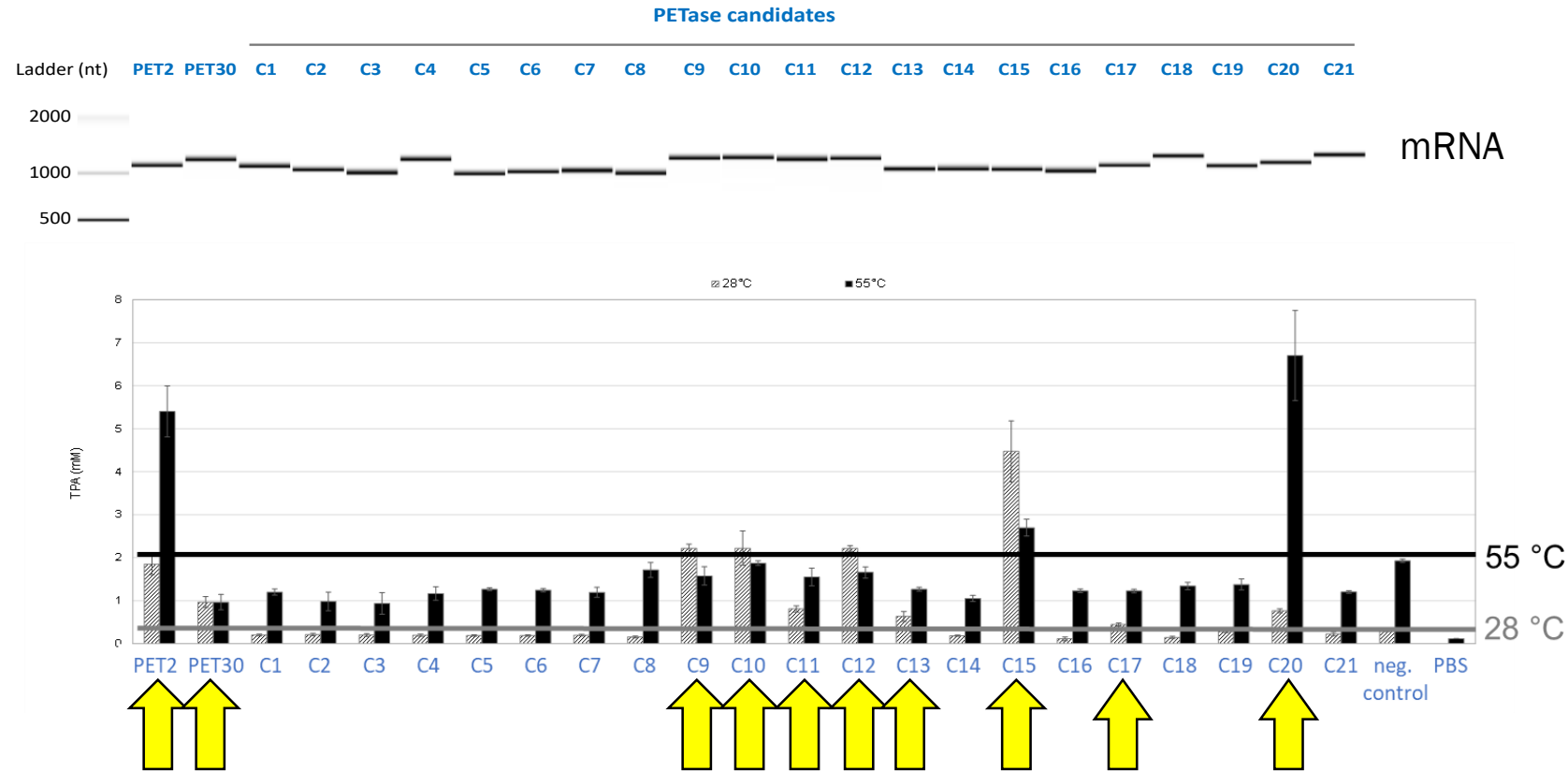


Activity assay



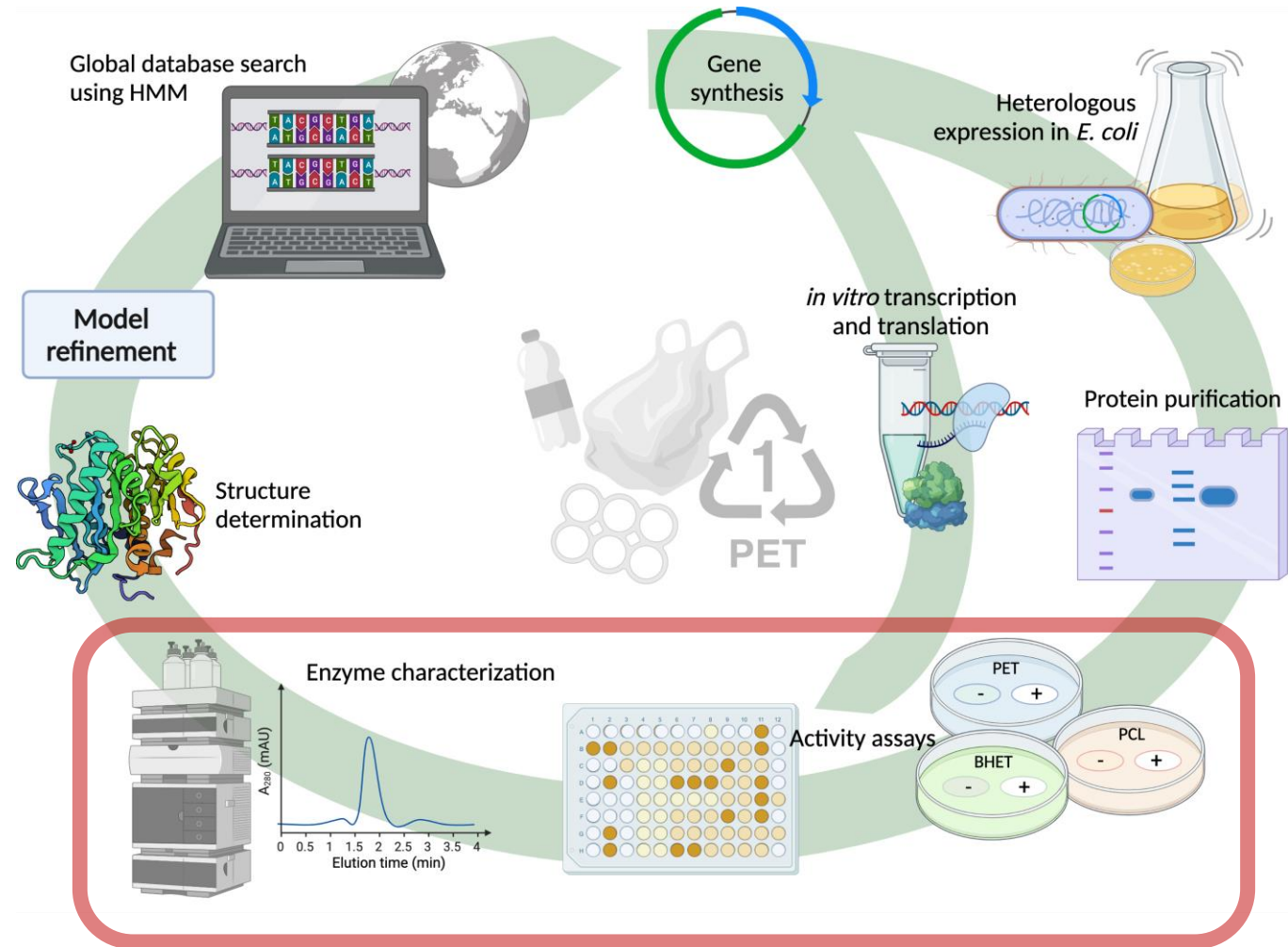


# *In vitro* transcription and translation of PETases





# Fluorescent reporter strain for fast activity measurements





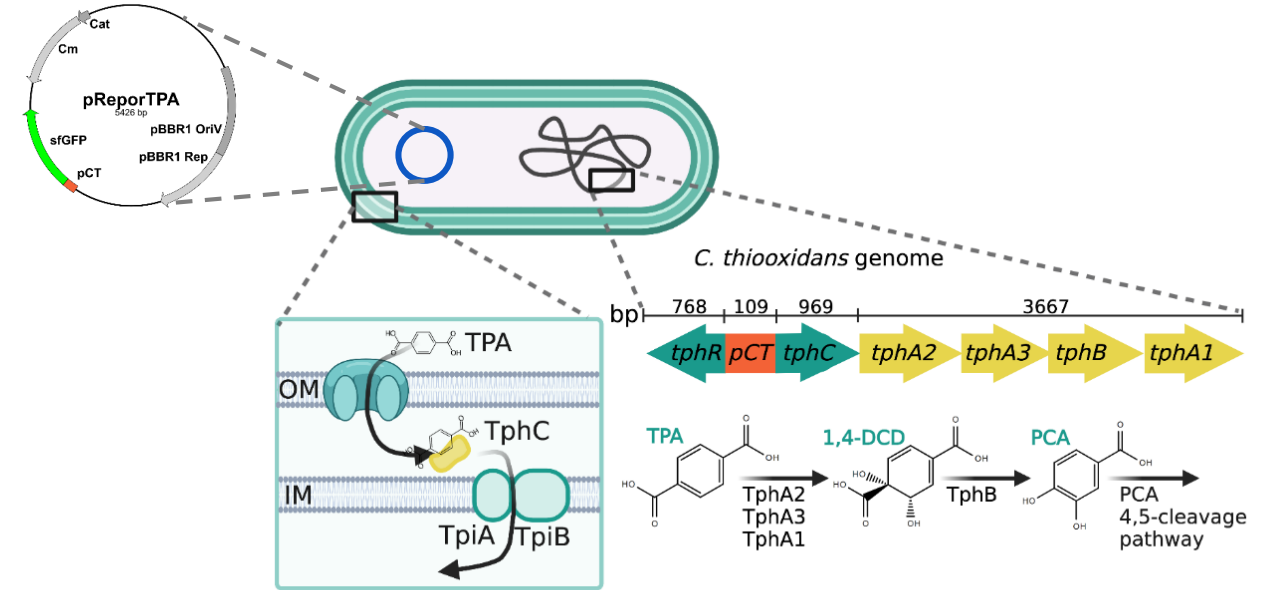
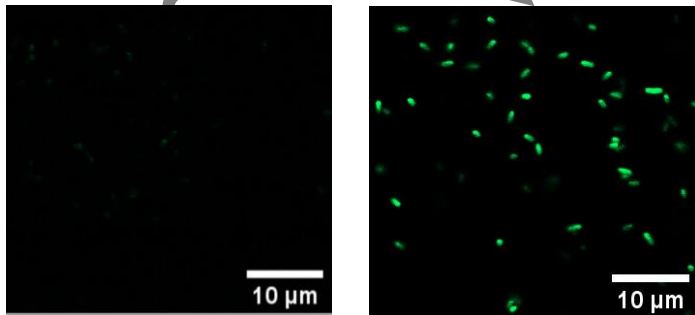
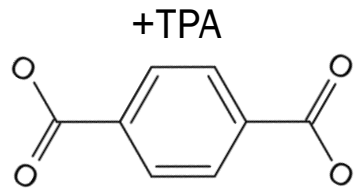


# ReporTPA™: A fast and sensitive TPA reporter

Reporter strain based on *Comamonas thiooxidans*

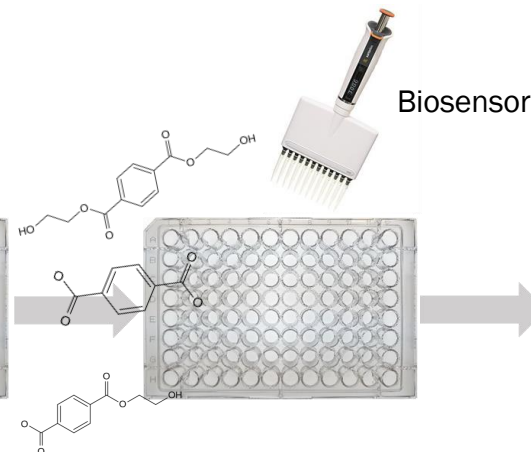
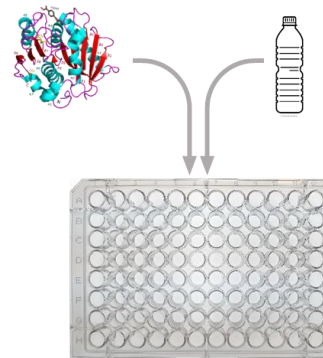
Contains gene cluster for TPA catabolism

TPA-degrading capability is used as starting point for Reporter



Dierkes et al., 2022 - Manuscript in preparation

