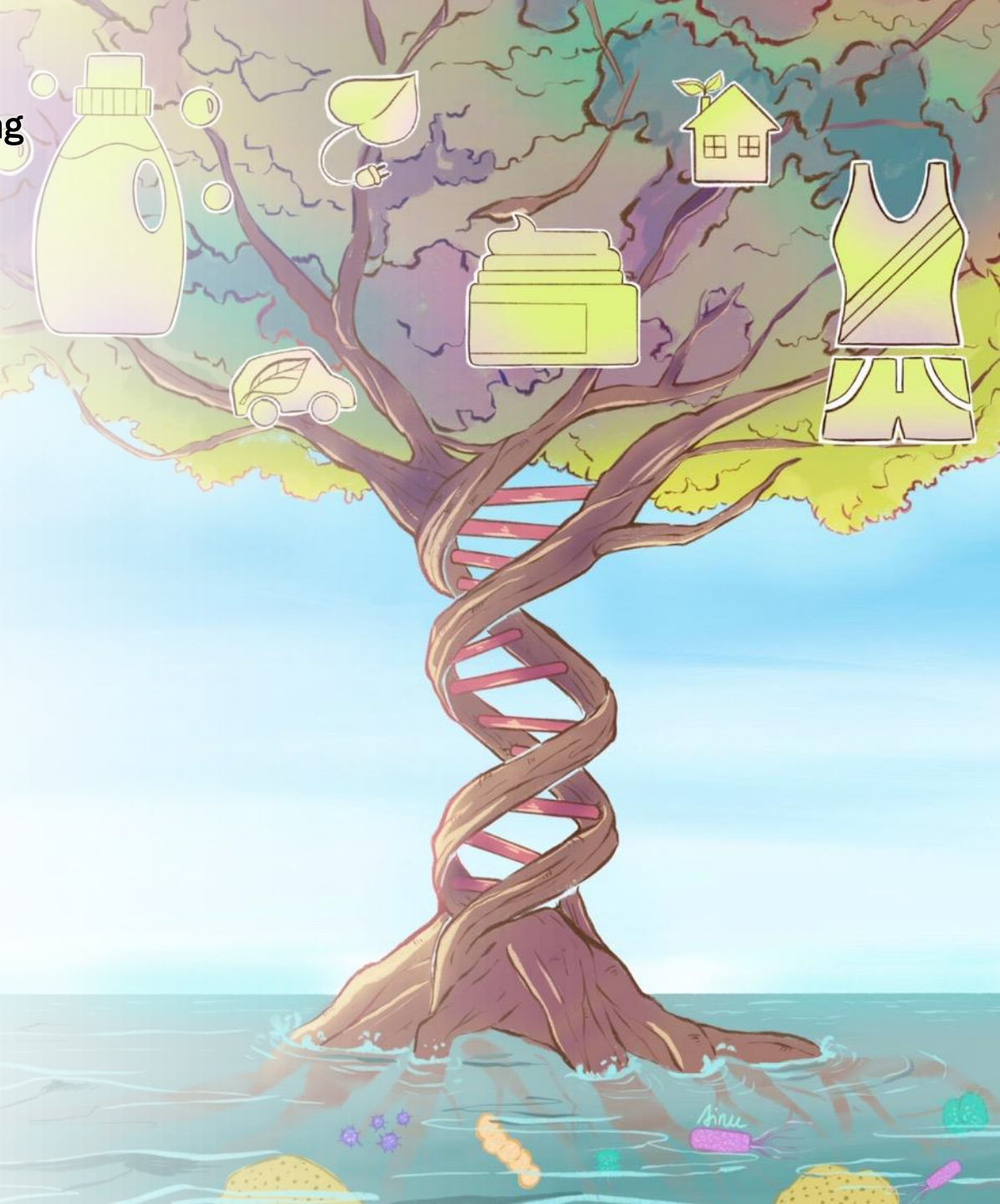
FuturEnzyme
Technologies of the FUTURe for low-cost ENZYMEs for environment-friendly products



FuturEnzyme: First Reporting Period
Start date: 1 June 2021 - End date: 31 May 2025
Proposal number: 101000327 - Consortium: 16 partners
Requested EU Contribution: 5,995,035.13 €

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

WP5 - Enhancing enzymes through innovative engineering

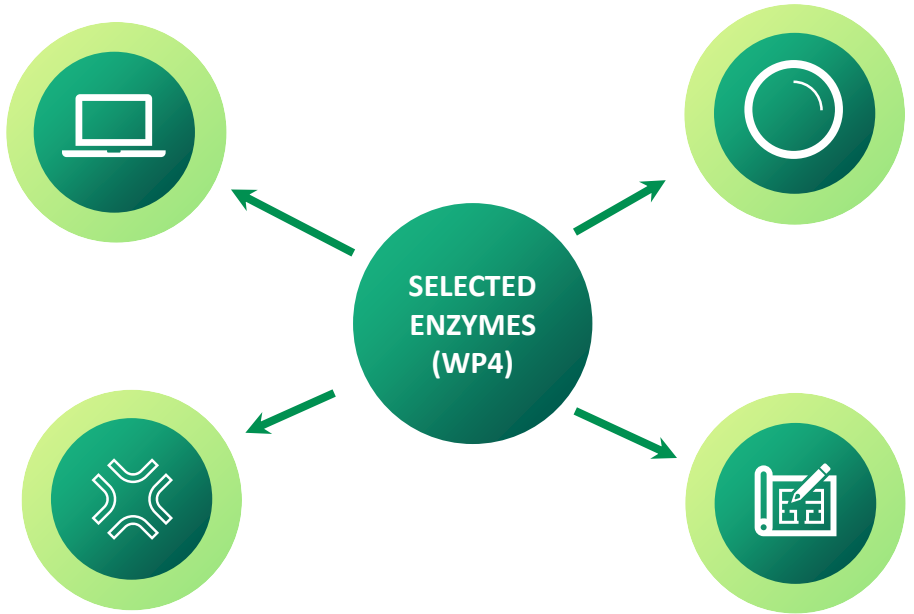
- OBJECTIVE: To **generate enhanced enzyme variants with real potential** for manufacturing the target products.

Task 5.1 Disruptive engineering computational tools

Lipase engineering for detergent and textile applications
Engineering FE_Lip5 by lid domain remodeling

Task 5.2 PluriZymes with multipurpose activities

In-One Protease-Esterase, Transaminase-Esterase and Xylanase-Esterase PluriZymes
Asite design
Fragaceatoxin

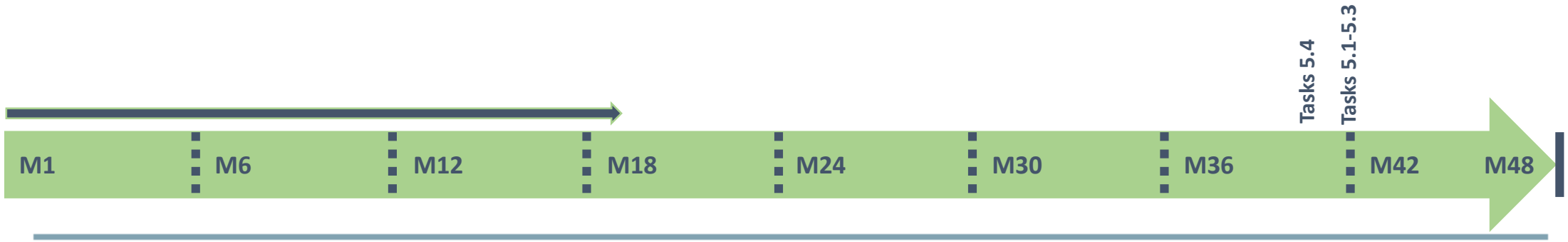


Task 5.4 Empowering enzymes by immobilization-guided supramolecular engineering

Supramolecular engineering using artificial chaperones
Expanding the range of supports
Improving immobilization-guided engineering for reduced production costs

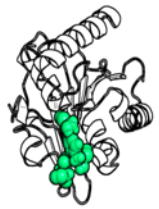
Task 5.3 Other advanced and classical methods

Lipase engineering
PETase engineering



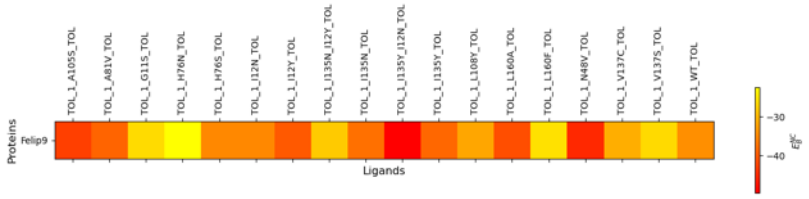
Task 5.1: Disruptive engineering computational tools

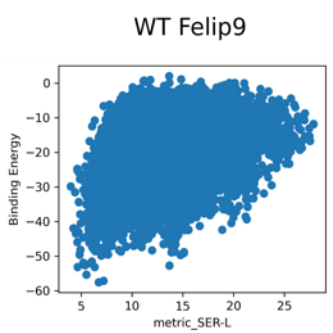
- Objective: The lipase Felip9 is very active against different long substrates and stains, but we want to increase the activity towards long triglycerides

