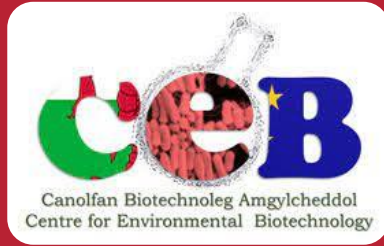




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# Discovery of Novel Metagenomic Carboxylesterases from Extreme Environments for Recycling Synthetic Polyesters

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**Supervisors: Prof. Peter Golyshin, Prof. Alexander Yakunin, Dr. Anna Khusnutdinova**

# Polyester plastics

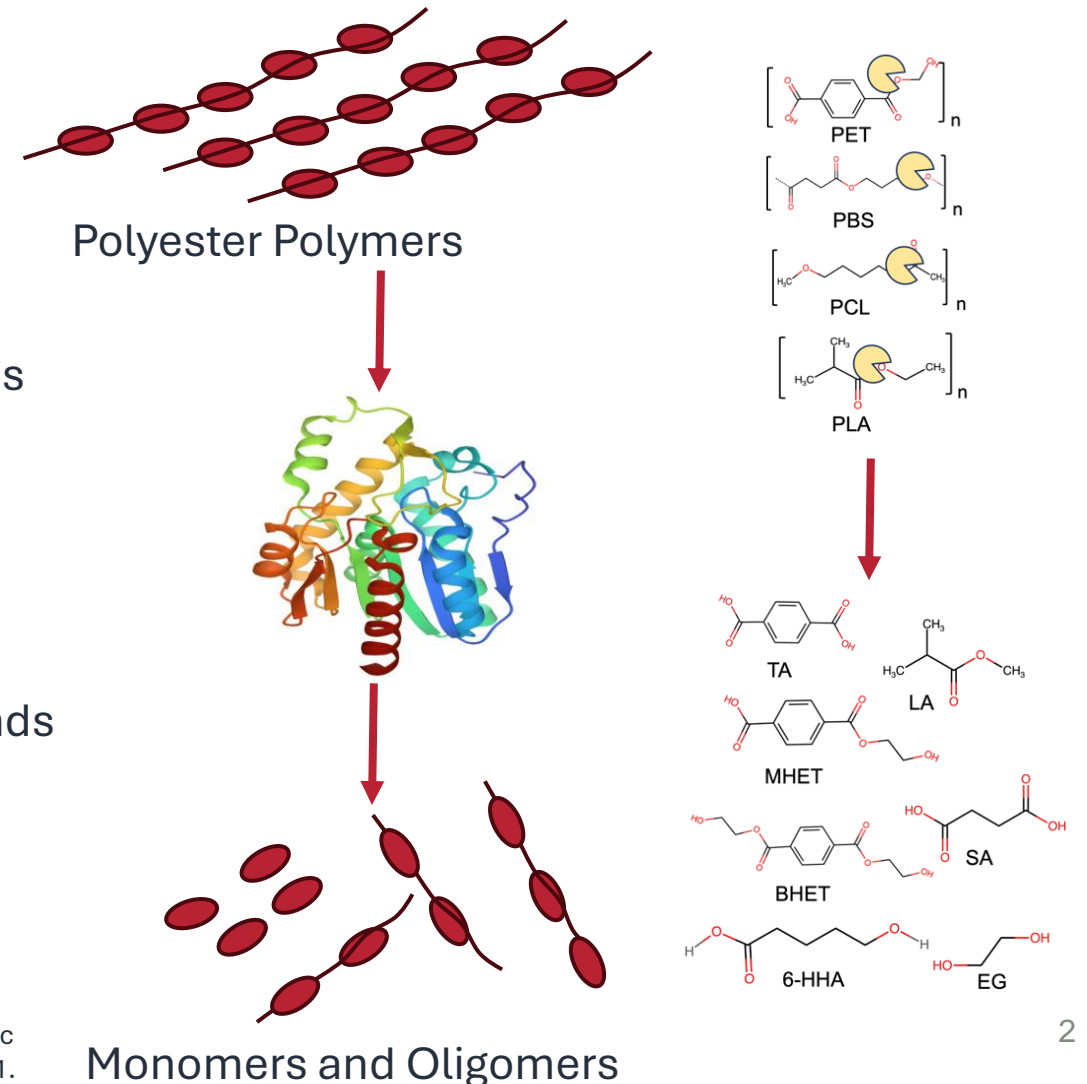
## 1. Productions

- polyester plastics production 40 million tonnes in 2023
- The production is expected to grow at a rate of 7% per year.
- Widely used in the manufacturing of fibres for clothing, home furnishings, packaging materials (like plastic bottles).

## 2. Waste Generation

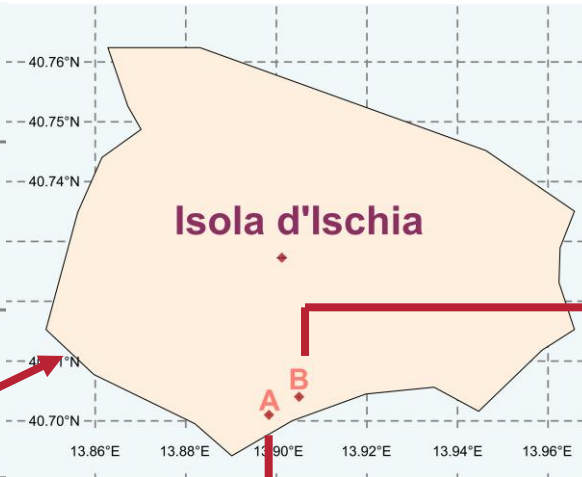
- Production of 350 million tonnes of total plastic waste annually
- A significant portion of waste comes from polyester plastics
- Only 23% of plastic recycled, 42% to landfills and 35% is incinerated
- The remaining waste often ends up in natural environments, contributing to pollution

## 3. Enzymatic degradation of polyesters



# Thermostable Esterases

Previous work done in the CEB labs  
Hydrothermal vent of the volcanic island located in Italy



Structural Biology | Research Article | 31 January 2023

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Thermophilic Carboxylesterases from Hydrothermal Vents of the Volcanic Island of Ischia Active on Synthetic and Biobased Polymers and Mycotoxins

Authors: Marco A. Distaso, Tatyana N. Chernikova, Rafael Bargiela, Cristina Coscolin, Peter Stogios, Jose L. Gonzalez-Alfonso, Sofia Lemak, SHOW ALL (17 AUTHORS), Peter N. Golyshin | AUTHORS INFO & AFFILIATIONS

DOI: <https://doi.org/10.1128/aem.01704-22> | Check for updates

**SiteB (Cavascura hydrothermal vent)**  
Environmental conditions (pH 7.0-8.5, 45-55°C)

**SiteA (Maronti Beach)**  
Environmental conditions (pH 4.5-5.0, 75-85°C)

- The Ischia metagenome library prepared from **polyester enrichment culture**
- IS10, IS11, and IS12
- **IS12 esterase**, had excellent **thermal stability** and **plastic degrading** properties

\*Correlation of sequence identity to thermal stability

26 IS12 protein homologous sequences were selected and studied. We are looking for active and thermal stable enzymes, and how sequence identity is related to thermostability with characterised enzymes

