FuturEnzyme
Technologies of the FUTURe for low-cost ENZYMEs for environment-friendly products

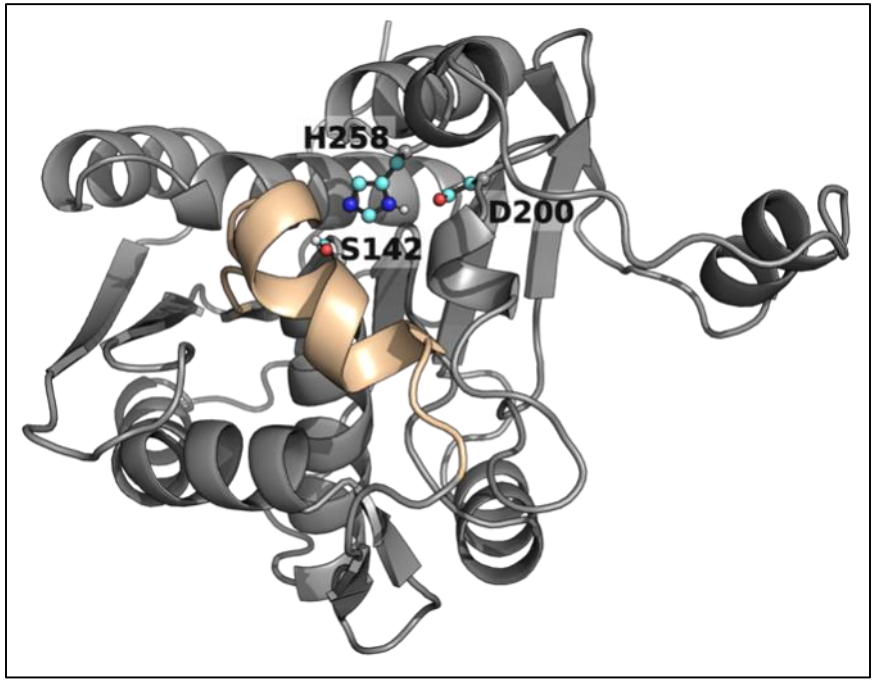
Executive Committee meeting

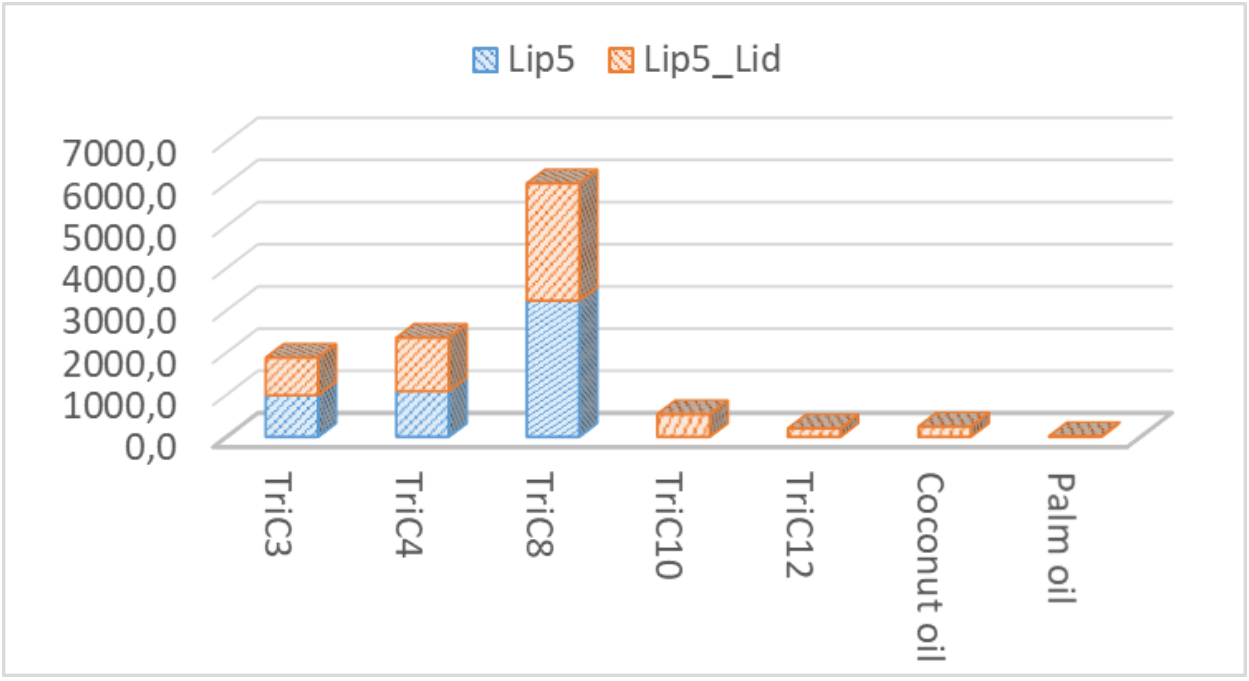
13TH of November 2022

Work Package 5

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

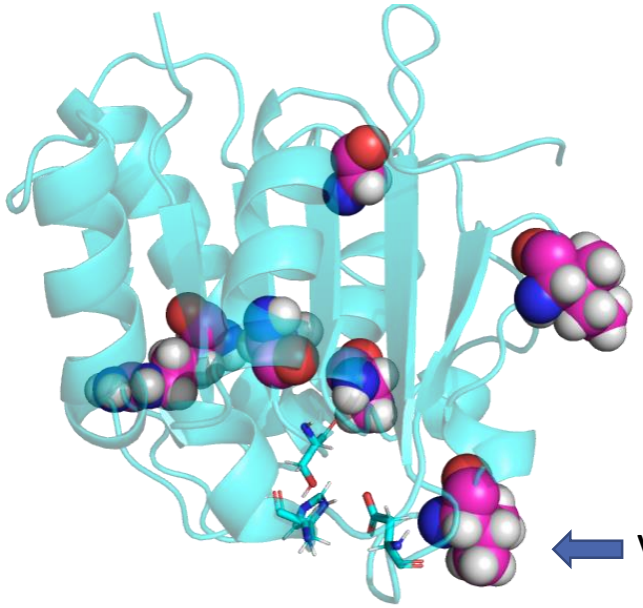
- CSIC and BSC contributed to improve the lipase character by lid domain design
  - Lip5: substituting “FRGTEITQIKDWLTDA” by “FRGTNSFRSAITDIVF”
  - Thus, it is possible to improve lipase character by lid domain substitutions





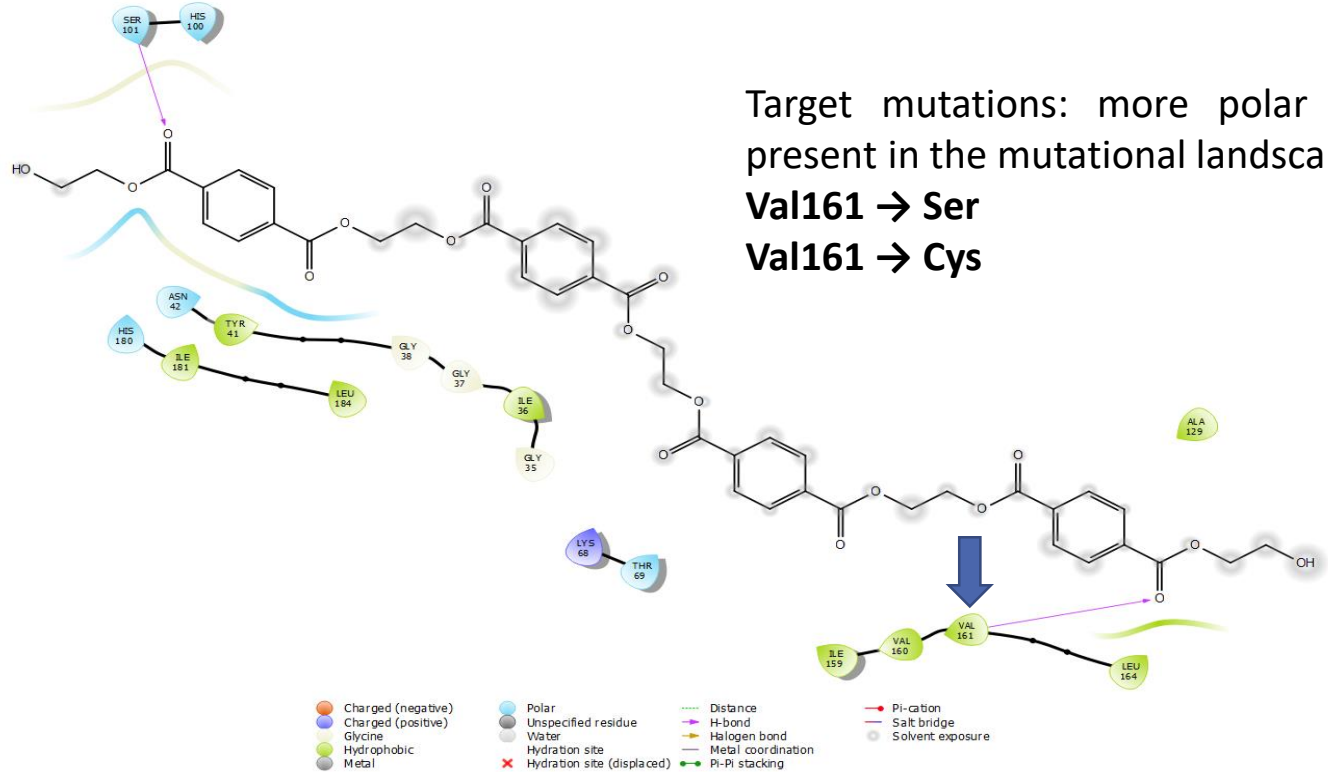
- CSIC and BSC set up engineering strategies for best 2 CSIC lipases: improving activity and stability
  - Lip9
    - Sequence:
      MAHHHHHHVGTGSNDDDDKSPDPMAEHNPVVMVHGIGGASYNFFSIKSYLATQGWDRNQLYAIDFIDKTGNNRNNGPRLSRFVKDVLDKTGAKK
      VDIVAHSMGGANTLYYIKNLDGGDKIENVVTIGGANGLVSSRALPGTDPNQKILYTSVYSSADLIVVNSLSRLIGARNVLIHGVGHIGLLTSSQVKGYIKEG
      LNGGGQNTN
    - Origin: Marine Metagenomics Database (MarRef)
    - Properties: Td 41,7ºC; Topt 30ºC; pHopt 9.0; stable in washing liquor (days), production 1 mg per L, acting on stained clothes and raw fabrics
  - ID9
    - Sequence:
      MAHHHHHHVGTGSNDDDDKSPDPMTNLSKPIPNPREYPILPPDMNYIYFENAHLFPFEPEKRDYSPVNAWWLSECAFLVYCHPGFARMAMALVGF
      DHFHFFQGKGTECMVSWNKDSIIVAFRGTEMKSLSAFHELRTDLNTAPVDFDKGSKVHKGFLKGLQEIWEGEEGLKLFLETLSAEAPSRSMWICGHSL
      GGALAALCFARLEKASGLYIYGAPRIGDGEFVRICDNRPVWRVEHGRDPIPLVPPDVPALNFNFKDMGKLIYIDYRGEILFERPLVTVEEEKSKVLLNISQQ
      RKRRESLSVEGFKGVLDKDRAKTLINGINEHIMQSRVEWKEYFDSLDKGIGLKIKDHMPIYYCAKLWNILIEGL
    - Origin: Metagenome from marine bone-degrading microbiome
    - Properties: Td 45.5ºC; Topt 40ºC; pHopt 9.5; stable in washing liquor (> weeks), production 63 mg per L, acting on stained clothes