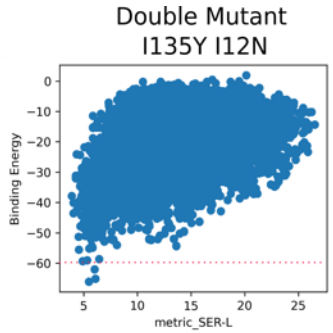


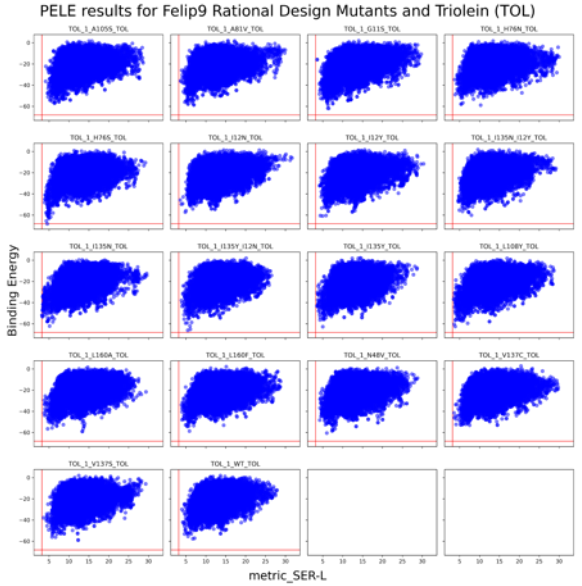
Native Binding Site

Input residues to mutate

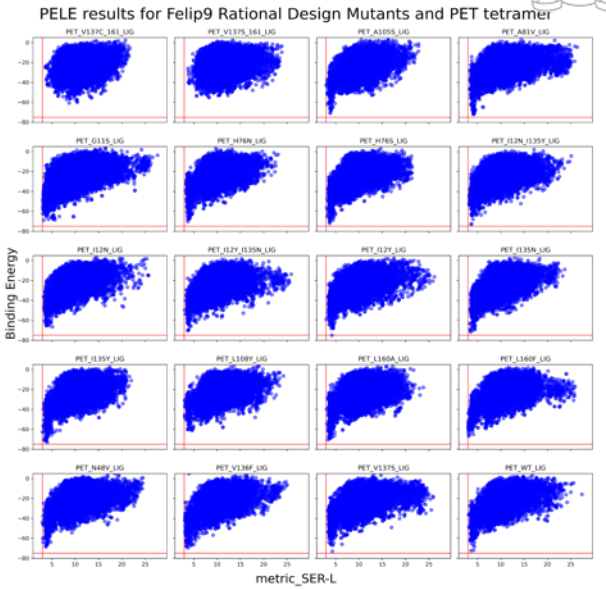








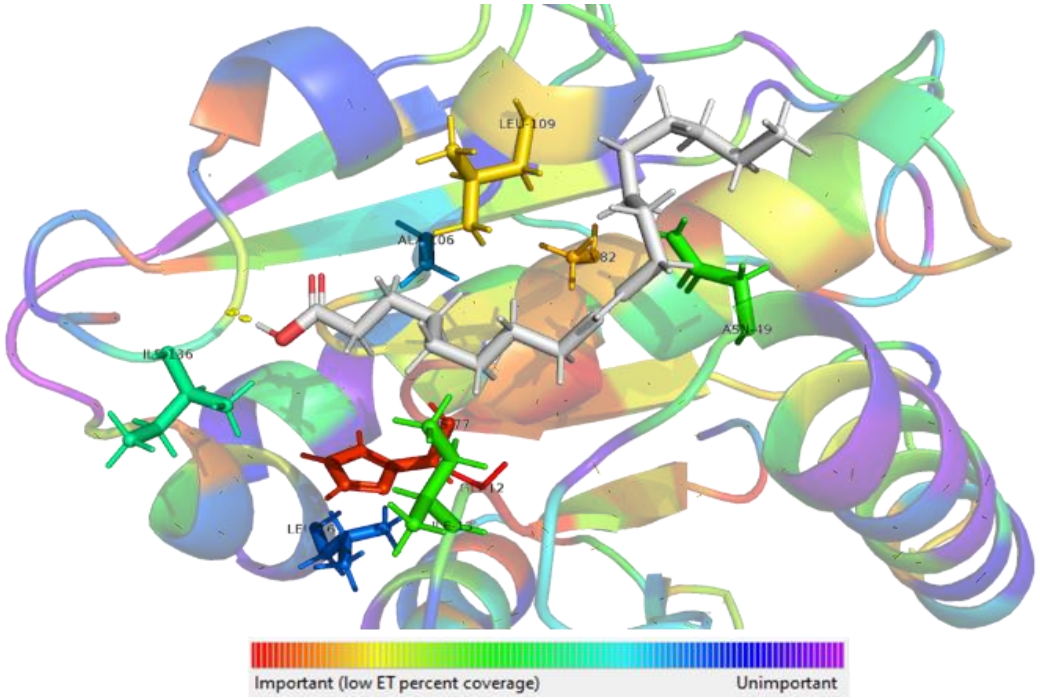
Multiplot of the energy profiles of the active site local exploration using triolein with PELE. Highlighted with a red line is the minimum catalytic distance serine-ligand. From all the simulations and the minimum binding energy or interaction energy (kcal/mol).



Multiplot of the energy profiles of the active site local exploration using BHET tetramer with PELE. Highlighted with a red line is the minimum catalytic distance serine-ligand. From all the simulations and the minimum binding energy or interaction energy (kcal/mol).

Task 5.1: Disruptive engineering computational tools

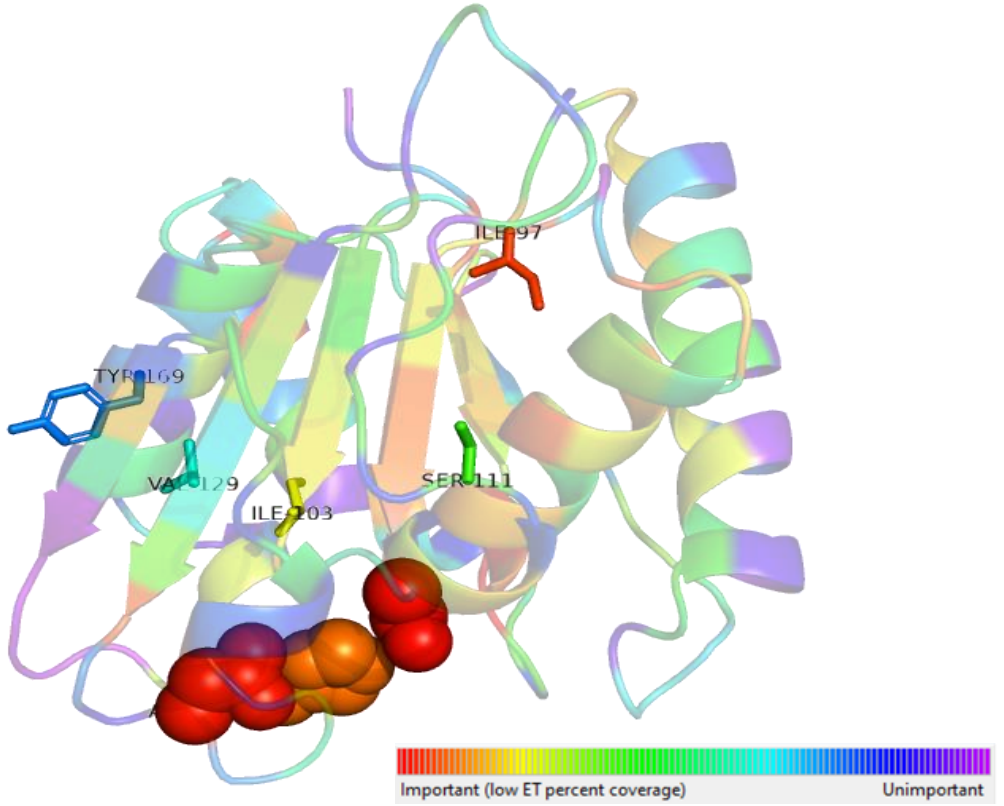
Residue	AA type	Coverage	variability
106	A	0.69444	.TMN**S**QLGDCIYAVP
136	I	0.54444	TEVILK.MAFQ
13	I	0.38333	VLI**N**QTF**Y**MW
109	L	0.17778	MLNF.REQA
82	A	0.15000	LPTNS**V**IAM
12	G	0.01667	GA
77	H	0.04444	HY**N**FLW
161	L	0.75000	VGIERPKMAT.LCNDS
49	N	0.40000	AINST**V**HLQRKM



(Left) table with selected positions, the coverage value obtained by evolutionary trace and the aminoacids present in the MSA of trace. Ana Robles suggested variants which are present in the variability found in multiple sequence alignment are in bold highlighted. (Right) Lip9 3D model colored by evolutionary trace coverage value (red-important, violet-less important), oleic acid (white) is positioned by swissdock, the positions suggested are shown as sticks. *Note: the numbering of residues in Ana Robles study differs 25 amino acids from the structure we currently use.*

Task 5.1: Disruptive engineering computational tools

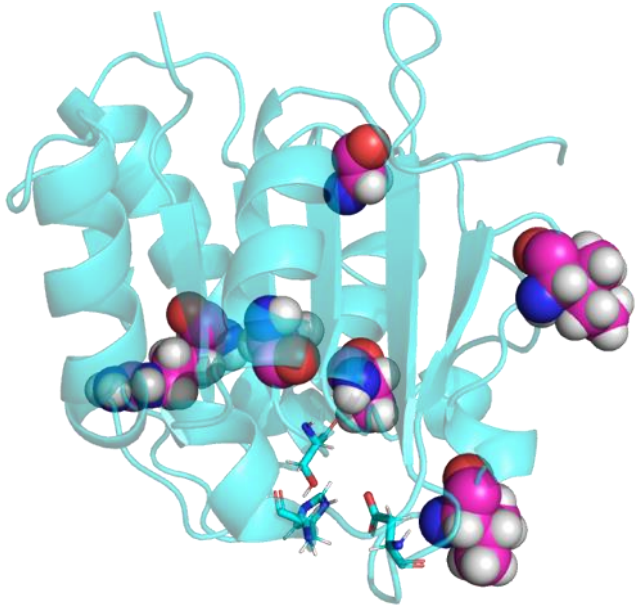
Position	Mutant suggested	Variability
152 Val	Trp/Asp	VLEIMFYW
134 Ser	His/Ala	VIACPG.LRTSYQ
126 Ile	Phe	VLIGTFM
120 Ile	Val	VIA
192 Tyr	Gln	GNYK.HAVTSILERQDM