# Target selections

## Previous work done in the CEB labs

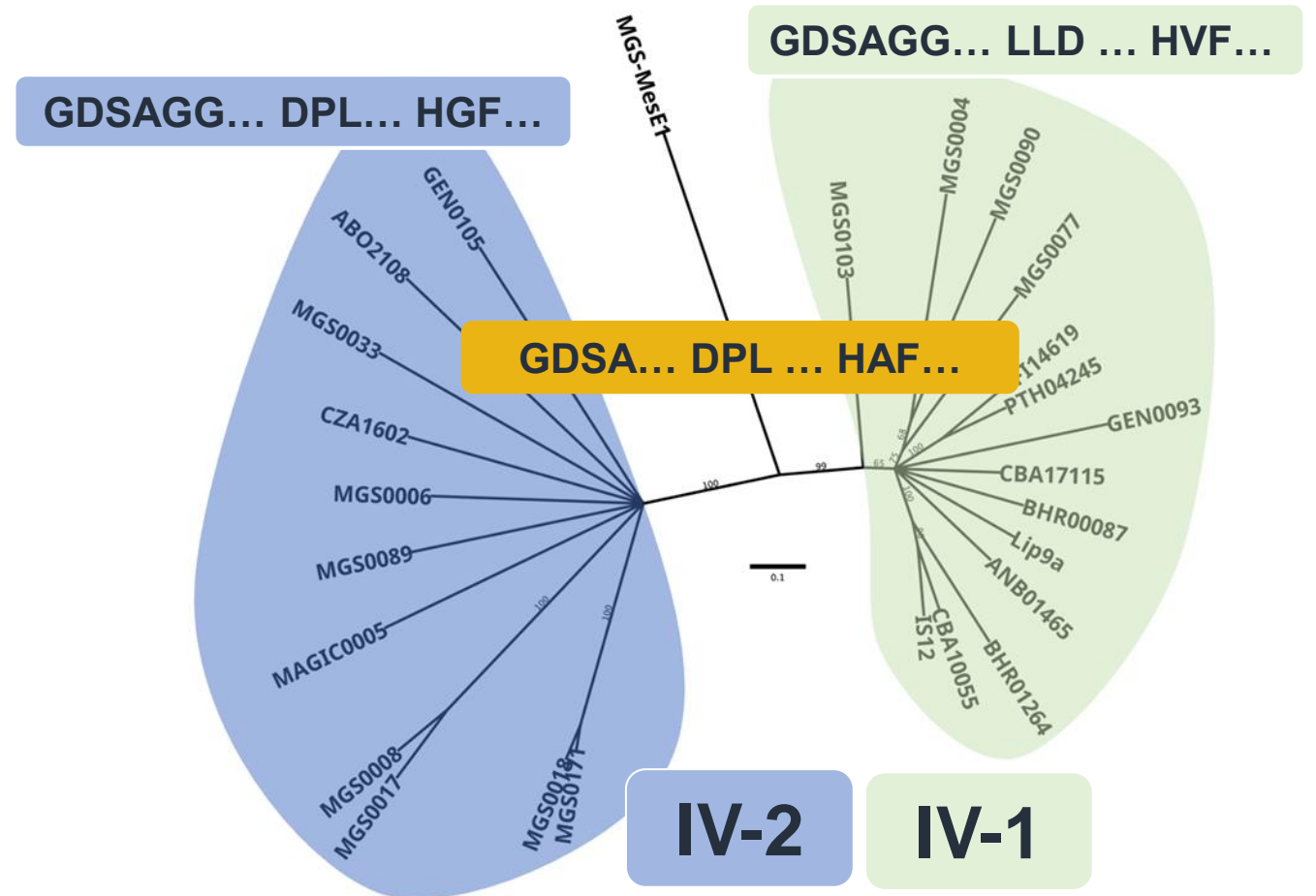
**IS12** esterase, had excellent **thermostability** and **plastic degrading** properties (from terrestrial hydrothermal vents located on the volcanic island of Ischia Italy). Sequences selected with sequence identity around 20% to 80%

| Protein name  | ORF/gene name    | Uniprot ID   | Protein length | % identity | Origin                                 |
|---------------|------------------|--------------|----------------|------------|--|
| 1. IS12       | ENO43_03940      | A0A7C2Y9X0   | 318 aa         | 100%       | Metagenome (Chloroflexota bacterium)   |
| 2. ABO2108    | ABO_2108         | Q0VMP2       | 316 aa         | 25.9%      | Alcanivorax borkumensis SK2            |
| 3. ANB01465   | ENQ48_01465      | A0A7C1F4L0   | 305 aa         | 60.8%      | Anaerolinea bacterium                  |
| 4. BHR00087   | HRbin25_00087    | A0A2H5YLC6   | 308 aa         | 59.4%      | Metagenome (bacterium HR25)            |
| 5. BHR01264   | HRbin23_01264    | A0A2H5YDR5   | 313 aa         | 65.3%      | Metagenome (bacterium HR23)            |
| 6. CBA10055   | ENP32_10055      | A0A7C2D648   | 317 aa         | 78.9%      | Metagenome (Chloroflexota bacterium)   |
| 7. CBA17115   | ENQ04_17115      | A0A7C1KEJ6   | 313 aa         | 59.7%      | Metagenome (Chloroflexota bacterium)   |
| 8. CZA1602    | CYCME_1602       | S5TGE2       | 298 aa         | 28.7%      | Cycloclasticus zancles 78-ME           |
| 9. CFI14619   | PBF_14619        | W7KS99       | 315 aa         | 57.8%      | Cytobacillus firmus DS1                |
| 10. GEN0093   | n/a <sup>a</sup> | A0A0G3FEG6   | 341 aa         | 46.7%      | Metagenome (Evry anaerobic digester)   |
| 11. GEN0105   | n/a <sup>a</sup> | A0A0G3FJ39   | 322 aa         | 25.2%      | Metagenome (Evry anaerobic digester)   |
| 12. Lip9a     | lip9A            | B8Y559       | 311 aa         | 54.0%      | Metagenome (uncultured bacterium)      |
| 13. MAGIC0005 | KKH72_12785      | A0A947SHF8   | 289 aa         | 34.0%      | Metagenome (Arreo Lake)                |
| 14. MGS0004   | n/a <sup>a</sup> | A0A0G3FL07   | 325 aa         | 45.5%      | Metagenome (Messina harbour)           |
| 15. MGS0006   | n/a <sup>a</sup> | A0A0G3FES7   | 297 aa         | 30.1%      | Metagenome (Messina harbour)           |
| 16. MGS0008   | n/a <sup>a</sup> | A0A1S5QLY1   | 360 aa         | 22.9%      | Metagenome (Messina harbour)           |
| 17. MGS0017   | n/a <sup>a</sup> | A0A0G3FET2   | 361 aa         | 21.8%      | Metagenome (Milazzo harbour)           |
| 18. MGS0018   | n/a <sup>a</sup> | T1VZD5       | 277 aa         | 24.2%      | Metagenome (R. exoculata gill chamber) |
| 19. MGS0033   | To be submitted  | WP_025895805 | 305 aa         | 22.9%      | Metagenome (Milazzo harbour)           |
| 20. MGS0077   | n/a              | A0A0G3FJ64   | 312 aa         | 50%        | Metagenome (composting plant)          |
| 21. MGS0089   | n/a              | A0A0G3FH39   | 296 aa         | 26.1%      | Metagenome (MT Haven sunken shipwreck) |
| 22. MGS0090   | n/a              | A0A0G3FEJ0   | 313 aa         | 45.7%      | Metagenome (MT Haven sunken shipwreck) |
| 23. MGS0103   | n/a              | A0A0G3FEU6   | 327 aa         | 49.7%      | Metagenome (Sobeslav soil)             |
| 24. MGS0171   | n/a              | A0A0G3FL37   | 264 aa         | 22.6%      | Metagenome (R. exoculata gill chamber) |
| 25. MGS-MesE1 | n/a              | A0A1S5QKE7   | 319 aa         | 25.5%      | Metagenome (Messina harbour sediment)  |
| 26. PTH04245  | DCC39_04245      | A0A2U1K574   | 309 aa         | 58.9%      | Pueribacillus theae                    |

# Phylogenetic analysis of selected proteins

## Carboxylesterases (E.C. 3.1.1.1)

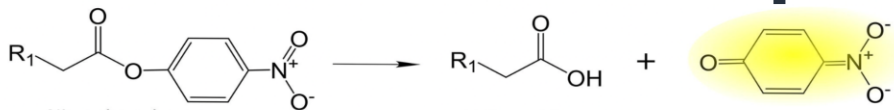
- A class of enzyme from  $\alpha/\beta$  hydrolases
- Bearing a catalytic **Ser-His-Asp**
- Esterases show preference for **short-chain** (<10-12 carbon atoms)
- Frequently show catalytic promiscuity
- Esterases have many industrial applications (i.e. food and paper; additive in detergent, biodiesel fuel, etc.)