- Lip9 functional hot spots found through hotspot wizard server (https://loschmidt.chemi.muni.cz/hotspotwizard)
- Lip9-PET_4 docking performed with Swissdock server (http://www.swissdock.ch/).



Val161 (hots

All functional hot spots detected seem far from the catalytic pocket except: Ala105 and Val161



Target mutations: more polar and are present in the mutational landscape

Val161 → Ser

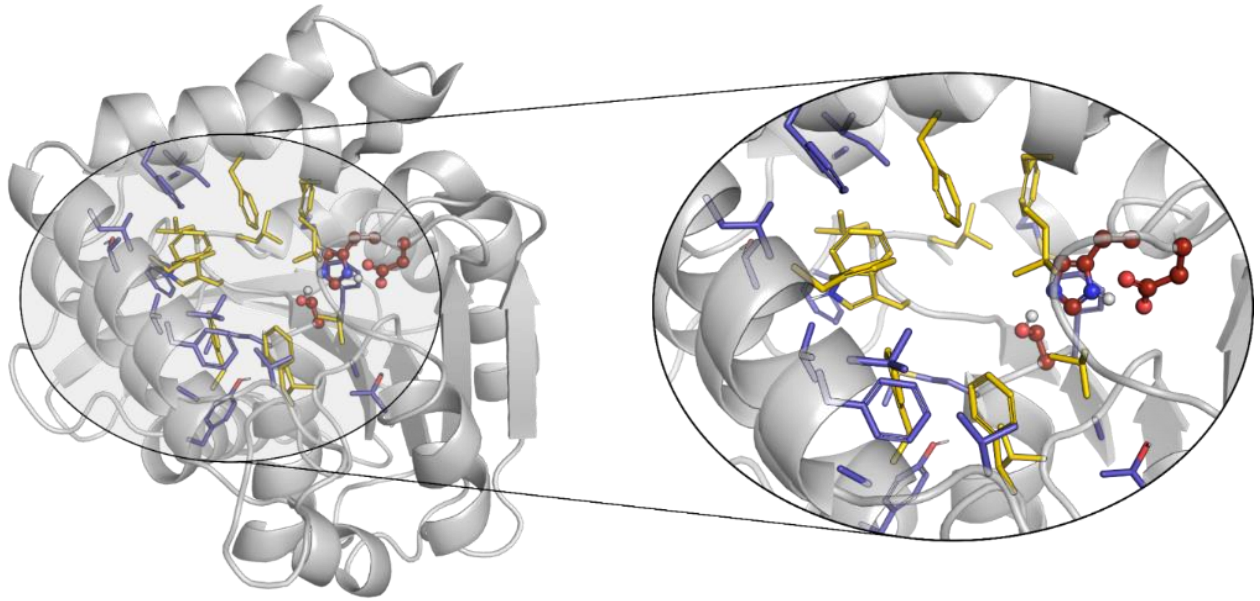
Val161 → Cys

ASiteDesign: An heuristic algorithm to design and redesign active sites

AsiteDesign

PELE

Rosetta Commons



ASiteDesign: An heuristic algorithm to design and redesign active sites

Results:

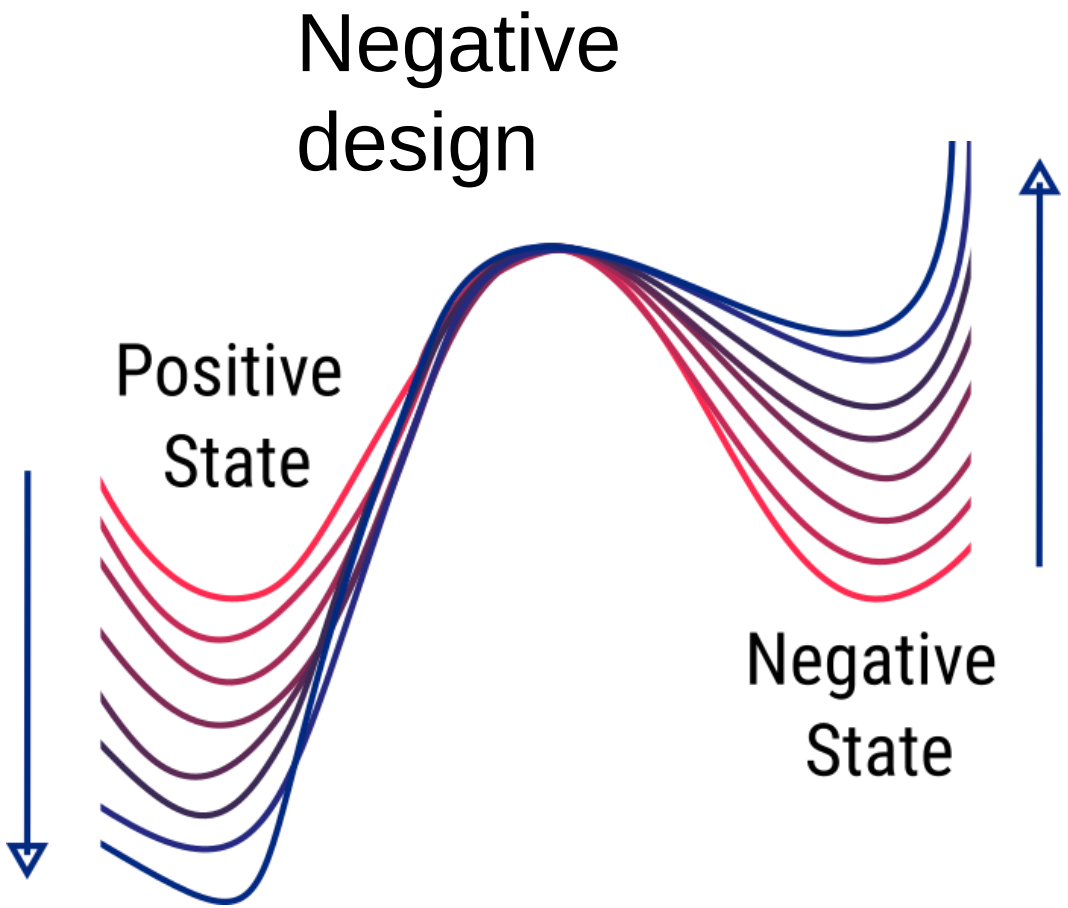
Table 1. Experimental measured activities for the catalytic designs in the hydrolysis of substrate **1**. The activity is reported as conversion. The residue numbering corresponds to the 1VA4 structure. [a]: after 24 hours, [b]: after 1 hour.