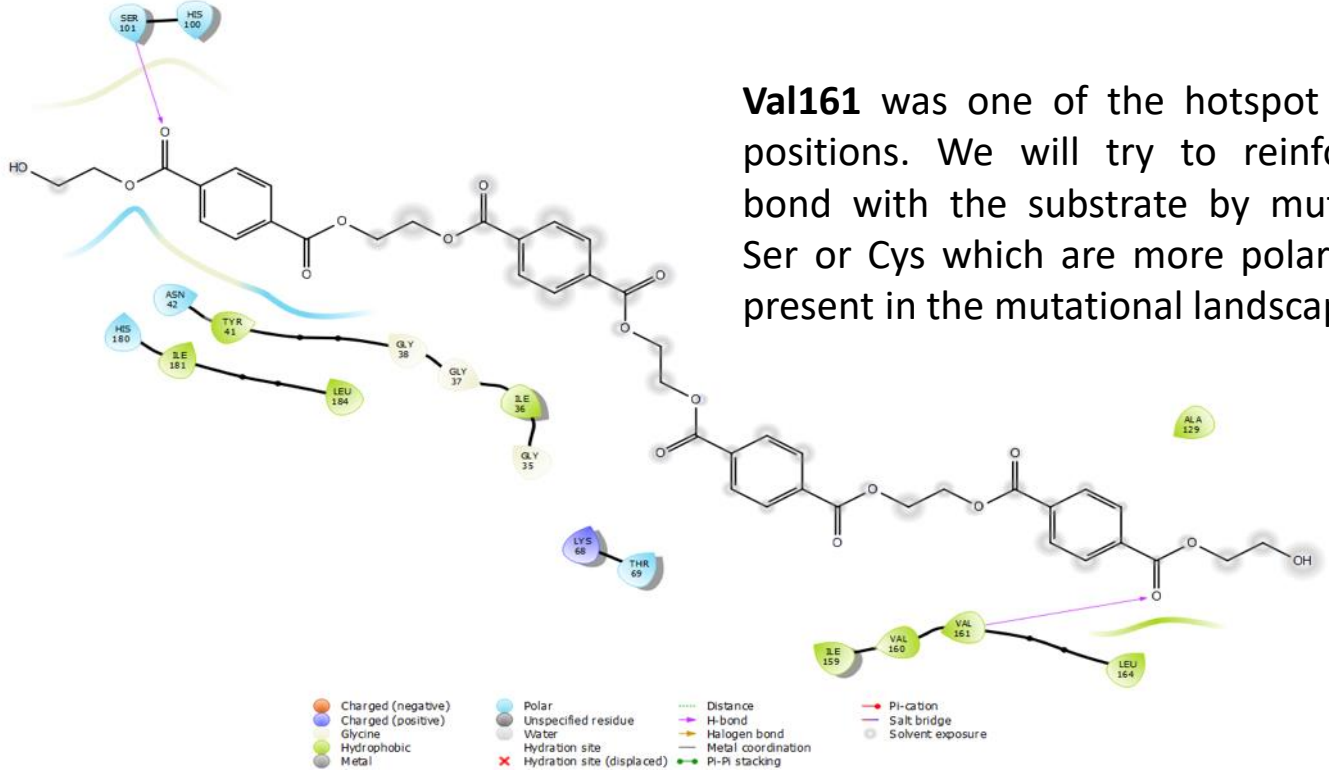
Thermostability hot spots found by sequence consensus (hotspot Wizard). (left) table with Lip 9 selected positions for rising thermostability, mutant variant suggested for each position and the variability of amino acids present in the multiple sequence alignment. (right) 3D model of Lip9 colored by evolutionary trace relevance, violet less important and red most important, catalytic triad is shown as balls and selected position for rising thermostability are shown as sticks.

Task 5.1: Disruptive engineering computational tools

- Lip9 functional hot spots found through hotspot wizard server (https://loschmidt.chemi.muni.cz/hotspotwizard/).
- Lip9-PET4 docking performed with Swissdock server (http://www.swissdock.ch/).



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



Val161 was one of the hotspot selected positions. We will try to reinforce the bond with the substrate by mutating to Ser or Cys which are more polar and are present in the mutational landscape.

Task 5.1: Disruptive engineering computational tools

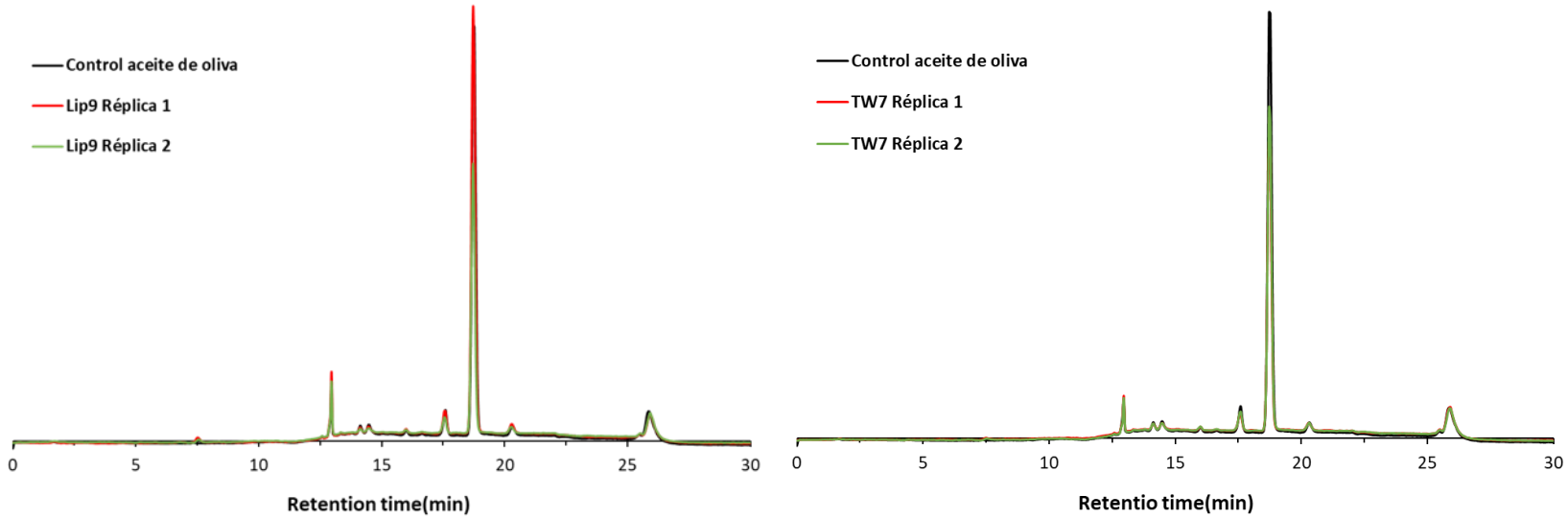
				Relative activity (%)							Degradation products (µM)	
Enzyme	Target	Solubility	Expression (mg/L)	Tri-C_8	Tri-C_{10}	Tri-C_{12}	Tri-C_{14}	Olive oil	Palm oil	Coconut oil	BHET	nPETc
Lip9 WT		Yes	1.0	100	100	100	100	100	100	100	2971±24	2238±40
Lip9 Val161Cys	Engineering PETase character	Yes	2.0	118.9	115.1	84.1	111.4	80.0	192.8	150.7	2880±13	1581±41
Lip9 Val161Ser	Engineering PETase character	Yes	3.0	123.9	98.1	91.7	102.3	168.9	204.8	179.2	3003±29	3342±63
Lip9 I36N-I159Y	Engineering lipase character	Yes	0.4	2.2	5.3	8.8	0.0	0.0	8.5	2.0	77±22	0
Lip9 H100S	Engineering lipase character	Yes	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	0
Lip9 A105F	Engineering lipase character	Yes	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	0
Lip9 I36Y	Engineering lipase character	Yes	1.5	7.9	30.4	21.2	14.4	0.0	30.7	21.8	0	0
Lip9 N72Y	Engineering lipase character	Yes	2,1	117.6	95.8	183.6	57.6	35.6	236.4	140.0	251±10	110±10
Lip9 A105V	Engineering lipase character	Yes	4.8	3.9	0.4	0.3	0.0	0.0	0.0	0.6	0	0
Lip9V152W	Thermostability	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lip9 V152E	Thermostability	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lip9 S134H	Thermostability	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lip9 S134A	Thermostability	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lip9 I126F	Thermostability	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lip9 I120V	Thermostability	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lip9 Y192Q	Thermostability	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Task 5.1: Disruptive engineering computational tools

HPLC chromatography olive oil

Reaction conditions: (24h) 30° 900 rpms

100 µl of HEPES 40mM, 200mM NaCl, pH 7
+
4 µl of olive oil half diluted in DMSO
+
2 µl of enzyme (1 mg/ml) (0,018mg/ml)