According to classification of lipolytic enzymes, selected proteins belong to the carboxylesterase family IV (hormone sensitive-like) with the characteristic catalytic Ser motif **GDSAGG**.



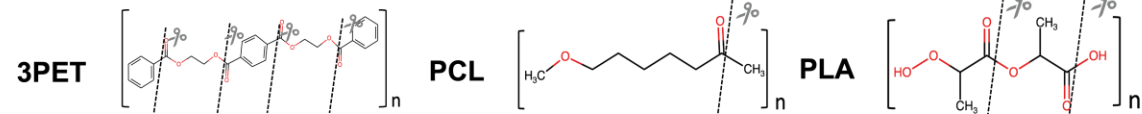
# Mono- and polyesterase activity screening



**$\rho$ -Nitrophenyl ester      Fatty acid       $\rho$ -Nitrophenolate**

|             | C2    | C4    | C8   | C12  | C14  | C16  | C18  |
|-------------|-------|-------|------|------|------|------|------|
| IS12        | 28.24 | 1.54  | 0.57 | 0.28 | 0.98 | n.d. | 0.63 |
| ABO2108     | 7.24  | 11.45 | 0.70 | 1.00 | 0.19 | n.d. | n.d. |
| ANB01465    | 3.61  | 3.83  | 0.48 | n.d. | 0.16 | n.d. | n.d. |
| BHR00087    | 11.81 | 15.10 | 0.88 | 0.32 | 0.11 | n.d. | 0.63 |
| ★ BHR01264  | 13.23 | 0.43  | 0.12 | 0.63 | 0.37 | n.d. | 0.20 |
| ★ CBA10055  | 39.48 | 3.77  | 1.96 | 0.82 | 0.39 | 0.24 | 0.17 |
| CBA17115    | 70.87 | 2.42  | 0.40 | n.d. | 0.19 | n.d. | n.d. |
| CFI14619    | 32.88 | 0.55  | 0.89 | n.d. | 0.82 | 0.86 | 0.68 |
| CZA1602     | 4.00  | 0.81  | 0.20 | 0.49 | 0.21 | 0.36 | 0.48 |
| GEN0093     | 87.53 | 63.17 | n.d. | 0.30 | 0.79 | n.d. | 0.13 |
| ★ GEN0105   | 1.92  | 19.54 | 2.18 | 1.56 | 0.78 | 0.23 | 0.12 |
| Lip9a       | 10.62 | 18.55 | 0.93 | 0.25 | 0.59 | 0.16 | n.d. |
| MAGIC0005   | 15.53 | 33.12 | 1.17 | 0.35 | 0.64 | n.d. | n.d. |
| ★ MGS-MesE1 | 15.80 | 58.85 | 2.67 | 0.50 | 0.81 | 0.18 | n.d. |
| MGS0004     | n.d.  | n.d.  | 0.58 | 0.14 | 0.64 | 0.27 | 0.18 |
| MGS0006     | 5.58  | 1.86  | 0.65 | 0.94 | 0.47 | n.d. | n.d. |
| MGS0008     | 2.36  | 11.41 | 1.13 | 0.19 | 0.51 | 0.17 | 0.26 |
| MGS0017     | 1.25  | 1.93  | 0.28 | 0.74 | 0.22 | 0.33 | 0.18 |
| MGS0018     | 1.49  | 8.50  | 0.55 | 0.15 | 0.33 | 0.15 | 0.42 |
| MGS0033     | n.d.  | 10.33 | 0.55 | 0.19 | n.d. | n.d. | 0.27 |
| MGS0077     | 14.24 | 11.24 | n.d. | 0.30 | 0.69 | n.d. | n.d. |
| MGS0089     | 0.90  | 2.50  | 0.99 | 0.50 | n.d. | n.d. | n.d. |
| MGS0090     | 16.35 | 21.69 | 0.76 | 0.19 | 0.52 | 0.17 | 0.16 |
| MGS0103     | 14.89 | 3.90  | 1.46 | 0.31 | 0.12 | 0.18 | 0.78 |
| MGS0171     | 0.38  | 7.63  | 1.16 | 0.16 | 0.72 | n.d. | n.d. |
| PTH04245    | 10.13 | 0.28  | 0.42 | 0.13 | 0.29 | 0.53 | 0.30 |