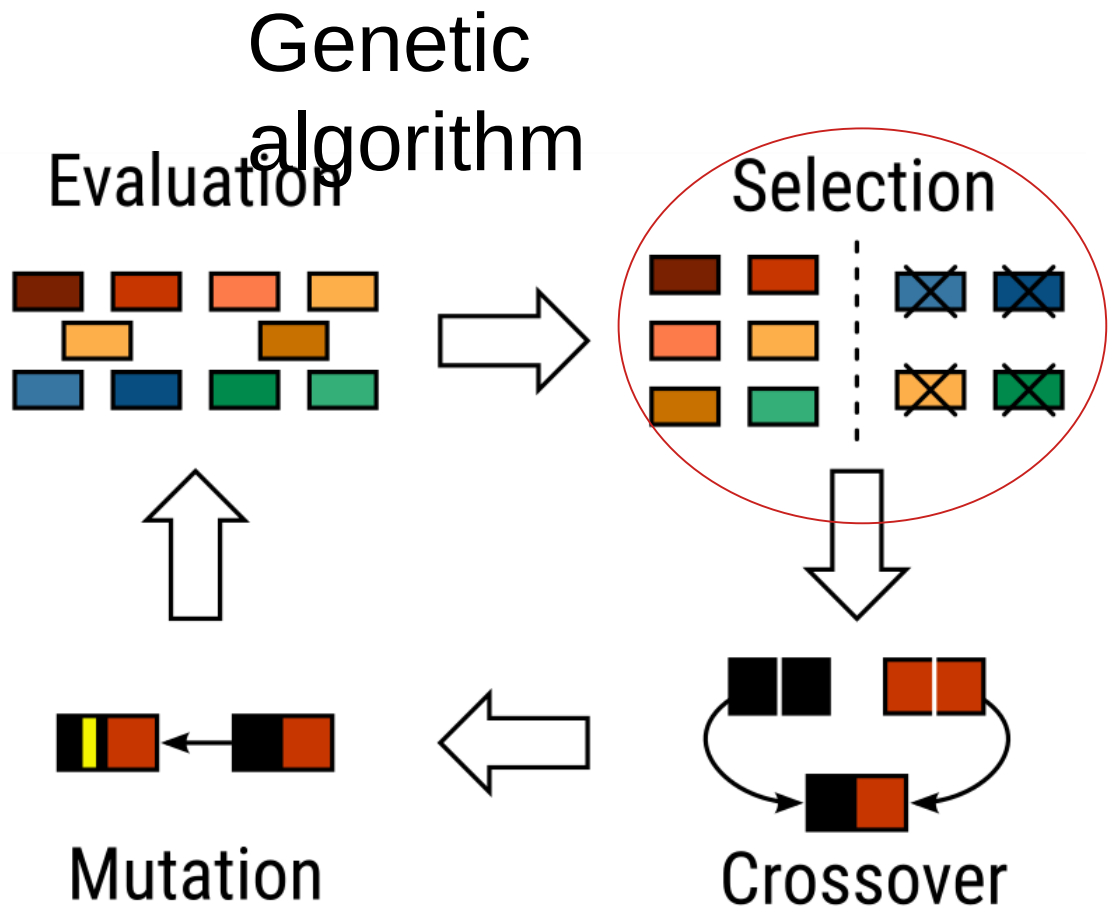
PFE variants	Mutations	Substrate **1**	Predicted selectivity
WT	-	8.9 % (8 %*ee* (*R*), E 1)[a]	-
PFE_1	W28S/L29H/T191D/ S94A	2.3 % (13 %*ee* (*S*), E 1)[a]	(*S*)
PFE_2	W28S/L29H/T191D/ S94A/C194T	Not detectable	(*S*)
PFE_3	W28S/L29H/T191D/ S94A/V195M	Not detectable	(*S*)

Table 2. Experimental measured activities for the binding pocket redesigns in the hydrolysis substrate **1**. The activity is reported as conversion. The residue numbering corresponds to 1VA4 structure. [a]: after 24 hours, [b]: after 1 hour, [c]: after 2 hours.

PFE variants	Mutations	Substrate **1**	Predicted selectivity
WT	-	8.9 % (8 %*ee* (*R*), E 1)[a]	-
PFE_4	W28A/F158L/F198A	6.0 % (3 %*ee* (*S*), E 1)[a]	-
PFE_5	F158L/F198A	66.7 % (32 %*ee* (*S*), E 4)[a]	(*S*)
PFE_6	W28A/F125A/F158L/F198A	3.3 % (9 %*ee* (*S*))[a]	(*S*)
PFE_7	W28A/F158L/F198A/I224L	5.2 % (8 %*ee* (*R*), E 1)[a]	(*S*)
PFE_8	F125A/F158L	23.4 % (55 %*ee* (*S*), E 4)[a]	(*R*)
PFE_9	F125A/F158L/I224L	1.4 % (29 %*ee* (*R*))[a]	(*R*)
PFE_10	F125A/F158L/F198A	16.3 % (60 %*ee* (*S*), E 4)[a]	(*R*)
PFE_11	V121A/F125A/I224L	2.9 % (38 %*ee* (*S*))[a]	(*R*)
PFE_12	V121A/F158A/F198V	1.6 % (100 %*ee* (*S*))[a]	(*R*)