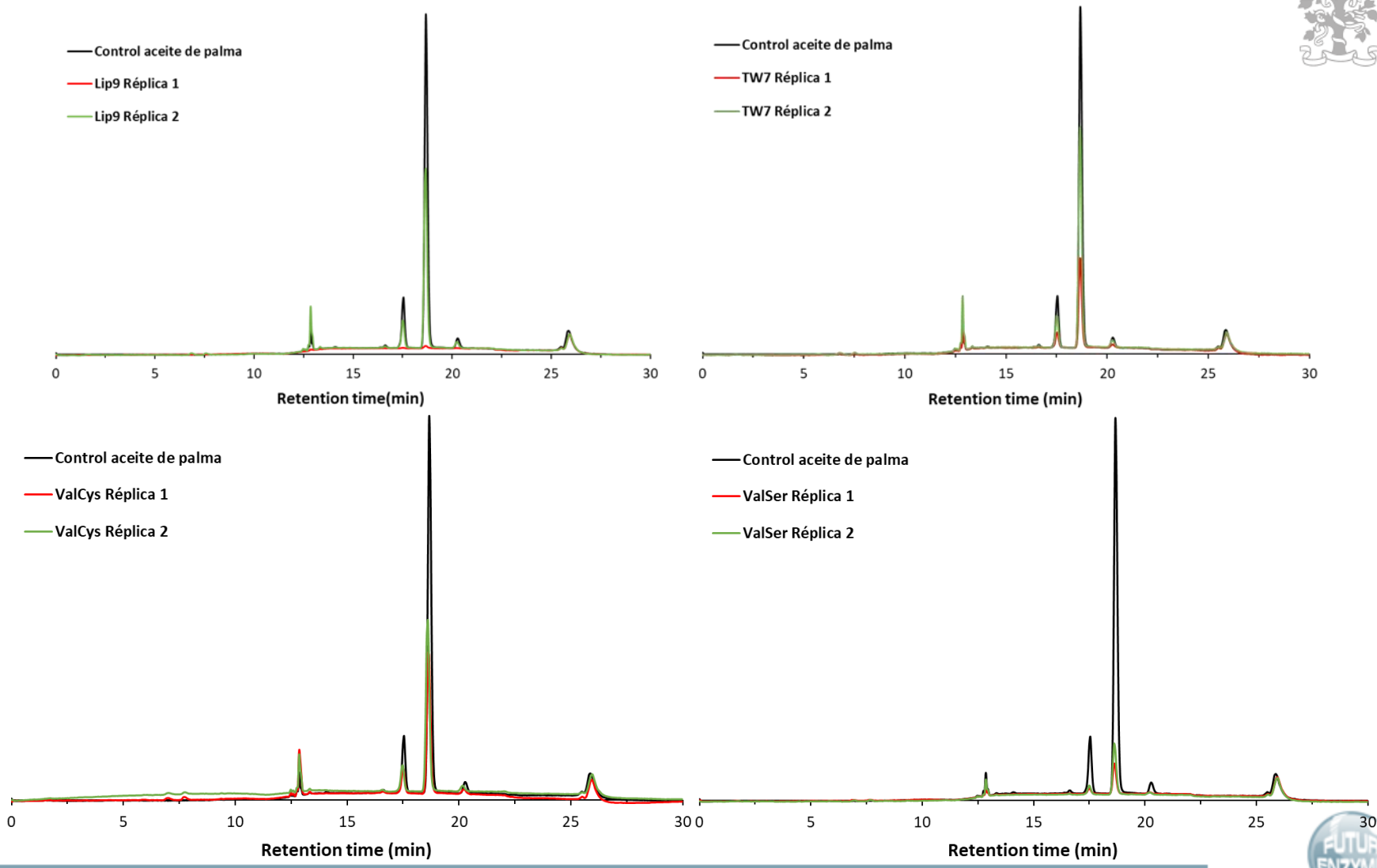


Task 5.1: Disruptive engineering computational tools

HPLC chromatography palm oil

Reaction conditions: (24h) 30° 900 rpms

100 µl of HEPES 40mM, 200mM NaCl, pH 7
+
4 µl of palm oil half diluted in DMSO
+
2 µl of enzyme (1 mg/ml) (0,018mg/ml)

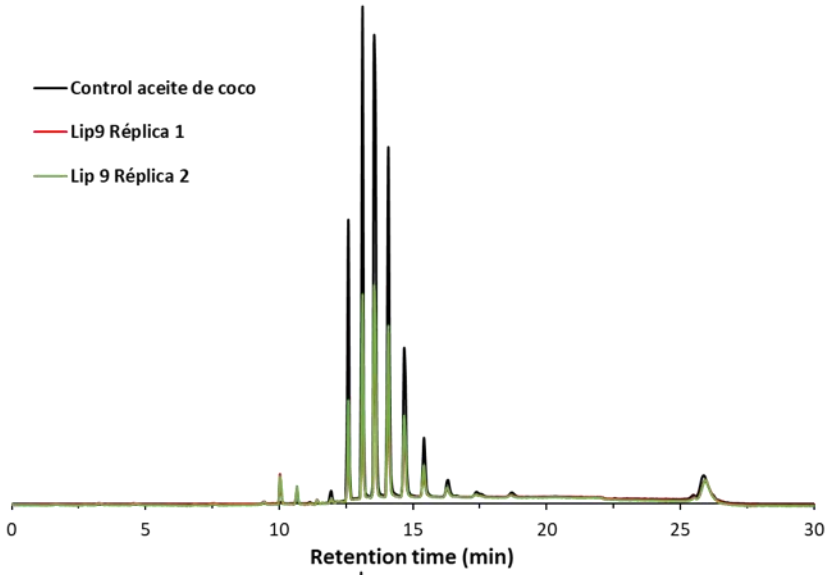


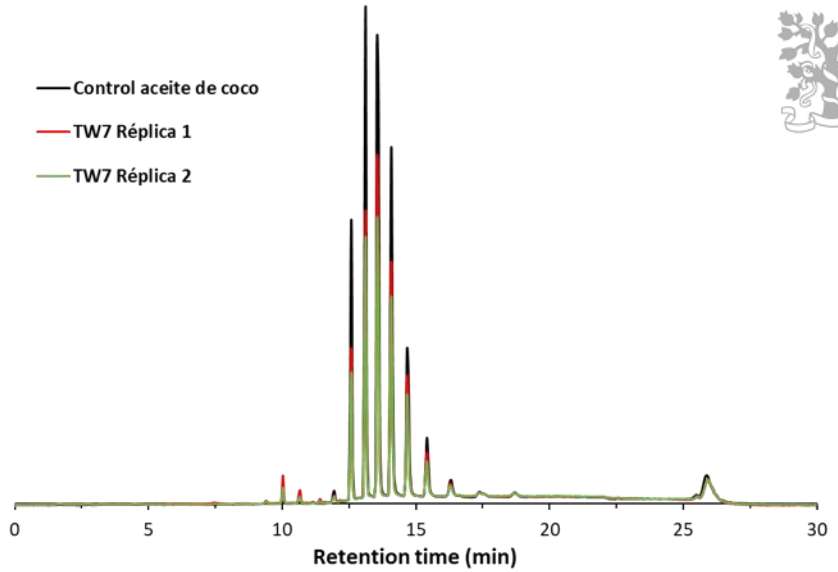
Task 5.1: Disruptive engineering computational tools

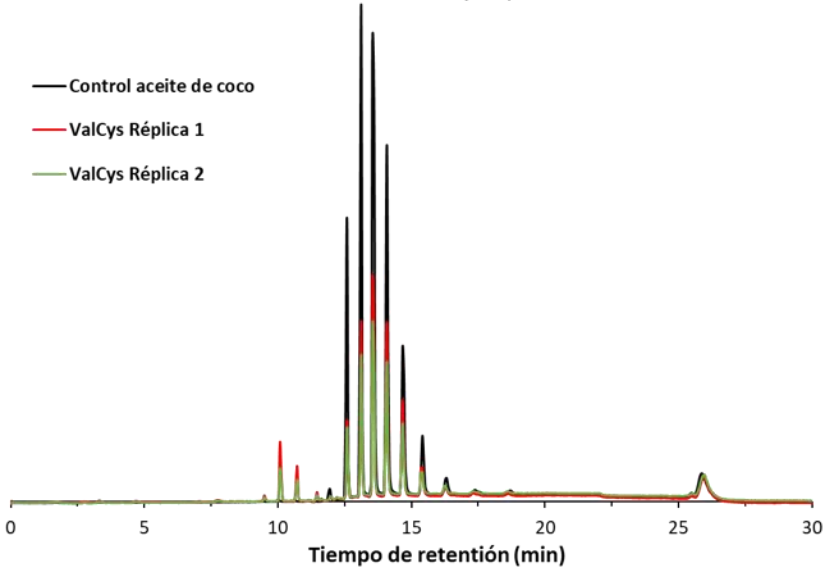
HPLC chromatography coconut oil

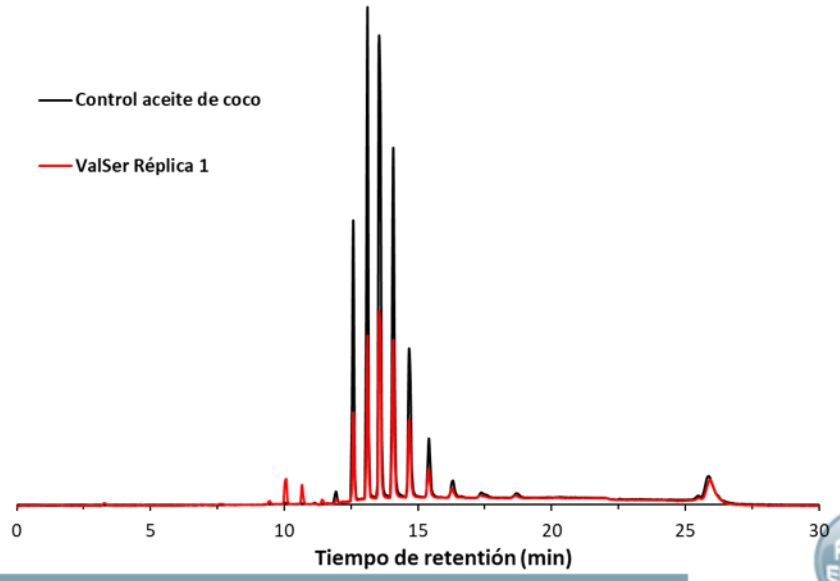
Reaction conditions: (24h) 30° 900 rpms

100 µl of HEPES 40mM, 200mM NaCl, pH 7
+
4 µl of coconut oil half diluted in DMSO
+
2 µl of enzyme (1 mg/ml) (0,018mg/ml)









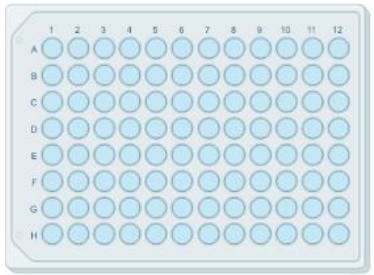
Task 5.1: Disruptive engineering computational tools

Free fatty acids assay (Stained clothes)

Pigment with oil on polyester/cotton PC-09	Mayonnaise on cotton C-S-05S	Lipstick, pink on polyester/cotton P-S-16	Fluid make up on cotton C-S-17	High discriminative sebum BEY on polyester/cotton PC-S-132	Beef fat on cotton C-S-61	Butterfat on cotton C-S-10