Carboxylesterase activity (U/mg), 0.5 1.0 2.0 5.0 10.0 20.0 80.0

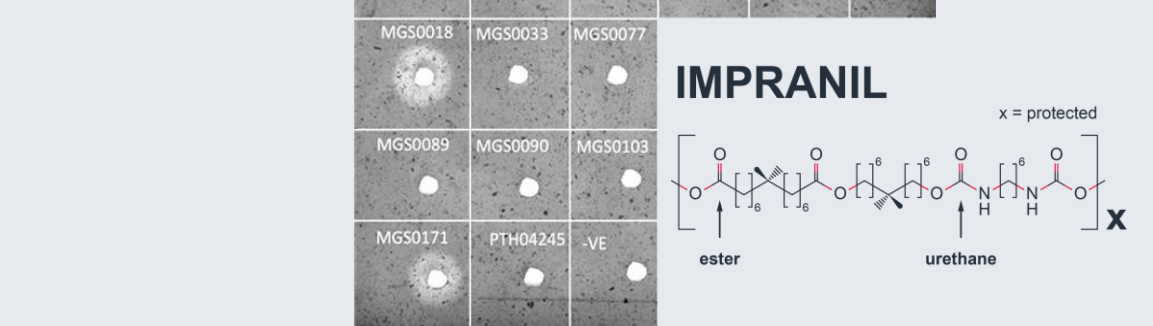
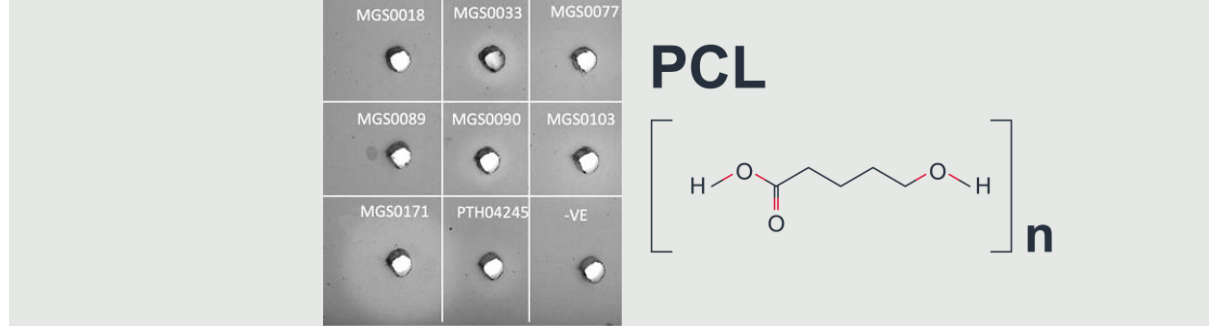
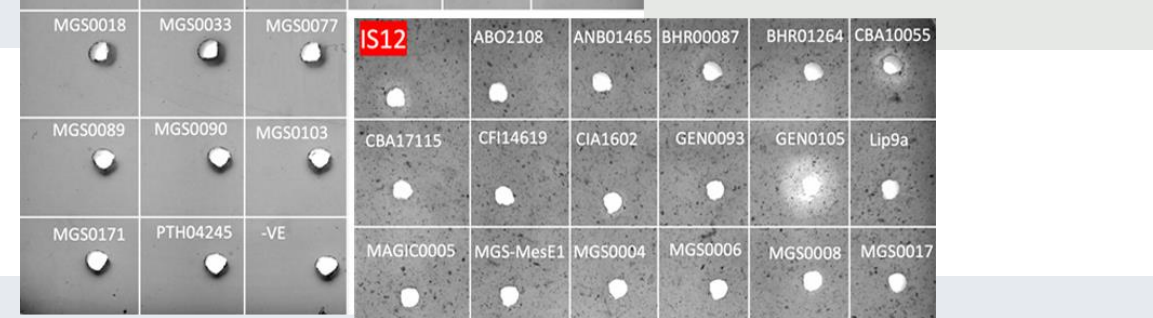
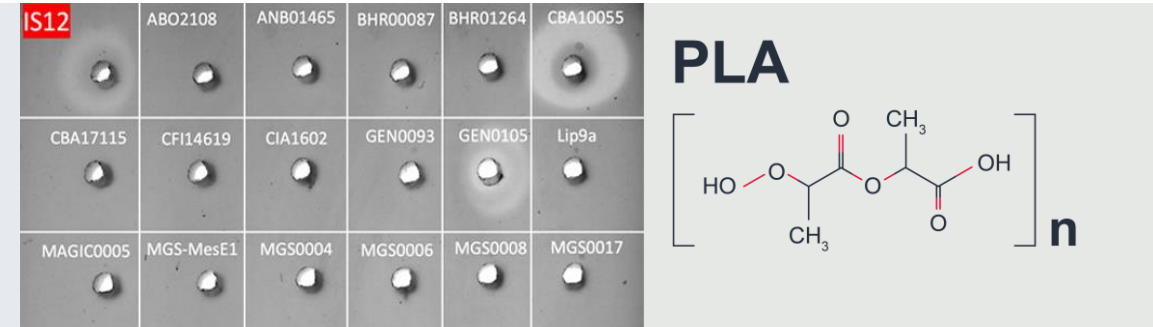
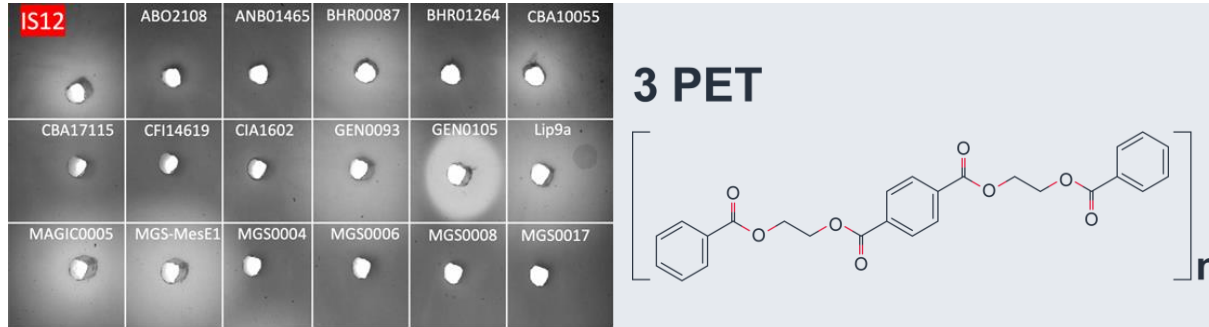


|             | Gel | BA     | BHET | MHET  | TA   | Gel       | 6-HHA | Gel    | LA        |     |       |
|-------------|-----|--------|------|-------|------|-----------|-------|--------|-----------|-----|-------|
| IS12        | ++  | 17.29  | 1.85 | 2.24  | n.d. | IS12      | -     | 5.56   | IS12      | ++  | 1.19  |
| ABO2108     | +   | 2.18   | n.d. | 0.61  | n.d. | ABO2108   | -     | 5.25   | ABO2108   | -   | 0.38  |
| ANB01465    | -   | 2.74   | n.d. | 2.92  | n.d. | ANB0146   | -     | 9.94   | ANB0146   | -   | 0.86  |
| BHR00087    | ++  | 2.58   | 0.21 | 0.27  | n.d. | BHR00087  | +     | 4.44   | BHR00087  | -   | 0.17  |
| ★ BHR01264  | -   | 60.20  | n.d. | 11.05 | n.d. | BHR01264  | -     | 2.49   | BHR01264  | -   | 2.55  |
| ★ CBA10055  | ++  | 41.02  | 8.61 | 11.83 | n.d. | CBA10055  | +++   | 74.16  | CBA10055  | +++ | 11.51 |
| CBA17115    | -   | 1.40   | n.d. | 0.73  | 0.03 | CBA17115  | ++    | 6.97   | CBA17115  | -   | 0.40  |
| CFI14619    | -   | 0.77   | 0.12 | 0.21  | n.d. | CFI14619  | ++    | 9.69   | CFI14619  | -   | nd    |
| CZA1602     | -   | 0.92   | 0.02 | 0.47  | n.d. | CZA1602   | +     | 8.37   | CZA1602   | +   | 6.11  |
| GEN0093     | ++  | 2.58   | n.d. | 1.08  | 0.01 | GEN0093   | +     | 13.56  | GEN0093   | -   | 0.31  |
| ★ GEN0105   | +++ | 112.66 | n.d. | 85.70 | 1.60 | GEN0105   | +++   | 191.38 | GEN0105   | +++ | 1.93  |
| Lip9a       | ++  | 3.63   | 0.45 | 1.58  | 0.08 | Lip9a     | +     | 2.46   | Lip9a     | -   | 0.28  |
| MAGIC0005   | +++ | 2.63   | 0.36 | 0.25  | n.d. | MAGIC0005 | -     | 4.22   | MAGIC0005 | -   | 0.11  |
| ★ MGS-MesE1 | +++ | 94.03  | n.d. | 49.32 | 3.59 | MGS-MesE1 | -     | 4.85   | MGS-MesE1 | -   | 0.61  |
| MGS0004     | -   | 10.12  | n.d. | 4.25  | n.d. | MGS0004   | ++    | 44.25  | MGS0004   | -   | 1.36  |
| MGS0006     | -   | 0.62   | n.d. | 0.27  | n.d. | MGS0006   | +     | 1.49   | MGS0006   | -   | 0.52  |
| MGS0008     | -   | 2.83   | n.d. | 1.52  | n.d. | MGS0008   | ++    | 8.37   | MGS0008   | -   | 0.14  |
| MGS0017     | -   | 1.33   | n.d. | 1.45  | 0.06 | MGS0017   | ++    | 1.85   | MGS0017   | -   | 0.54  |
| MGS0018     | ++  | 3.79   | 0.18 | 0.85  | n.d. | MGS0018   | -     | 11.69  | MGS0018   | -   | 2.97  |
| MGS0033     | -   | 1.28   | n.d. | 0.78  | n.d. | MGS0033   | ++    | 8.77   | MGS0033   | -   | 0.89  |
| MGS0077     | ++  | 3.88   | n.d. | 1.61  | 0.02 | MGS0077   | +     | 2.25   | MGS0077   | -   | 0.85  |
| MGS0089     | -   | 5.79   | n.d. | 2.67  | 0.14 | MGS0089   | -     | 3.30   | MGS0089   | -   | 0.13  |
| MGS0090     | +   | 2.31   | n.d. | 0.92  | n.d. | MGS0090   | +     | 2.45   | MGS0090   | -   | 0.19  |
| MGS0103     | +++ | 3.59   | 0.46 | 0.63  | n.d. | MGS0103   | ++    | 19.34  | MGS0103   | -   | 1.19  |
| MGS0171     | ++  | 61.97  | n.d. | 19.73 | n.d. | MGS0171   | +++   | 18.42  | MGS0171   | -   | 0.96  |
| PTH04245    | +   | 4.47   | n.d. | 0.81  | n.d. | PTH04245  | +     | 2.64   | PTH04245  | -   | 0.18  |