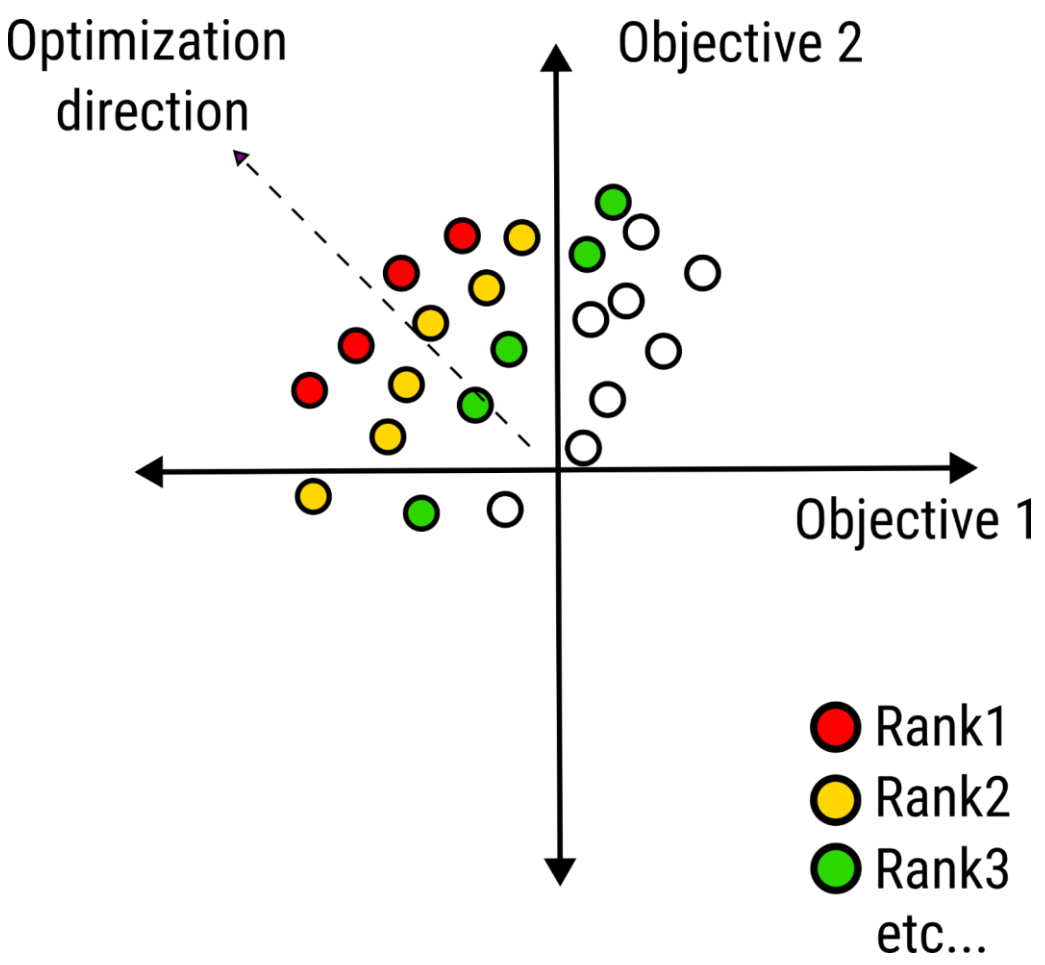
Multistate Design approach

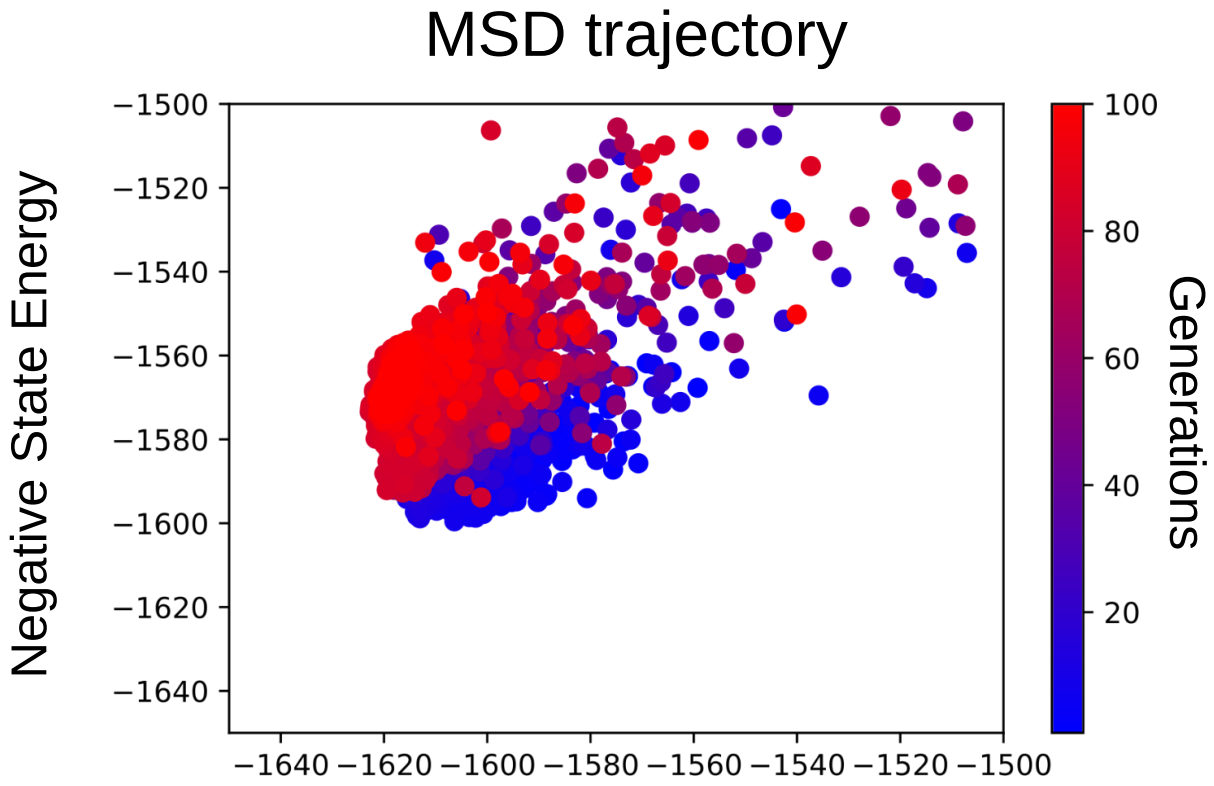




Task 5.1. Disruptive engineering computational tools M3-M42

Multistate Design approach



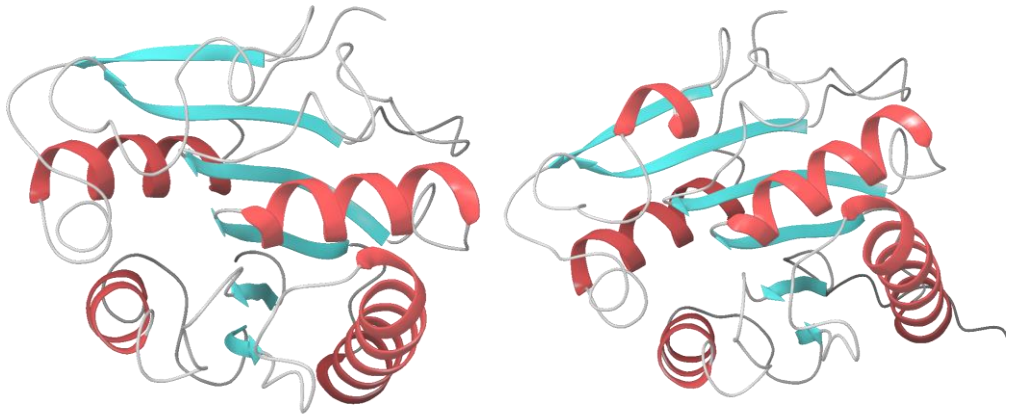


Multistate Design & AsiteDesign

Improve Activity Against Stain Compounds:

- **Olive oil** (Tri-C16:1)
- **Coconut oil** (triglycerides of C8:0, C10:0, C12:0, C14:0, C-16:0, C18:0, C18:1, C18:2)
- **Glyceryl tridodecanoate** (Tri-C12)
- **Palm oil** (mainly C16:0, C18:0, C18:1, C18:2 and C18:3)
- **Glyceryl trimyristate** (Tri-C14)

FElip9 ID9



Task 5.1. Disruptive engineering computational tools M3-M42

Ongoing work: increase thermostability esterase EH37

Statistical Potential Approach

CUPSAT

Empirical Potential Approach

FOLDX EVOEF

Machine Learning Approach

MAESTRO AUTOMUTE INPS MUPRO I-MUTANT

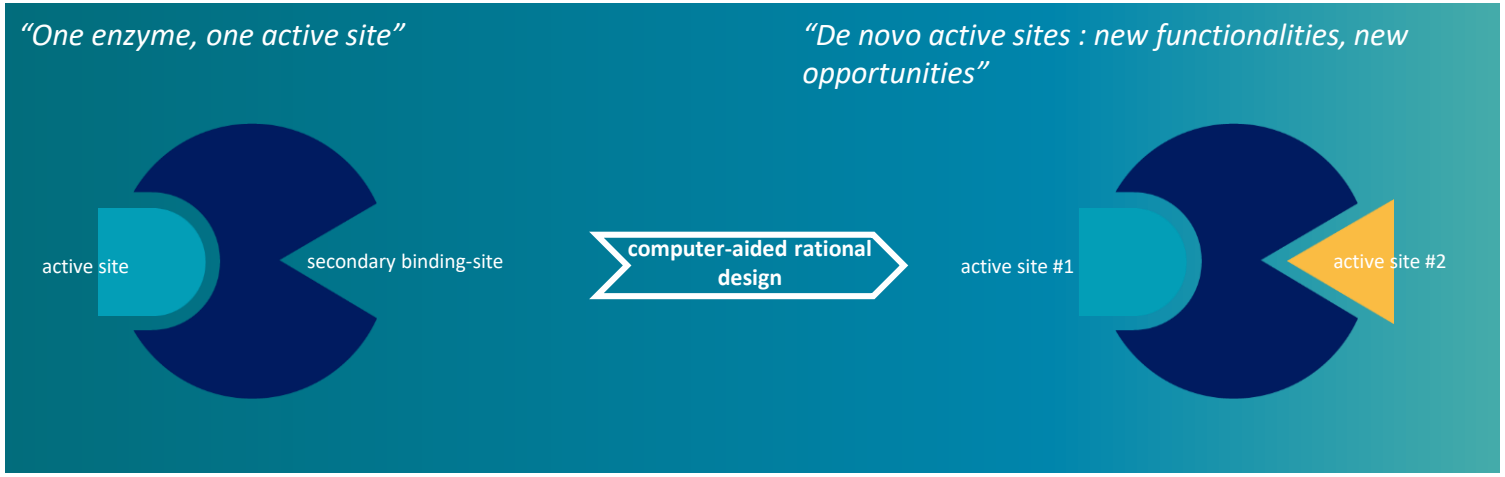
- METAPREDICTOR

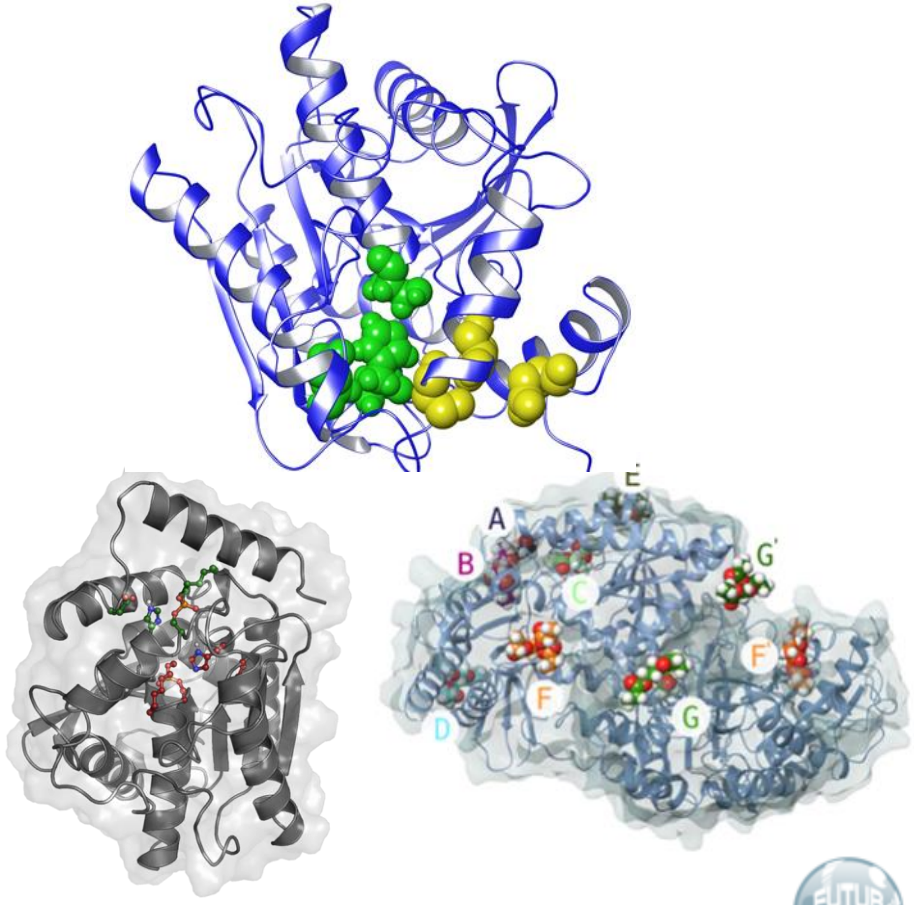
SCOT: Stability **Co**nsensus Me**t**apredictor

RANKING	MUTATION	RFC %	RFR ΔΔG (Kcal/mol)
1	44L	98	-0.5004
2	284M	97	-0.6562
3	302A	97	-0.3168
4	45I	96	-0.2228
5	283I	95	-0.8898
6	284L	95	-0.8608
7	265M	95	-0.7479
8	26M	95	-0.439
9	265I	94	-1.1778
10	152L	94	-0.8301
11	62L	94	-0.7276
12	284V	94	-0.406
13	22L	94	-0.3843
...	...	...	...
350	55I	71	-0.0056