100 µl Washing liquor (2.5 g/L in water)
24 hours
30°C
950 rpm

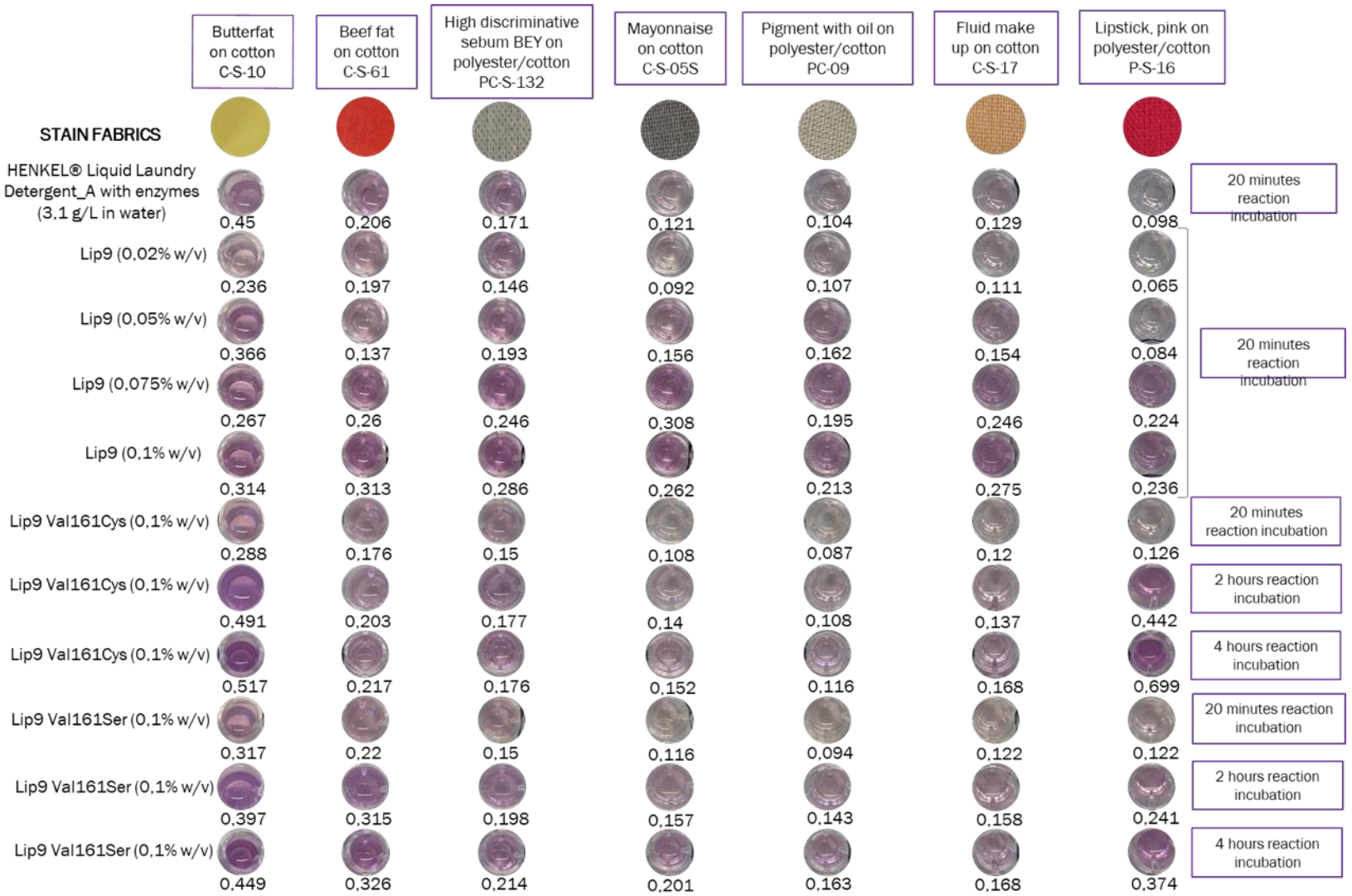
- Lip9 (0,02% - 0,1 w/v)
- Lip9 mutants (0,1% w/v)
- HENKEL® Liquid Laundry Detergent_A with enzymes (3,1 g/L in water)



NEFA –Kit of Wako

1. 10 µl "wash test" + 100 µl of R1a → 6 min, 37 °C
2. Add 50 µl of R2a → 6 min, 30 °C
3. Measure absorbance at 550 nm

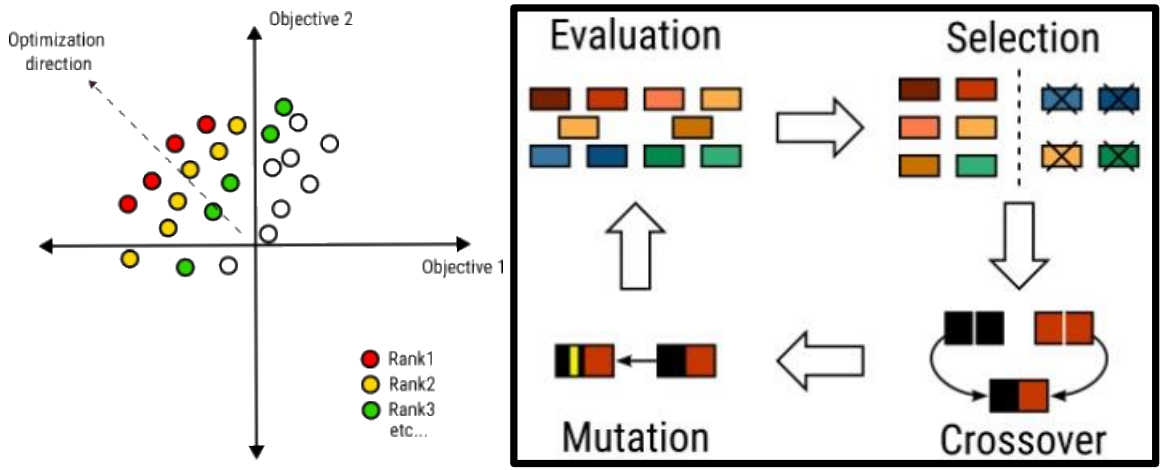
Task 5.1: Disruptive engineering computational tools



Task 5.1: Disruptive engineering computational tools

Mutagenesis Softwares

Best mutants from one round are parents for the next one, making use of a genetic algorithm.

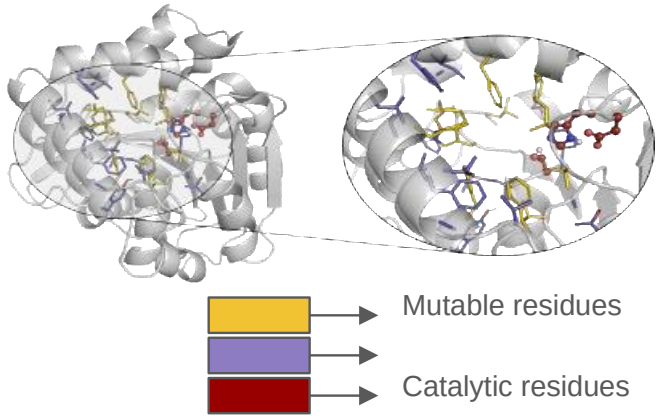


During the simulation, mutations are introduced and energies are evaluated.

AsiteDesign

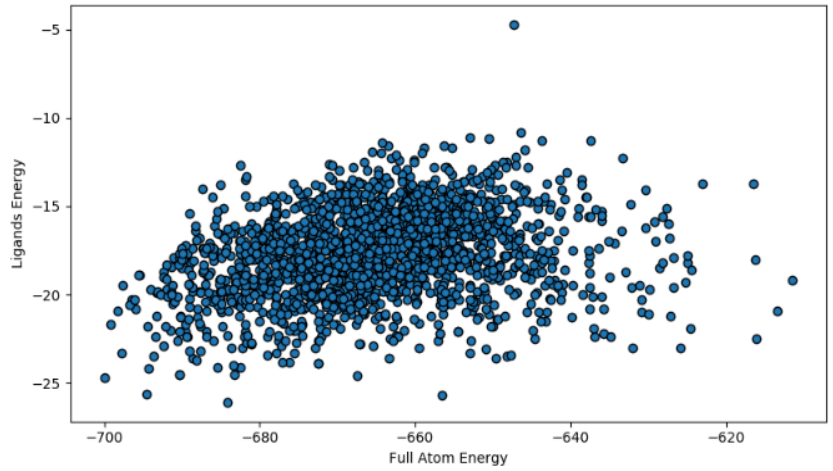
P E L E

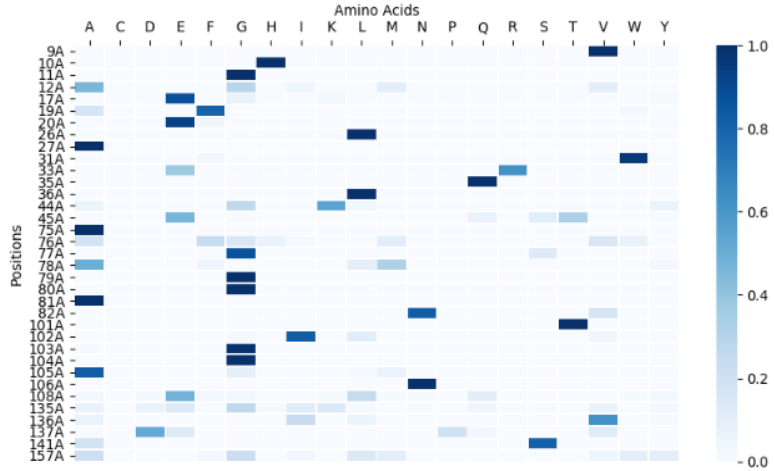
Rosetta Commons

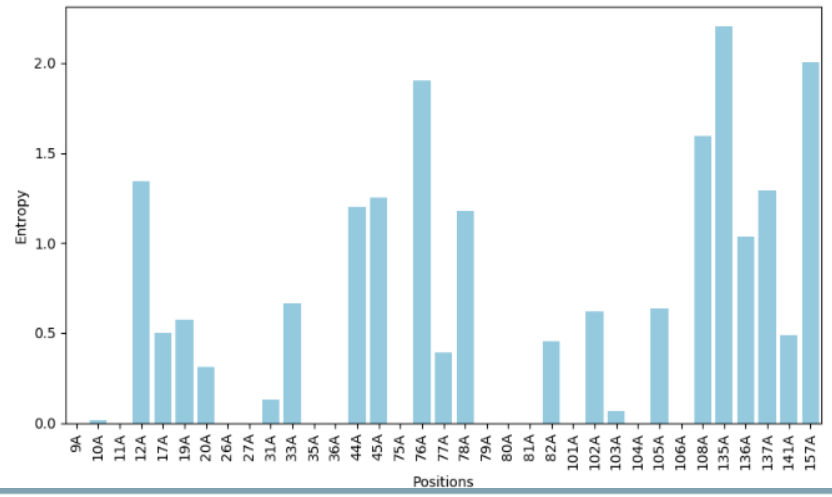


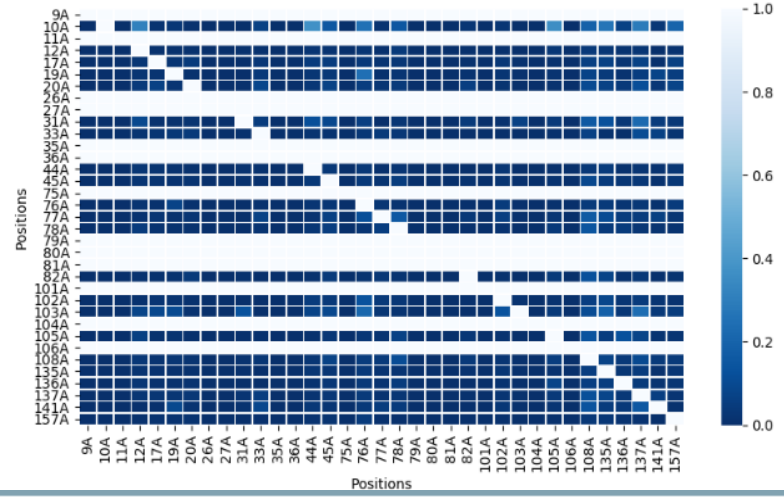
Task 5.1: Disruptive engineering computational tools