Activity (mU/mg) 1 2 5 10 50 100 5 10 50 1 2 5

**\* GEN0105 highly active**

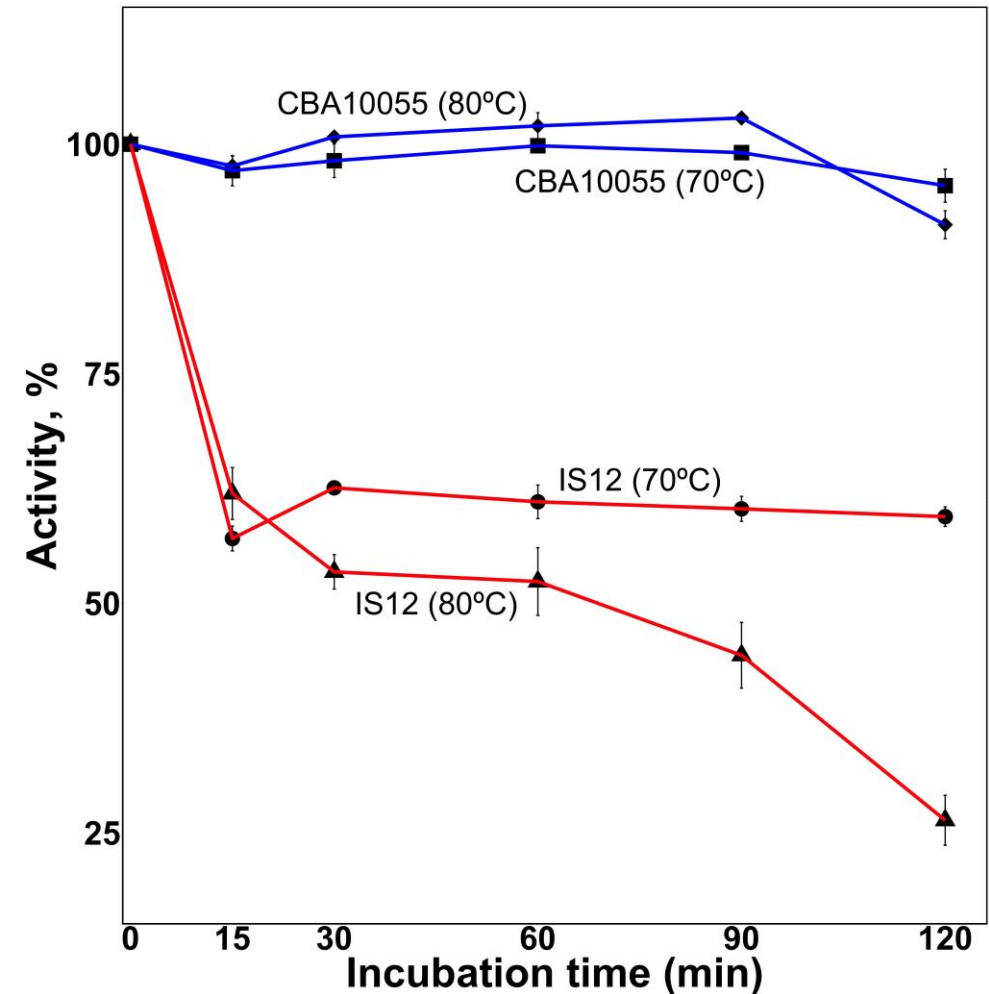
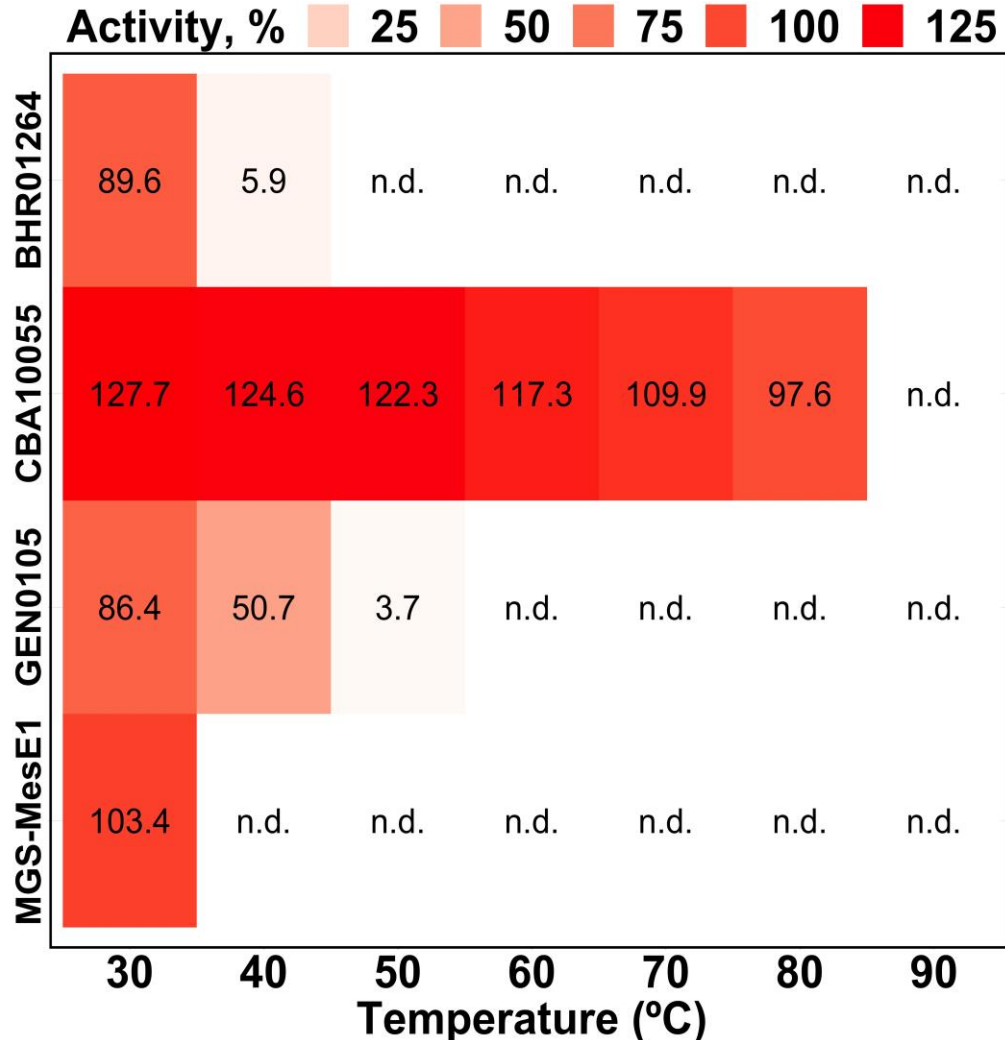
# Agarose gel based activity analysis