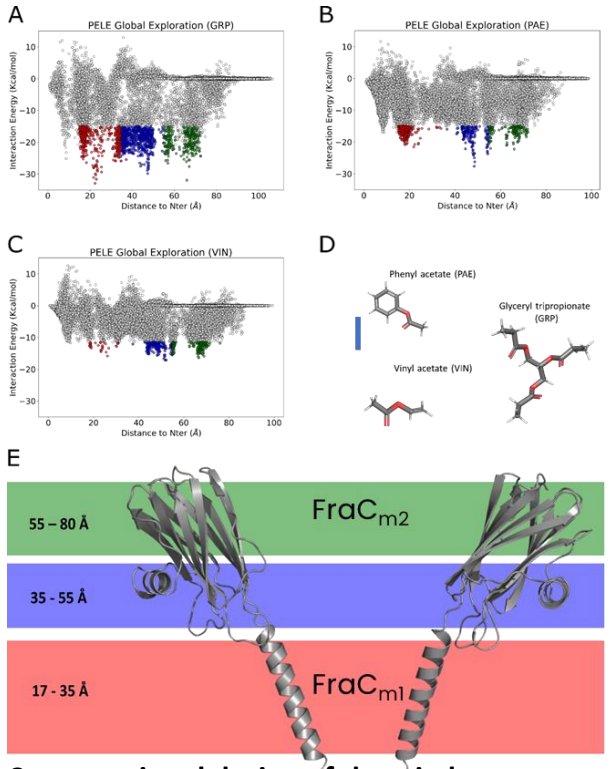
Task 5.2 Developing disruptive PluriZymes with multipurpose activities M6-M42

- CSIC & BSC established a platform for designing PluriZymes
- It confers higher activity and increase substrate range because synergy between active sites
- The *PluriZymer* module is available at BSC
  - PluriZyme EH_{1AB1}: native esterase + artificial esterase
  - PluriZyme EH_{1AB1C}: native esterase + artificial protease
  - PluriZyme TR_2E_2: native transaminase + artificial protease

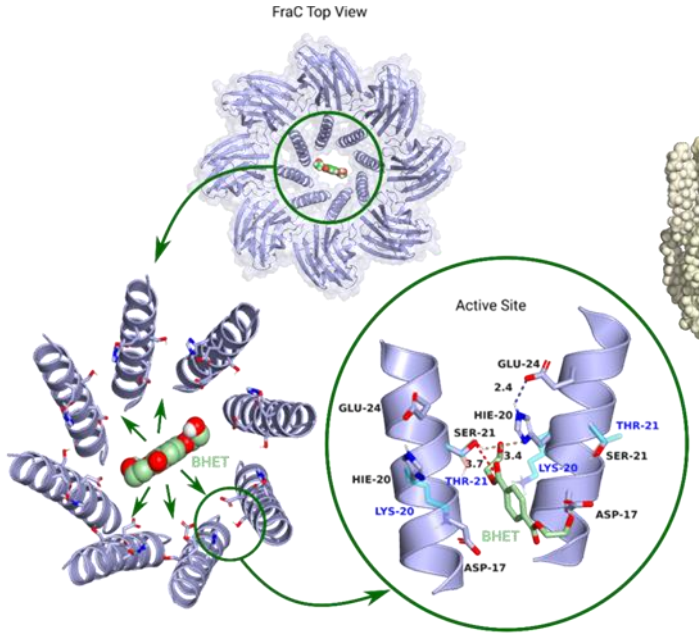


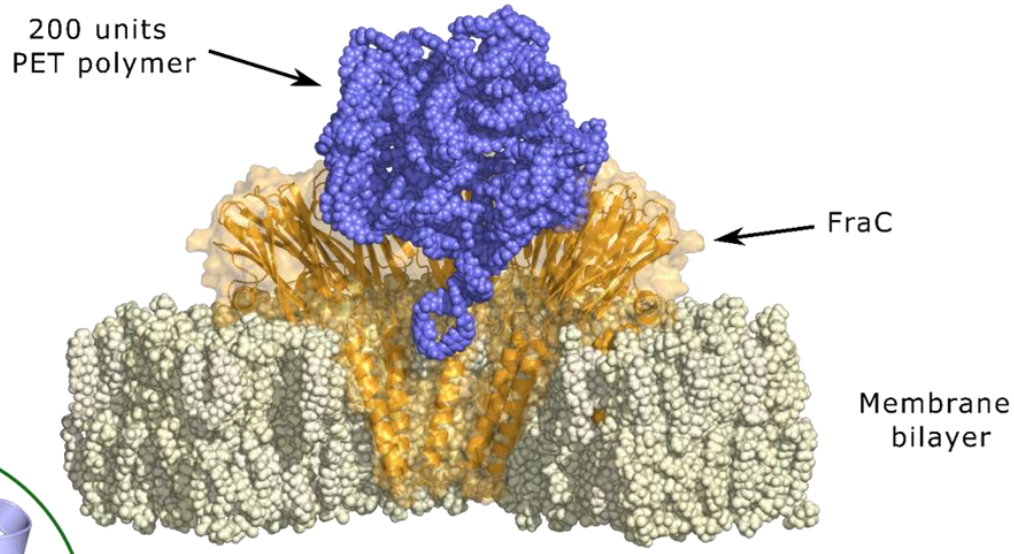


- CSIC & BSC established a platform to design artificial enzymes others than PluriZymes
  - Pore forming protein FraC selected as target
  - Two FraC mutants were designed, efficient for micro-plastic degradation through introducing PETase sites



Computational design of the triad



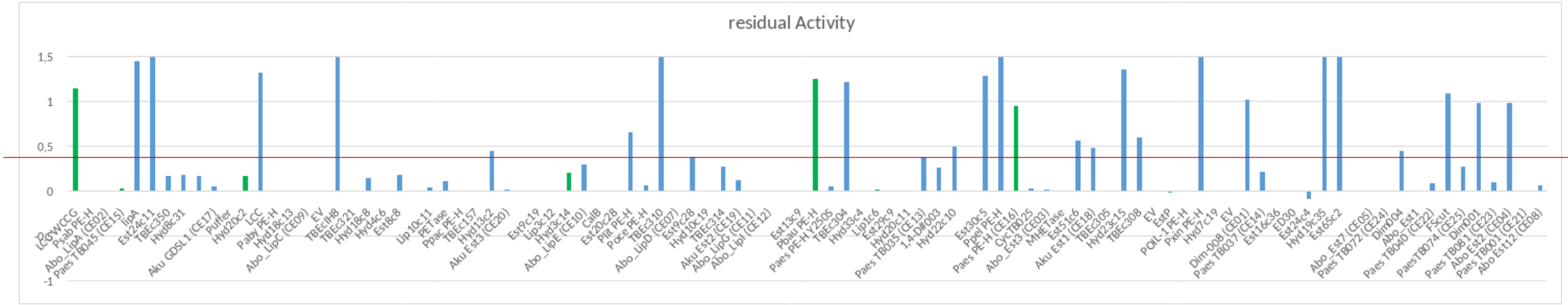


With **Álvaro del Pozo/Sara García** (UCM)

Task 5.3 Other advanced and classical mutation methods M4-M42

Characterization of selected candidate enzymes – stability

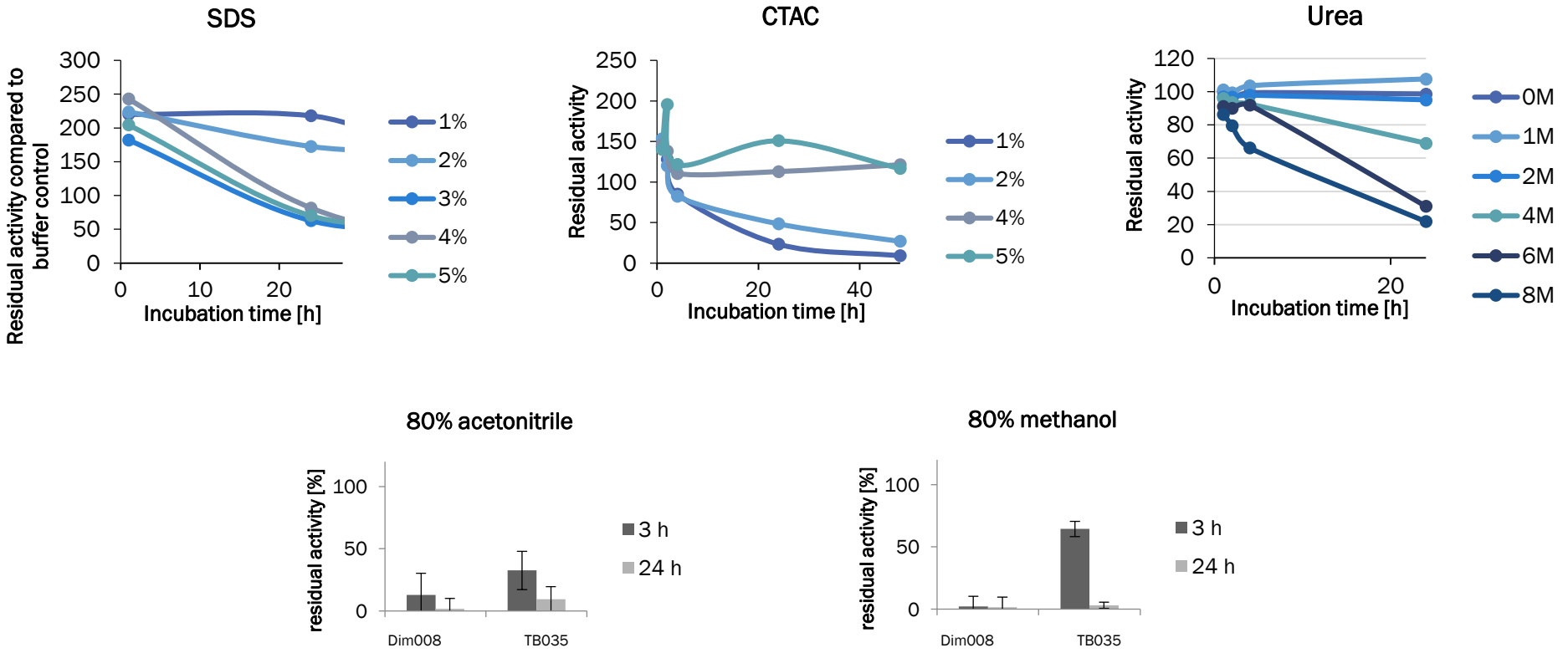
Identification of thermostable enzymes



- 30 enzymes with residual activity >25% after incubation at 60°C for 30 min
- 22 enzymes with residual activity >50% after incubation at 60°C for 30 min, e.g., Dim-008, lipA, TBEc304, TBEc310, Hyd23c15