Enzyme engineering of Lip9 - AsiteDesign



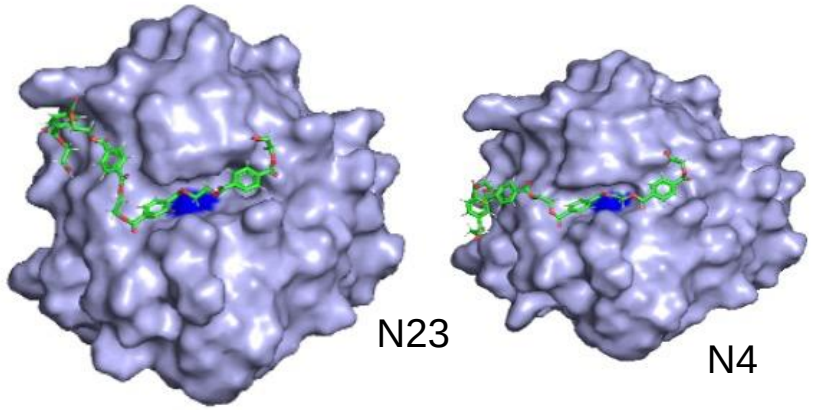






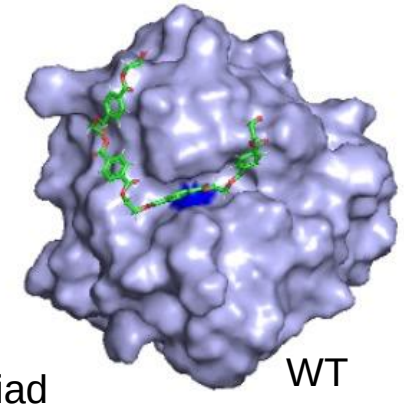
Task 5.1: Disruptive engineering computational tools

Enzyme engineering of Lip9 - AsiteDesign



N23

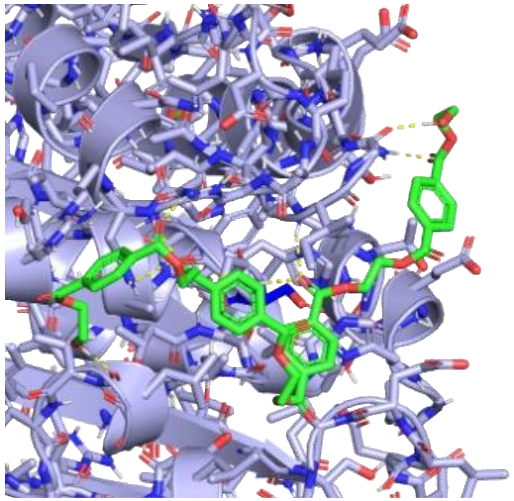
N4



WT

Additional Filters:

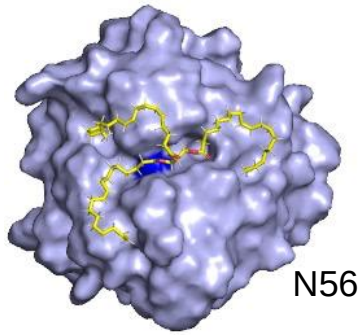
- By SER-L distance (minimum 4 A)
- By Conservation of the Catalytic Triad



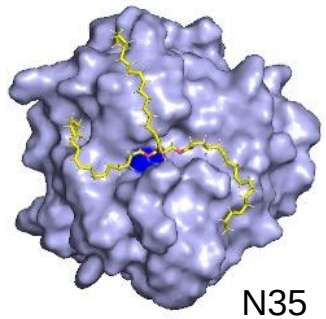
	Mutations	DnAtoms	# mutations	Energy (kcal/mol)	SER-L	HIS-ACID	SER-HIS	list_len
Felip9_PET2_I_N16_-676.7	[I_12_A, T_45_G]	-16	2	-676.7	2.979666	2.850549	2.830216	34.000000
Felip9_PET2_I_N23_-674.7	[I_12_A, T_45_G, H_76_A]	-23	3	-674.7	3.488373	2.782102	2.974250	30.666667
Felip9_PET2_I_N43_-670.9	[I_12_A, L_108_A, I_135_V]	-21	3	-670.9	2.879757	2.913635	2.846405	20.666667
Felip9_PET2_I_N48_-670.3	[T_45_A, H_76_A, M_78_A]	-18	3	-670.3	3.378494	2.992044	3.981659	19.666667
Felip9_PET2_I_N56_-668.7	[I_12_G, T_45_G, A_105_G, I_135_E]	-26	4	-668.7	3.034616	2.832980	3.254137	19.500000
Felip9_PET2_I_N58_-668.2	[I_12_G, T_45_G, A_105_G]	-22	3	-668.2	2.891762	2.875315	3.223516	22.666667
Felip9_PET2_I_N73_-664.3	[I_12_G, I_135_D, V_137_G]	-28	3	-664.3	3.316825	2.819818	3.642555	5.666667

Task 5.1: Disruptive engineering computational tools

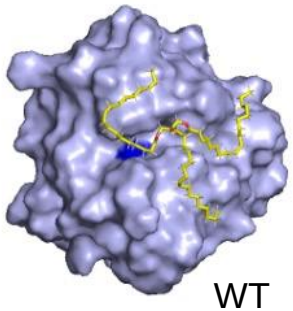
Enzyme engineering of Lip9 - AsiteDesign



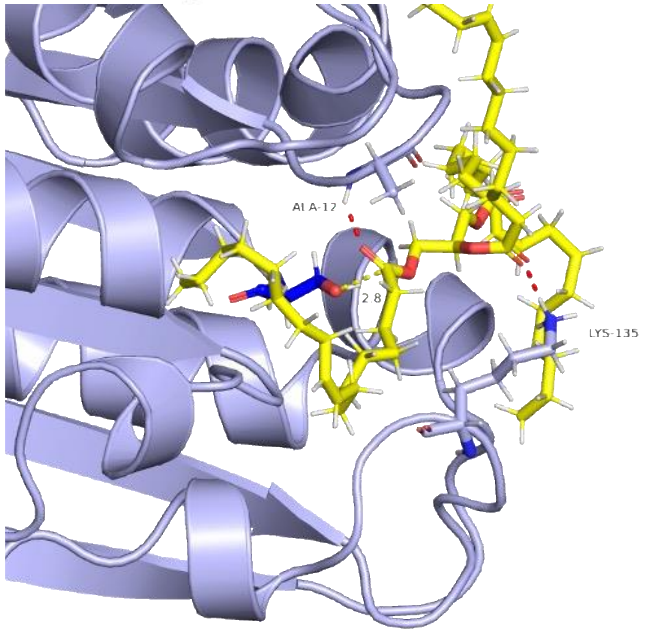
N56



N35



WT



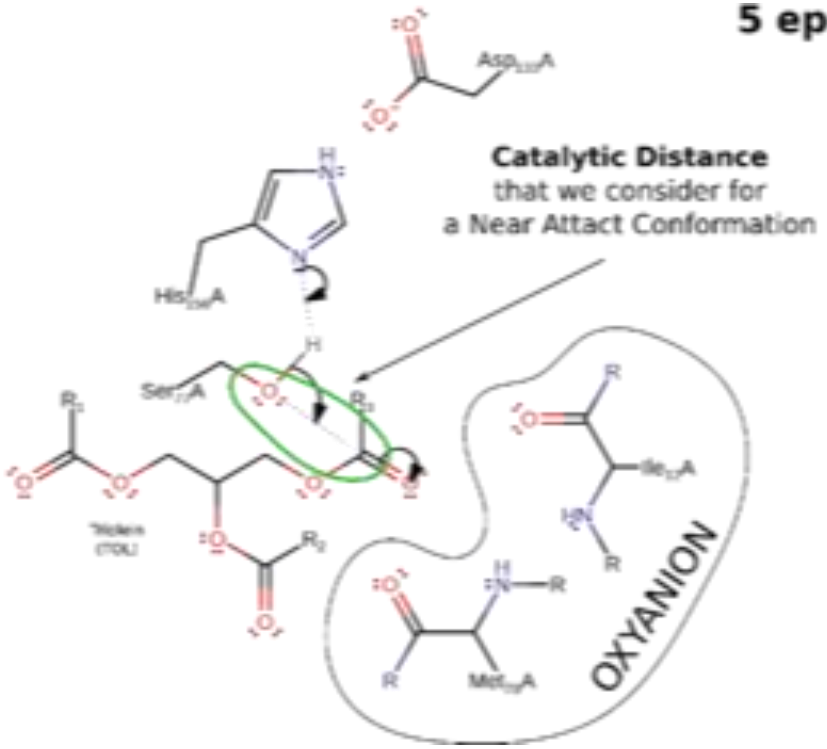
Additional Filters:

- By SER-L distance (minimum 4 A)
- By Conservation of the Catalytic Triad

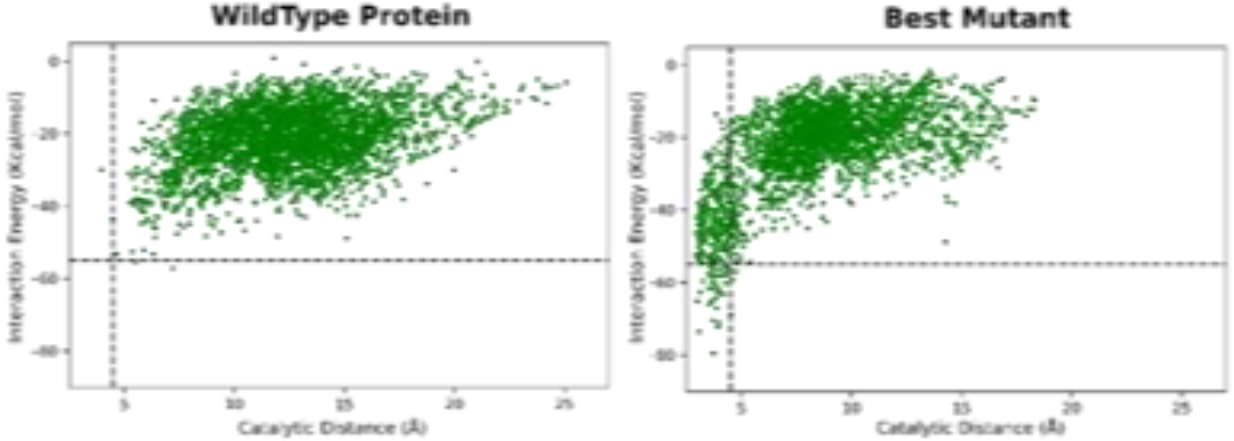
	Mutations	DnAtoms	# mutations	Energy (kcal/mol)	SER-L	HIS-ACID	SER-HIS	list_len
Felip9_optimization_I_N1_-680.5	[M_78_A]	-7	1	-680.5	3.656016	1.878617	3.768290	22.000000
Felip9_optimization_I_N4_-679.1	[I_12_M]	-2	1	-679.1	3.945704	1.848167	3.623794	5.000000
Felip9_optimization_I_N17_-672.9	[I_12_A, M_78_A]	-16	2	-672.9	3.345721	1.961689	3.920650	21.500000
Felip9_optimization_I_N25_-671.4	[I_12_V, Y_17_G]	-17	2	-671.4	3.762525	1.911687	3.707783	5.500000
Felip9_optimization_I_N34_-670.2	[I_12_A, F_19_A]	-19	2	-670.2	2.858579	1.821954	3.842225	17.000000
Felip9_optimization_I_N41_-669.2	[Y_17_G, I_135_K, I_157_G]	-23	3	-669.2	3.507518	1.854129	3.747090	9.333333
Felip9_optimization_I_N46_-668.7	[Y_17_G, I_157_G]	-26	2	-668.7	3.875139	1.888876	3.698335	9.000000
Felip9_optimization_I_N96_-660.9	[I_12_A, F_19_A, I_135_K]	-16	3	-660.9	2.757963	1.791450	3.516148	14.666667

Task 5.1: Disruptive engineering computational tools

Enzyme engineering of Lip9 - AsiteDesign



5 epochs of **100 PELEsteps** were performed using **32 CPUs** over **48 h**



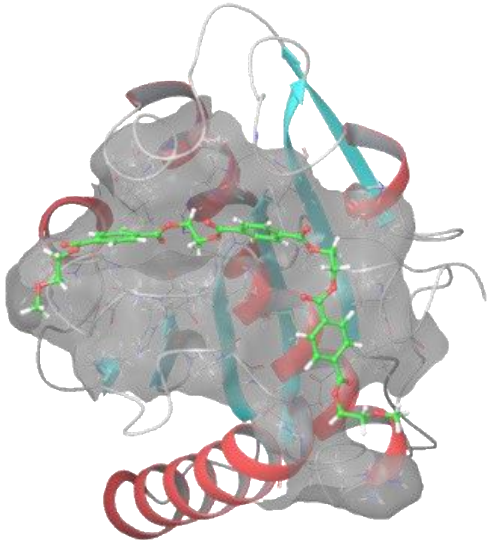
Four mutations were designed that decreased both the **interaction energy** and the **catalytic distance** (serine - substrate ester carbon).

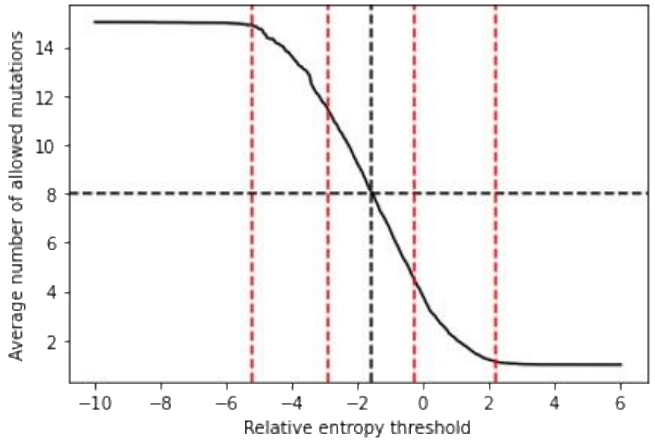
Task 5.1: Disruptive engineering computational tools

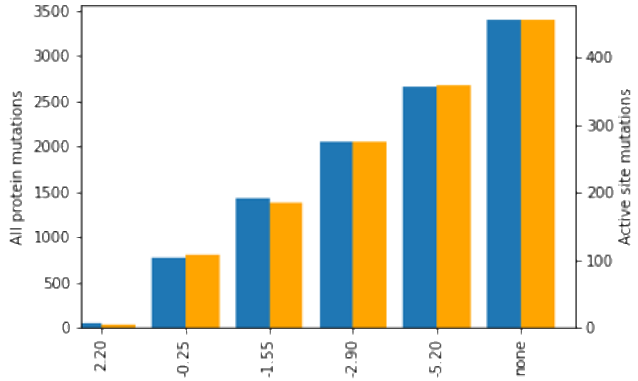
Enzyme engineering of Lip9

MultiOptimizationDesign input

Residues selection → MSA: restriction by entropic threshold → Final possible mutations

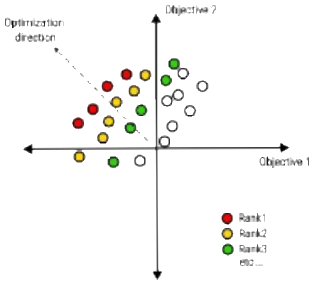






More restrictive restrictive ← Less

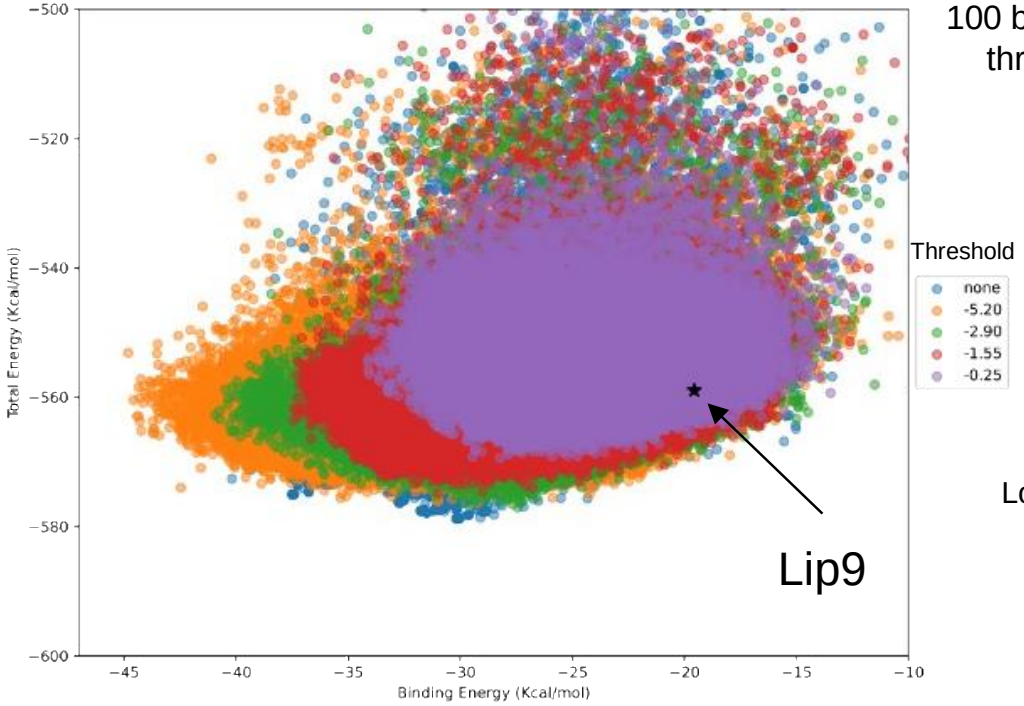
Task 5.1: Disruptive engineering computational tools



Enzyme engineering of Lip9

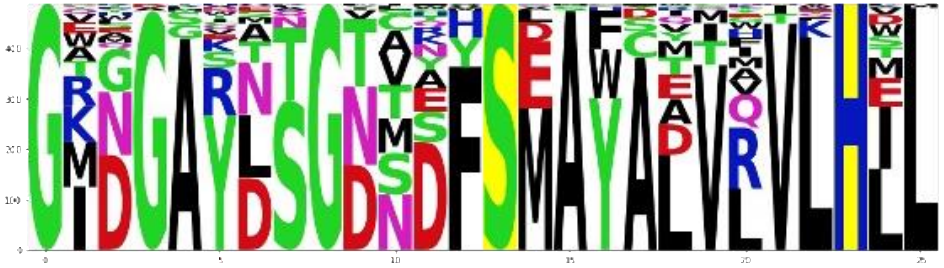
MultiOptimizationDesign Results

Energy vs Binding energy for all generated sequences



100 best mutants for each threshold is selected

Logo for the mutable residues of selected mutants (catalytic residues in yellow)