# Activity based analysis of thermostability

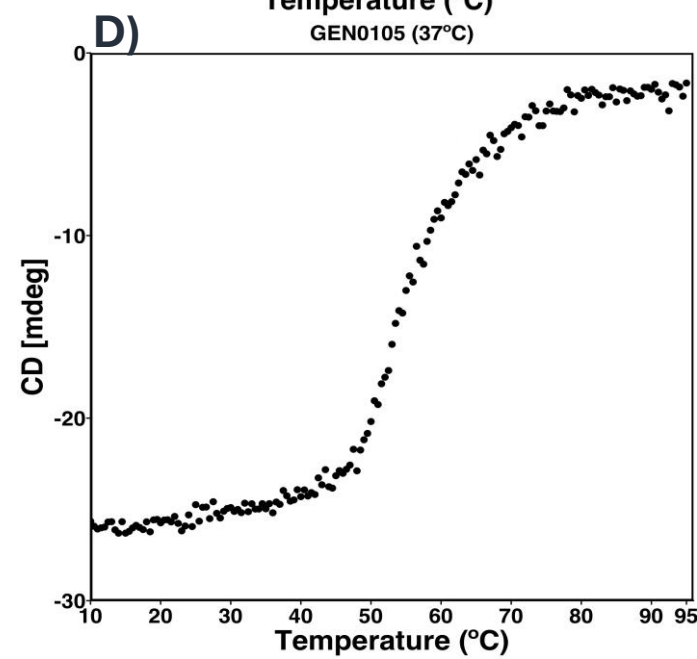
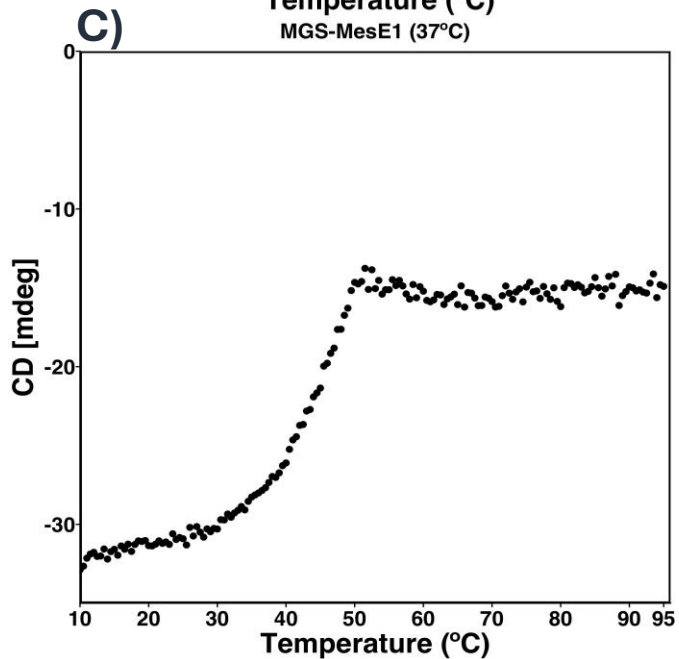
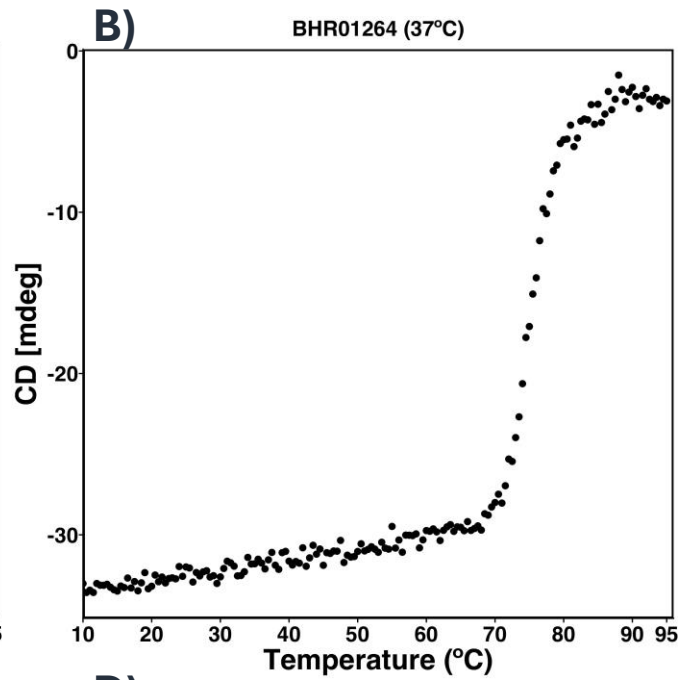
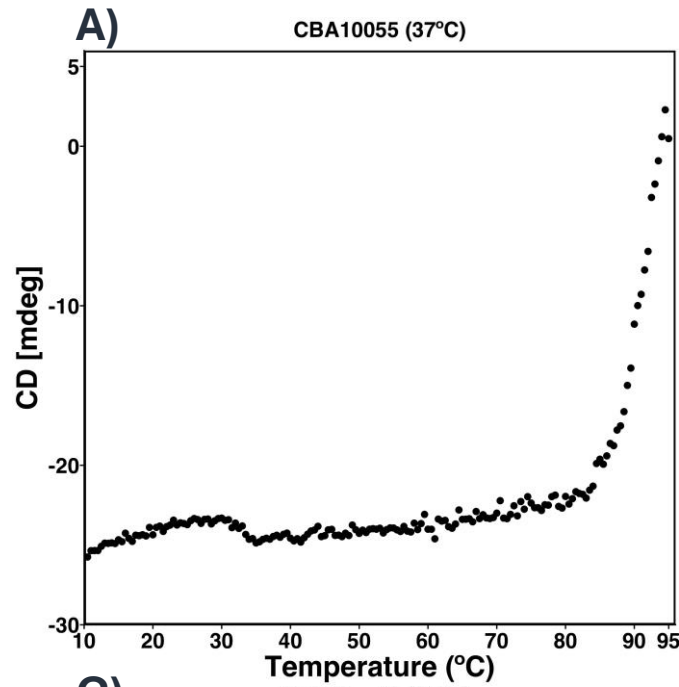
- Thermostability test for 4 selected enzymes incubated from 30°C to 90°C for 1h

- Comparison of the thermostability of **CBA10055** to **IS12** thermostable polyesterases at 70°C and 80°C