Characterization of selected candidate enzymes – stability

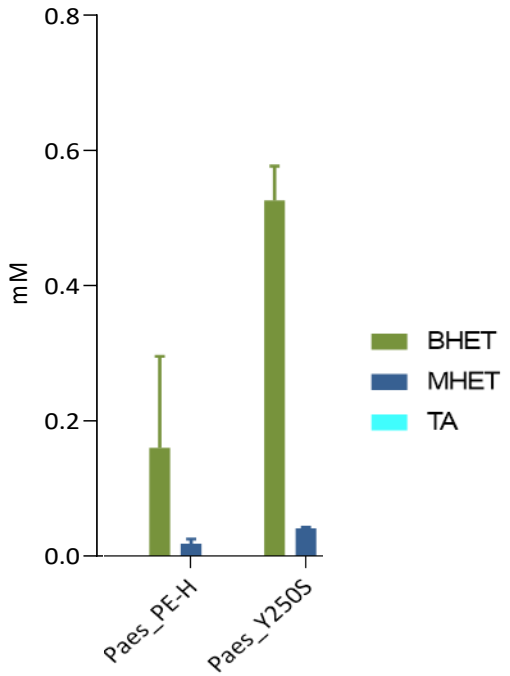
Identification of thermostable enzymes - Characterization of Esterase EstLip_Paes_TB035 as highly stable towards chemical agents



Textile application

PET Monomer release from Schoeller sample textile increased by Paes _PE-H mutant

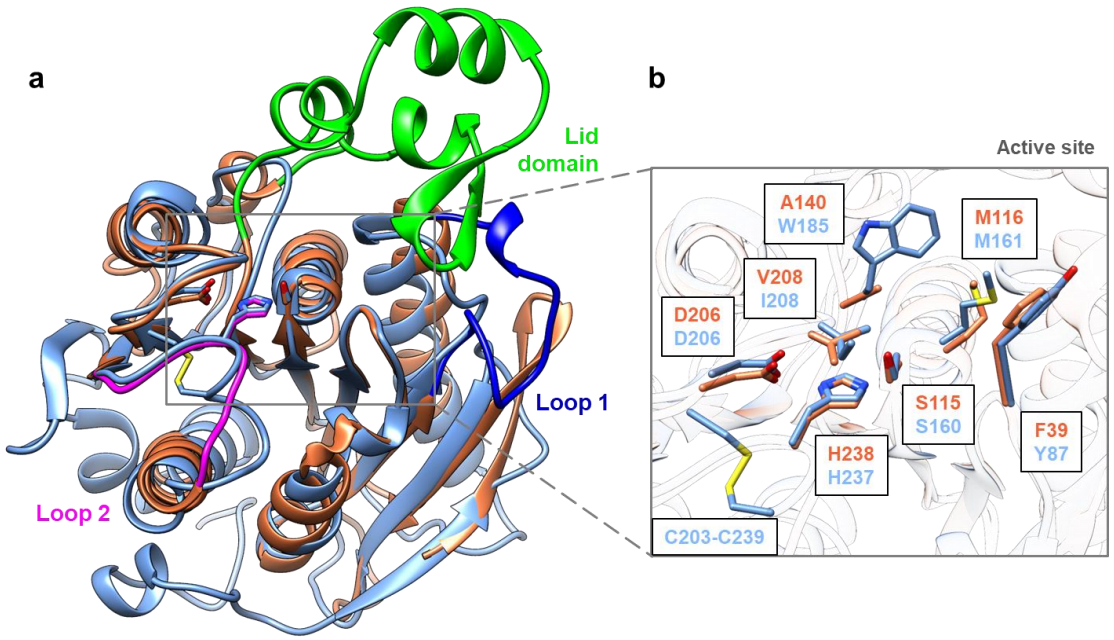
Substrate: sample textile 4-b 3X58 (VORB, 100% PES 100g/m^2) pretreated by alkaline boiling

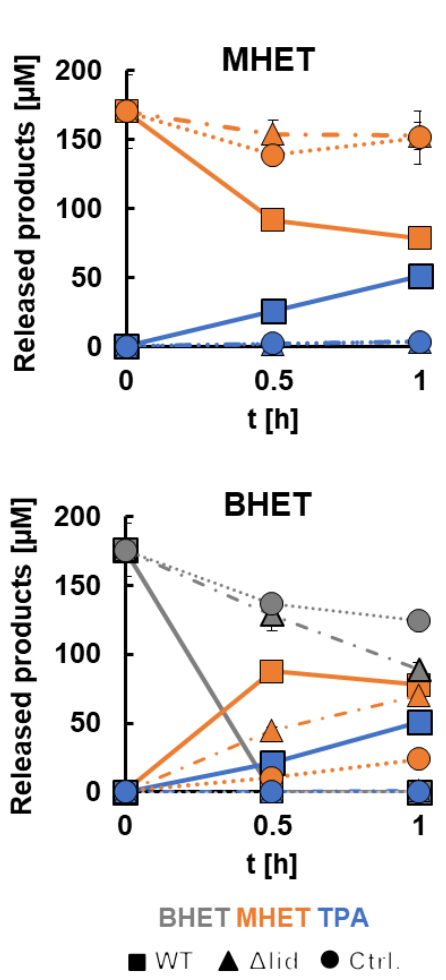


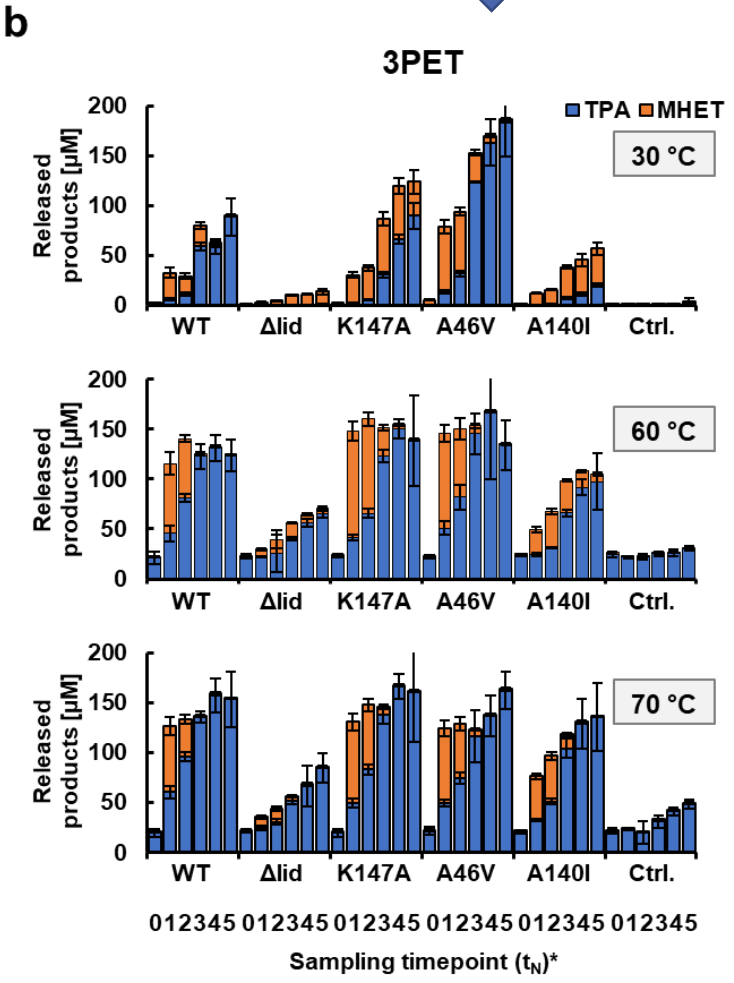
Task 5.3 Other advanced and classical mutation methods M4-M42

A46V!