Candidate enzymes + PET



Measurement





# Conclusions



- Microbes harbour an enormous potential on biocatalysts due to their metabolic versatility
- This potential can be accessed by metagenomics
- In bioindustries, hydrolases and esp. lipases and carboxylesterases have one of the highest shares
- UHAM built up an enzyme collection of >80 lipases by functional screenings
- With a sequence-based approach, >80 potential PET degrading enzymes have been found
- At UHAM, innovative tools are developed that help to fasten the discovery of novel enzymes: *In vitro* expression system and reporter strain for TPA detection

# Team in Hamburg



## External collaborators

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Birte Höcker, Bayreuth, Germany

Steven Hallam, UBC, Vancouver, Canada

Jürgen Pleiss, Stuttgart, Germany

Rolf Daniel, Göttingen, Germany

Ruth Schmitz-Streit, CAU Kiel, Germany

Alex Almeida, Sangre Centre; Hinxton UK

& others....



**RAHN-QUADE-STIFTUNG**



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European Union funding  
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...and thank you for your attention!



Hongli  
Zhang



Robert  
Dierkes



Pablo  
Pérez-García



Myllena  
Perreira



Sasipa  
Wong-  
wattananarat



Marno  
Gurschke



Golo  
Feuerriegel



Alan  
Wypych



Tabea  
Neumann

PLASTSEA