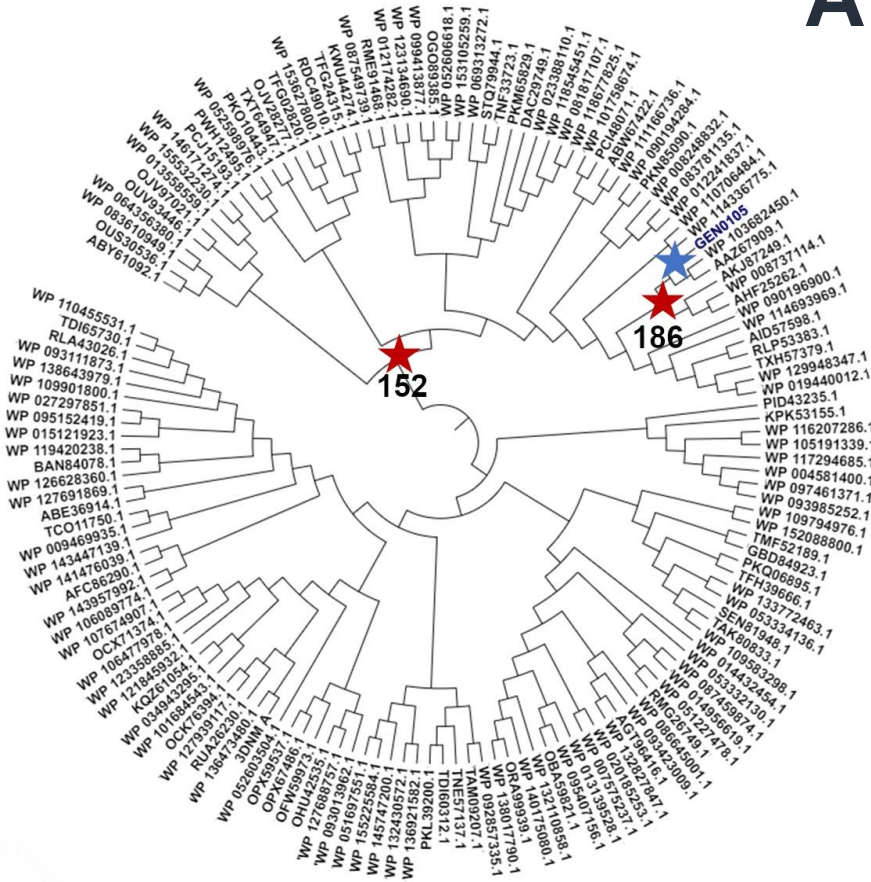


# Protein melting temperature ( $T_m$ )



| Protein      | $T_m$ (°C) |
|--------------|------------|
| 1. IS12      | 77.7       |
| 2. BHR01264  | 74.4       |
| 3. CBA10055  | 91.9       |
| 4. MGS-MesE1 | 43.3       |
| 5. GEN0105   | 54.0       |

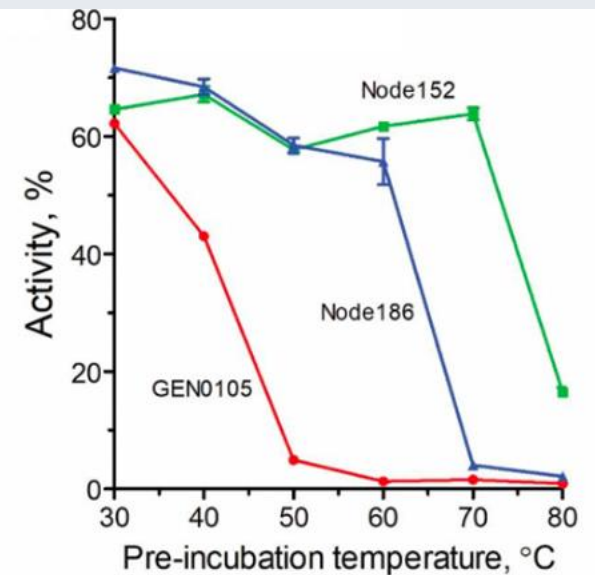
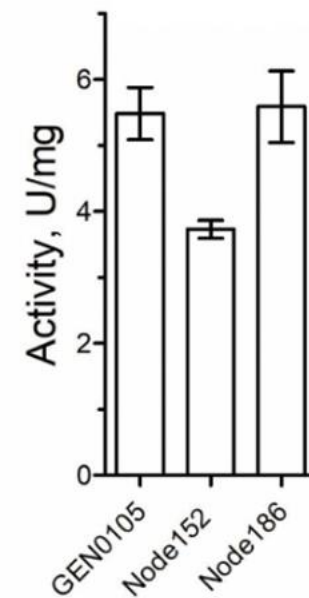
# Protein backbone engineering of GEN0105: Ancestral Reconstruction



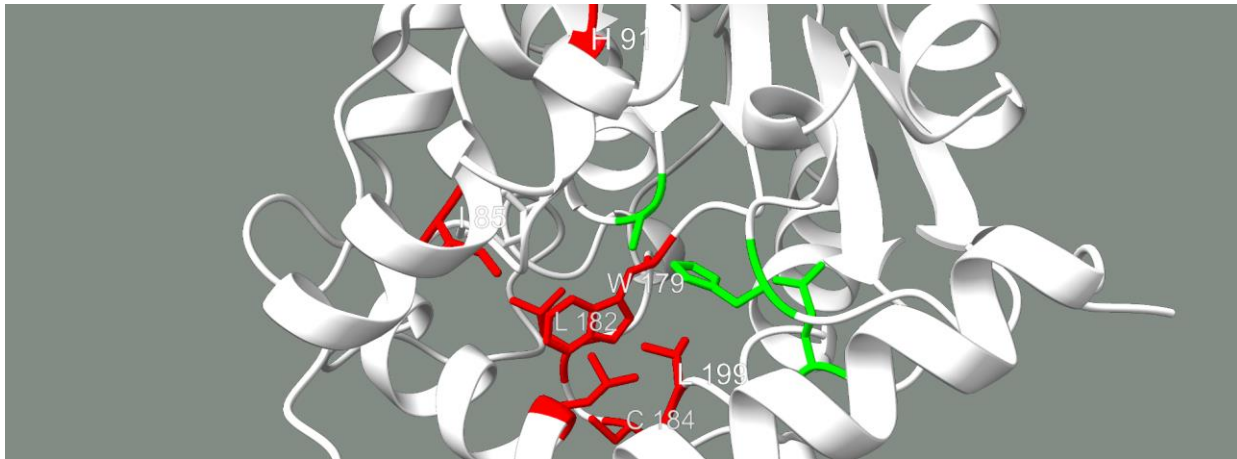
- Ancestral reconstruction approach aims to find protein with **increased thermostability**
- Phylogenetic tree was structured from past time
- Sequences selection based on **sequence identity**
- Two nodes with sequence identities of 42.1% (**NODE152**) and 51.8% (**NODE186**) to GEN0105 were selected