PET46 from Candidatus Bathyarchaeota



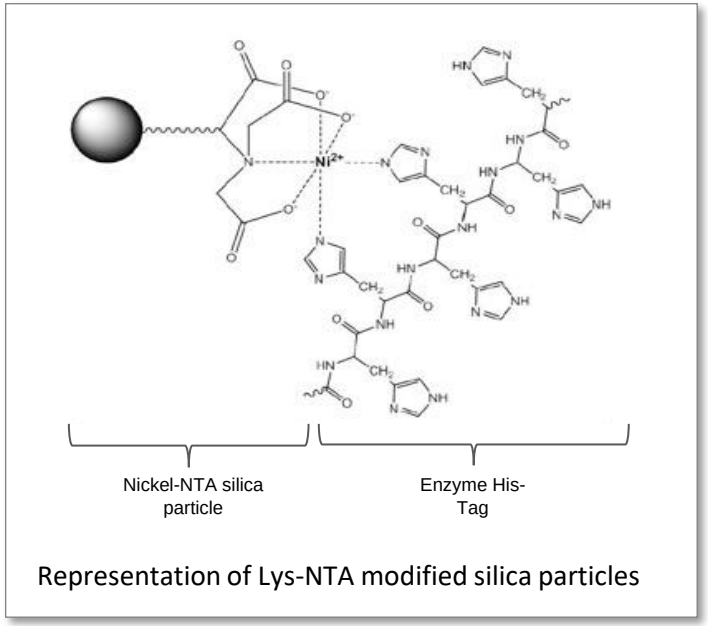




bioRxiv preprint DOI: https://doi.org/10.1101/2022.10.14.512230

Anchoring of enzymes through their His-tag on silica particles and shielding

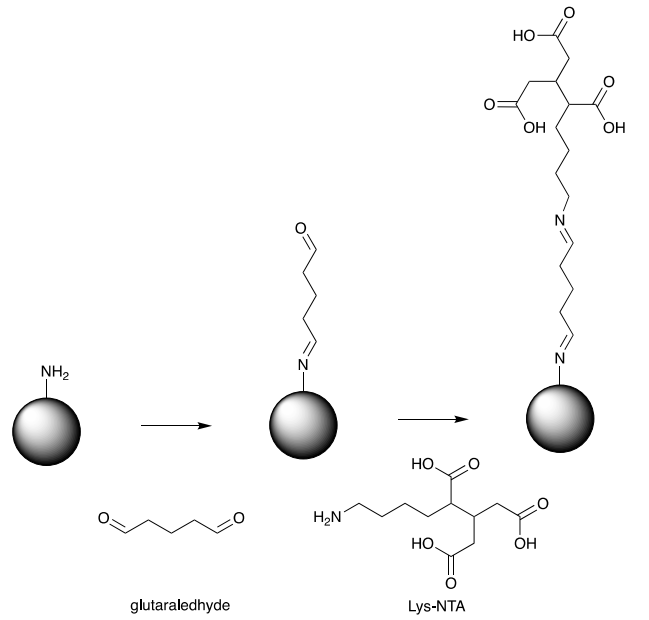
- Our method of enzyme immobilization is carried out using purified enzymes
- In order to improve cost-efficiency, we developped a method of surface immobilization using Ni-NTA-modified silica nanoparticles (diameter 300 nm).
- Layer growth conditions were optimized in order to avoid enzyme release.
- We will be testing the method with lipases sent by CSIC



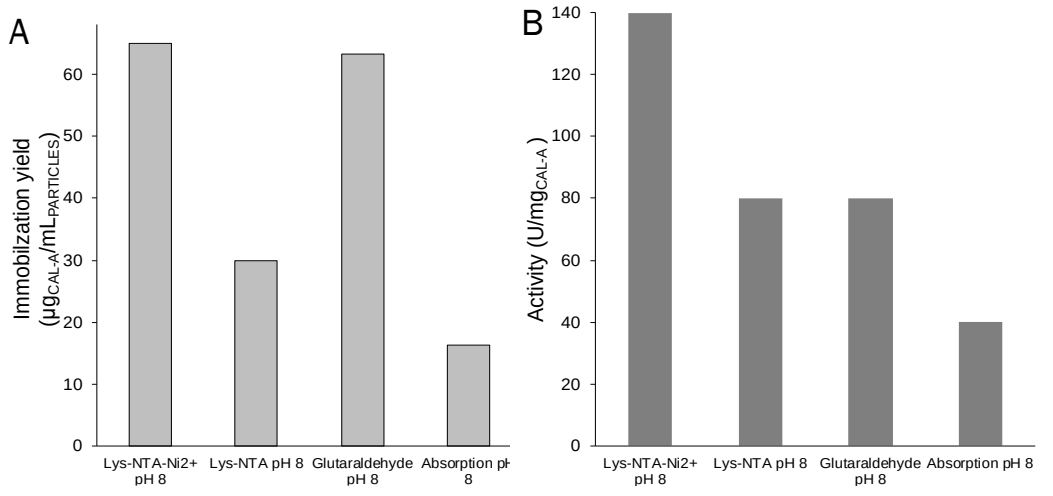
Representation of Lys-NTA modified silica particles

Task 5.4. Empowering enzymes by immobilization-guided supramolecular engineering M6-M40

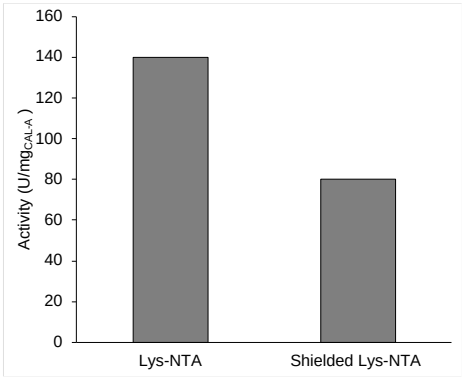
Anchoring of enzymes through their His-tag on silica particles and shielding



Lys-NTA grafting on silica nanoparticles through imine bond formation



A) Immobilization yield of a His-tag lipase anchored on silica particles carrying Lys-NTA motifs. B) Activity of a His-tag lipase anchored on particles with different strategies.



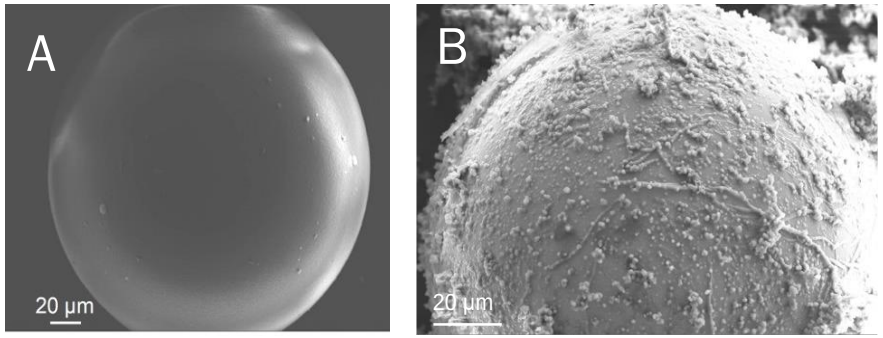
Activity of a His-tag lipase anchored on particles and shielded.

Shielding of enzymes immobilized on methacrylate resins

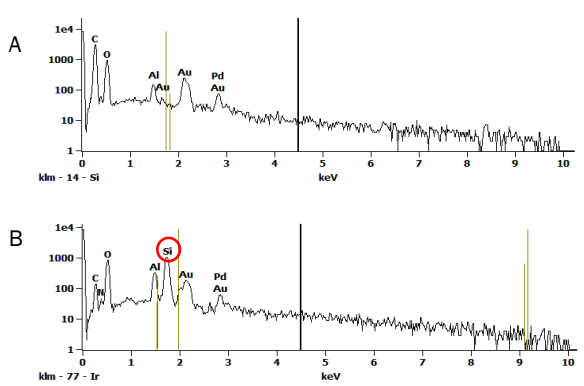
In order to enlarge the list of carriers available for different applications, the shielding strategy was optimized to favor the polycondensation of the organosilanes ((3-Aminopropyl)triethoxysilane and Tetraethyl orthosilicate) on non-silica-based carrier.

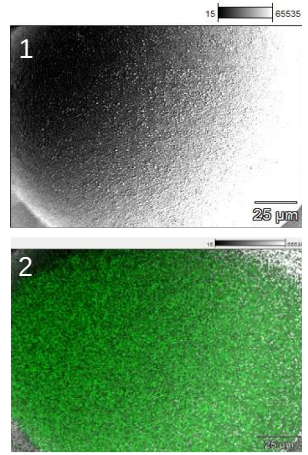
Commercially available Candida antarctica lipase A (Cal-A) was used as model enzyme.

Amino-modified methacrylated beads with diameter ranging 300 – 700 µm were selected.



SEM micrograph of bare methacrylate beads (A) and of methacrylate beads with Cal-A immobilized and shielded (B)





Energy dispersive X-ray spectroscopy analysis

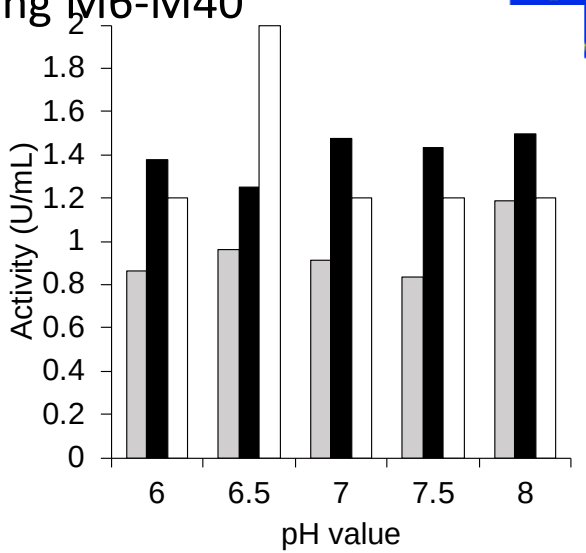
Task 5.4. Empowering enzymes by immobilization-guided supramolecular engineering M6-M40

Selected enzymes, immobilization and organosilica shielding

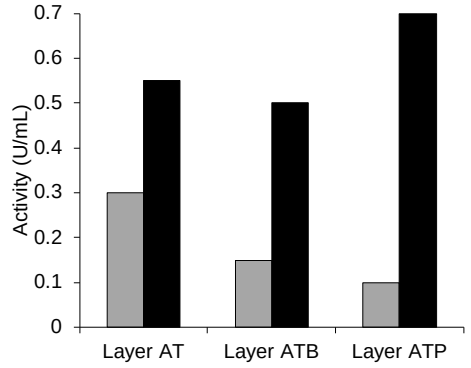
- *Candida antatica* lipase B (CalB), which finds application (among others) in textile pretreatment
- α-Amylase (Amplify 12L) for potential application in Laundry & Home Care (LHC) detergents
- FELip9 and FEPolur 1 (CSIC)

Study focus:

- Lipase immobilization/shielding efficiency has been show to strongly depend on:
  - Surface (chemical) composition of the carrier and reaction pH
  - Curring conditions*



Enzyme (CalB) activity after immobilization on silica particles with varying surface composition [amine: light grey, amine + aliphatic: black; amine + aromatic : white]

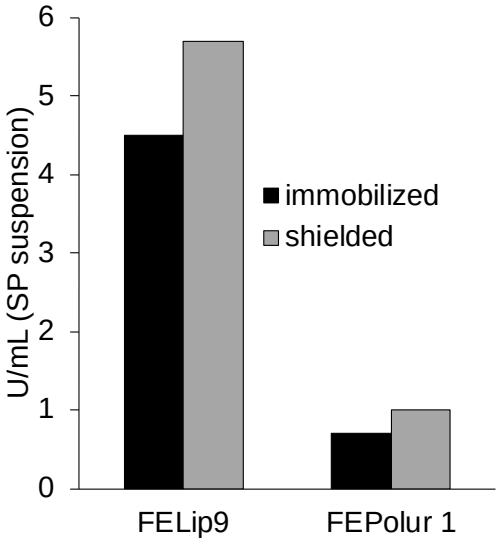


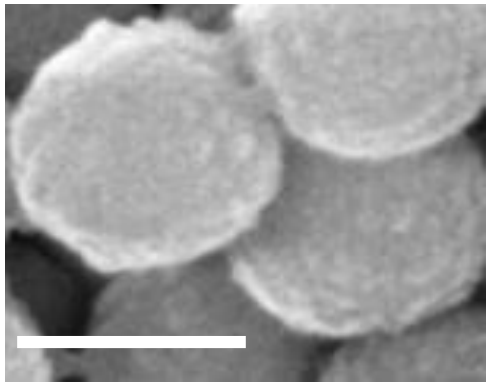
Enzyme activity after curing in buffer (grey) or organic solvent (black)

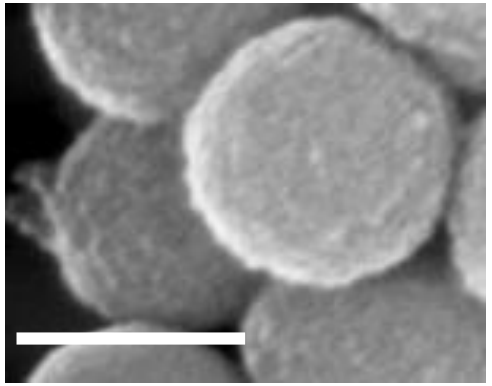
*We previously demonstrated that the organosilica layer undergoes a "curing" reaction at RT. This phase allows for layer stabilization and softenning, and enzyme activity recovery.

Immobilization and shielding on silica particles of FuturEnzyme-lipases

The lipases FELip9 and FEPolur 1 (CSIC) were immobilized on amino modified silica particles (SP, diameter 230 nm), and shielded with an organosilica layer. Glutaraldehyde was used as homo-bifunctional crosslinker.







The catalytic activities of FELip9 and FEPolur 1 were measured using using p-nitrophenyl butyrate (PNPB) as a substrate (**A**). The activity of the shielded enzymes were 21 % and 30% higher than the immobilized enzymes respectively. *SEM micrograph of SP with immobilized and shielded FELip9 (**B**) and FEPolur 1 (**C**); scale bars represent 200 nm*

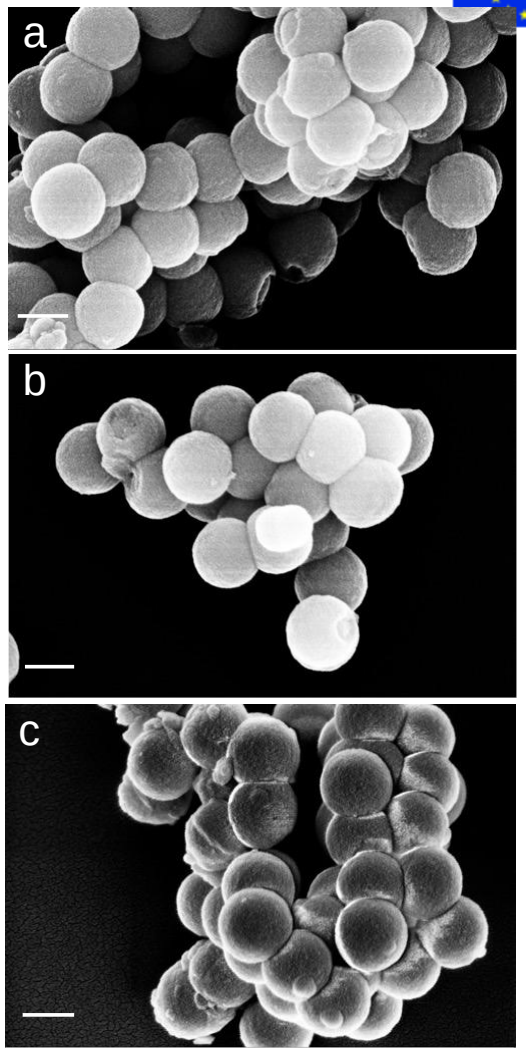
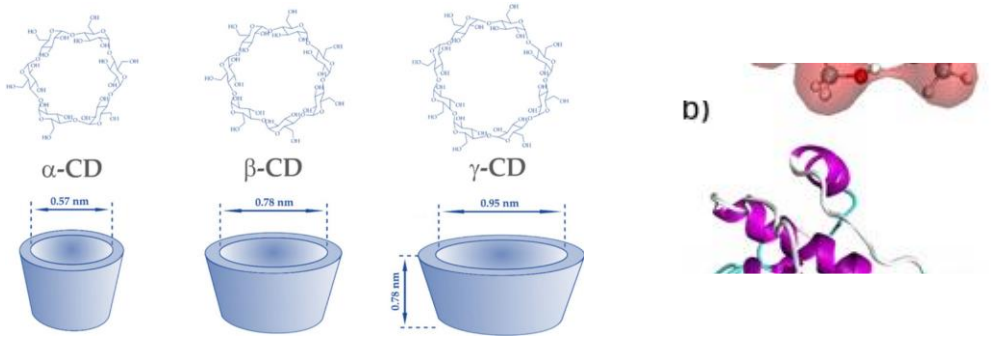


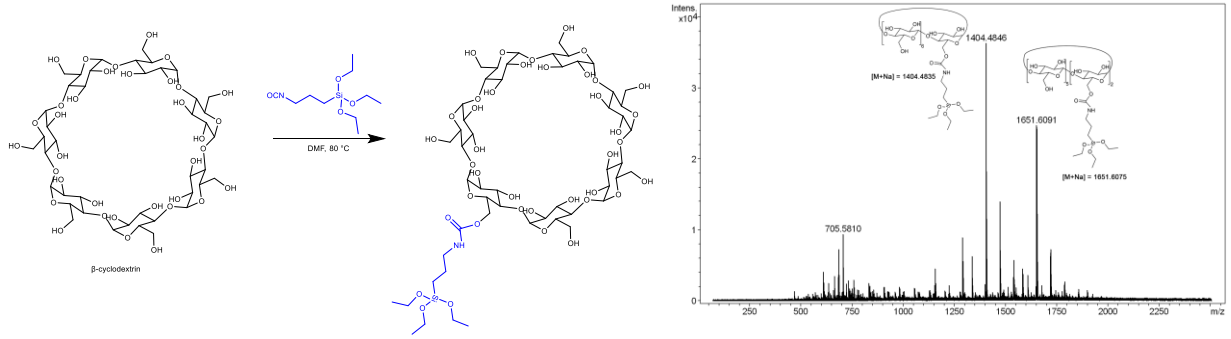
Figure: SEM micrographs of lipase-based nanobiocatalysts shielded with an organosilica layer of controlled thickness. All scale bars represent 200 nm

A new artificial building block mimicking natural protein chaperones

A new building block has been designed and synthesized for improving enzyme stability within organosilica protection shields.



Cyclodextrin are cyclic oligomers of glucose. Hydrophilic and water-soluble macrocycles, CDs display a hydrophobic cavity capable of forming inclusion complexes with aromatic amino-acids exposed at the surface of the immobilized enzyme.



Synthesis (left) and and MS characterization (right) of a cycldextrin bearing trialkoxysilane functions to be used in organosilica shield formation.

WP5 conclusions

- CSIC-BSC
  - Identify the “lid domain substitution” as efficient approach to engineer lipases
  - Stablish the PluriZymer module, which is available for designing PluriZymes and artificial Enzymes
  - Development of PluriZymes seems to be a robust procedure, with a nearly 90% success rate
  - Novel methodologies developed will allow faster and easier implementation of such engineering efforts
  - Multistate design offers new perspectives in specificity and other complex designs
  - Produce bout 200 mg of best two CSIC lipases for supramolecular engineering
- INOFEA-FHNW
  - Developed a method to shield enzymes in organosilica at the surface of polymeric nanoparticles, expanding the possibilities of the method
  - Developed a method (to be applied to best CSIC lipase enzymes) for the immobilization and organosilica shielding of enzymes using His-tag
  - A new artificial building block has been produced for enhancing enzyme stabilization effect within organosilica and will be tested for best-in-class lipases.
- UHAM
  - Generated and tested out several mutants of a PETase and one of them was more active at lower temperatures than the WT
- UDUS
  - Development of thermostable enzymes for textile applications
  - Candidate esterases with high solvent stability are available
  - Rational mutagenesis based on comparative literature analysis also improves activity on PET fibers