**FirePort ASR** : A Web Server for fully automated ancestral sequence reconstruction (Musil et al., 2021)

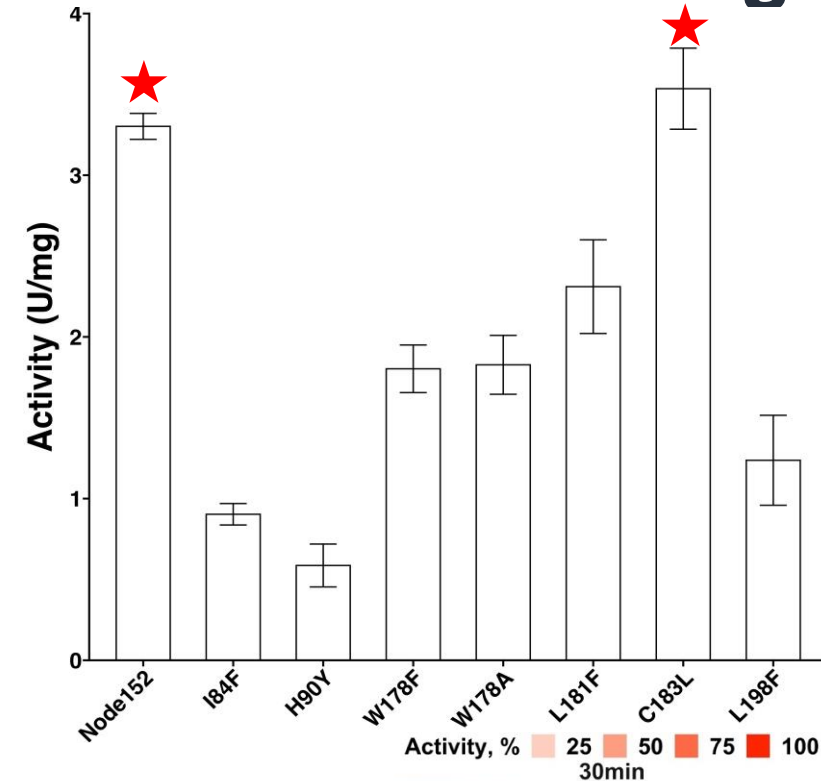
- Ancestral sequences were reconstructed using an evolutionary matrix.
- The wild-type protein GEN0105 was used as the input for ancestral reconstruction.



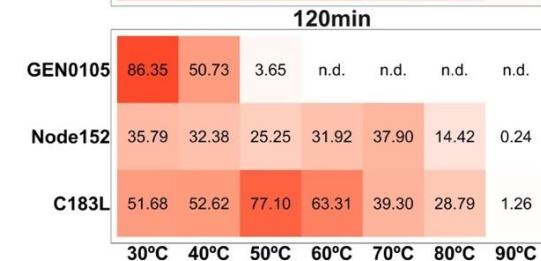
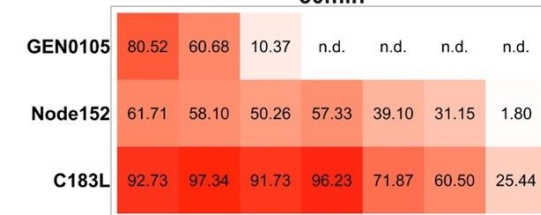
# Protein engineering of Node152: Site-directed Mutagenesis



|                 |     |   |                               |                               |                        |                            |          |               |        |      |     |
|-----------------|-----|---|-------------------------------|-------------------------------|------------------------|----------------------------|----------|---------------|--------|------|-----|
| Gen0105         | 1   | MSETSSASALPAYARIVVDKRAPFIRAILYLIILRYVIKRS | KPDA                          | DILKLRAMQLRADQKYAHPA          | ADAVMTFVD              | 74                         |          |               |        |      |     |
| Gen0105_Node152 | 1   | M   | ASFQARLFRQLLR                 | MMRKFQSSNSNTSIQQQRKHLEKMARMF  | PVPPGVQIEQVN           | 54                         |          |               |        |      |     |
| Gen0105_Node186 | 1   | MASW                                      | KASFQAKILNLLRLLVKPKMLRRPDPGLN | KIQRLRKHLARLDKRLAHP           | PAGVTVEEVR             | 64                         |          |               |        |      |     |
| Gen0105         | 75  | CDGVKANWITL                               | PGARPERVIFYLHGGAWMNFNPT       | YAAMLGRWARLLNARVLMVDYRLAPEHRY | PAGANDCETAYRWLLA       | 154                        |          |               |        |      |     |
| Gen0105_Node152 | 55  | IGGVPAEWITPKNASKSNKVI                     | LYLHGGGYVIGSPK                | THRNLSRLARASGMRVLVVDYRLAPEHFP | PAALDALAAYRWLLE        | 135                        |          |               |        |      |     |
| Gen0105_Node186 | 65  | VGGVPCWITAEENS                            | KADRTILYLHGGGFVHSPK           | TYRNFVSR                      | LAKALGARVLMVDYRLAPEHPY | PAAADCLAAAYRWLLE           | 145      |               |        |      |     |
| Gen0105         | 155 | QGIDSKQIVIGGDS                            | SAGGNLTLITLLRLKSNQPLP         | CAVALSPFVDF                   | TLSSPSMITNEKIDFMFTLE   | AMLGLRPHYLDPQD             | 235      |               |        |      |     |
| Gen0105_Node152 | 136 | QGYSPENIVIAGDS                            | SAGGNLTLATLLKREQGLP           | PAAVLSPWTD                    | LTCSGESFKTNAKRD        | MLSAESLQQCAKYYCGNQD        | 216      |               |        |      |     |
| Gen0105_Node186 | 146 | QGYDPKQIVIAGDS                            | SAGGNLTLATLLRIRDAGL           | PMPCAVLLSPATD                 | FTLSGPFQ               | TNEKADFMFSAEALQVFRKLYLGGQD | 226      |               |        |      |     |
| Gen0105         | 236 | FLNVDA                                    | SPIFGDFSGLPPIFFQSSNTE         | MLRDES                        | SVRAAARAHQ             | GVIVELELWQHLP              | HVFQAL   | QKLPQADAALQSI | IVRFIN | 315  |     |
| Gen0105_Node152 | 217 | PKNPLISPL                                 | FGDLSGLPMLIQVGSDE             | IELLDD                        | SVRLAERAK              | QAGVELEVWEDM               | HVFQIFAS | FLPEARQAI     | KIQIGK | FIR  | 297 |
| Gen0105_Node186 | 227 | PTNPLVSP                                  | IFGDFSGLPPMMFHVSSTE           | IELRDDS                       | SVRLAEKAR              | QAGVELEVQVQNM              | HVFPIF   | HFLPESKQ      | ALKQIV | KFIN | 306 |
| Gen0105         | 316 | SHTGWQA                                   |                               |                               |                        |                            |          |               |        |      |     |
| Gen0105_Node152 | 298 | QHLSQA                                    |                               |                               |                        |                            |          |               |        |      |     |
| Gen0105_Node186 | 307 | KHLSQA                                    | AASTAANRPA                    |                               |                        |                            |          |               |        |      |     |



Activity, % 25 50 75 100



| Sample  | Substrate     | Km (mM)   | Vmax | Kcat (s-1) | Kcat/Km (s-1 mM-1) |
|---------|---------------|-----------|------|------------|--------------------|
| Gen0105 | pNP-octanoate | 0.8±0.1   | 11.3 | 6.6±0.4    | 8.3                |
| Node186 | pNP-octanoate | 0.64±0.2  | 12.7 | 7.4±0.4    | 11.6               |
| Node152 | pNP-octanoate | 0.15±0.05 | 1.4  | 0.8±0.2    | 5.5                |
| C183L   | pNP-octanoate | 0.11±0.03 | 4.1  | 2.4±0.3    | 21.8★              |

**Highest efficiency**

# Summary

Polyesterase activity is widespread in the family IV carboxylesterases from bacteria

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- Characterised 25 enzymes from Carboxylesterase Family IV.
- Identified CBA10055 from *Chloroflexota* spp., a naturally occurring enzyme with remarkable thermostability ( $T_m = 91.9^\circ\text{C}$ ) and ~80% sequence identity to IS12.
- Discovered GEN0105 from the Evry (Paris) wastewater treatment plant, which demonstrated superior activity with plastic substrates but low thermostability, and lower sequence identity to IS12 (36%) making it a candidate for computational design.
- Engineered GEN0105\_Node151\_C183L, which improved thermostability while retaining wild-type activity.
- This study demonstrated that both natural gene library screening and computational design are effective strategies for developing industrially valuable enzymes.



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