Task 5.1: Disruptive engineering computational tools

Enzyme engineering of Lip9

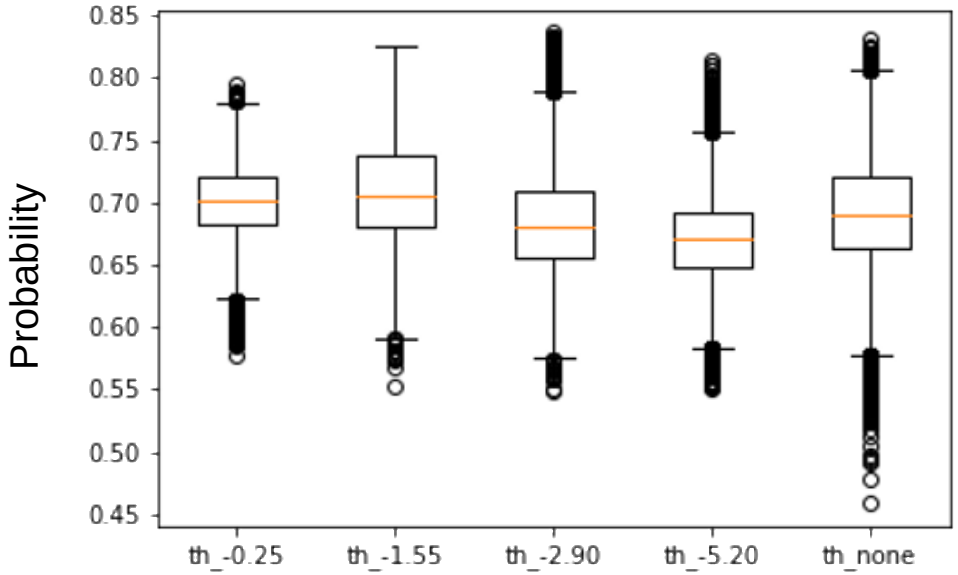
MultiOptimizationDesign Results

Expression Prediction: **SoluProt**

SoluProt is a Predictor of Expression trained with all the proteins in the PDB. It gives an estimate value of probability (between 0 and 1) of how likely will the protein be correctly expressed in **E. coli**.

Although it is the best predictor we have at the moment, it is just slightly better than randomness.

SoluProt Calculations for each threshold



More restrictive

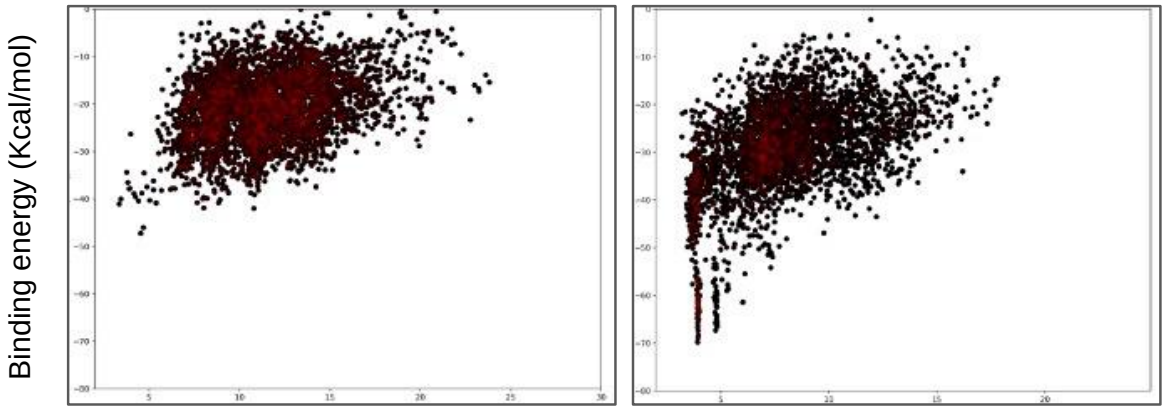
Less restrictive

Task 5.1: Disruptive engineering computational tools

Enzyme engineering of Lip9

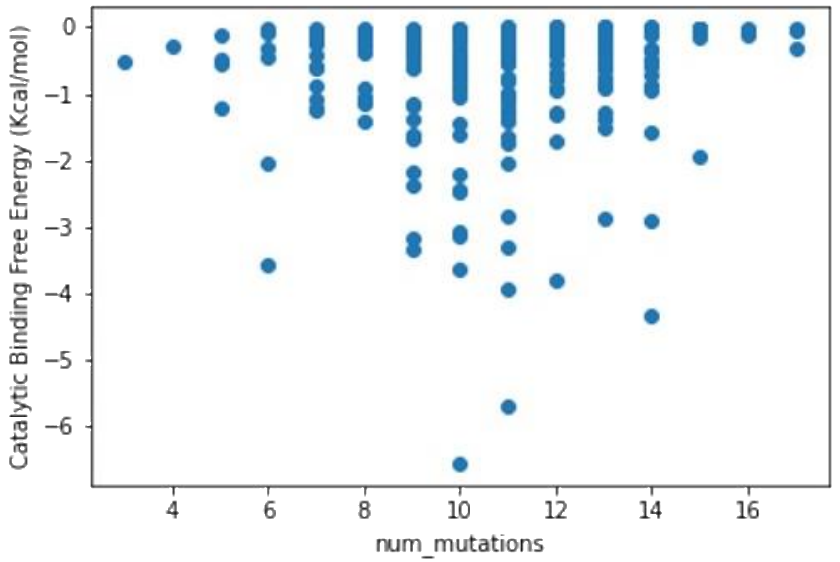
MultiOptimizationDesign **Preliminary** Results

Lip9 Best Mutant



Catalytic distance (serine to any of the target carbons of PET)

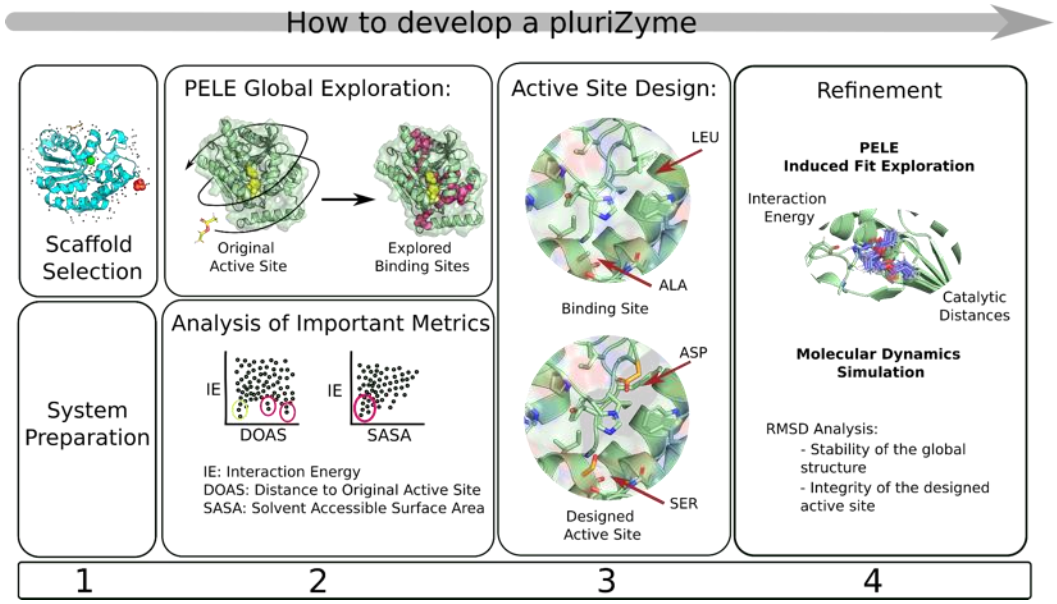
Catalytic Binding Free Energy vs number of mutations



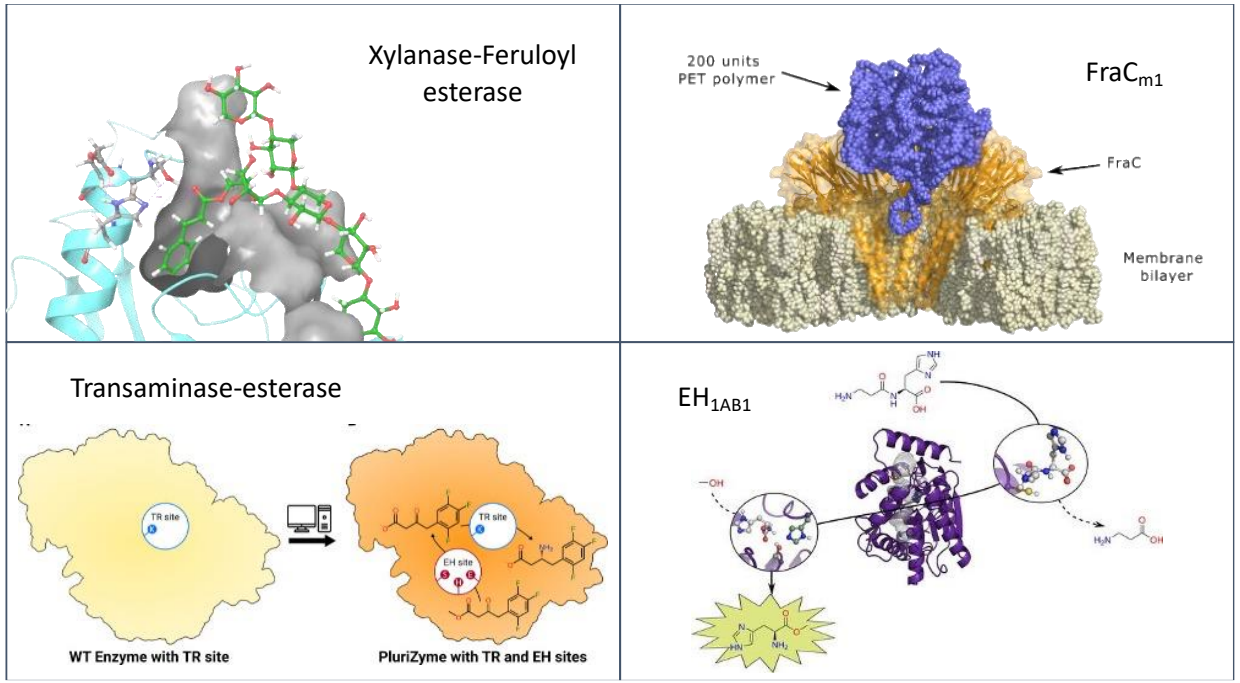
These are preliminary results, longer simulations are needed

Task 5.2: PluriZymes with multipurpose activities

- Objective
  - Engineered enzymes adding additional active sites: design of PluriZymes.



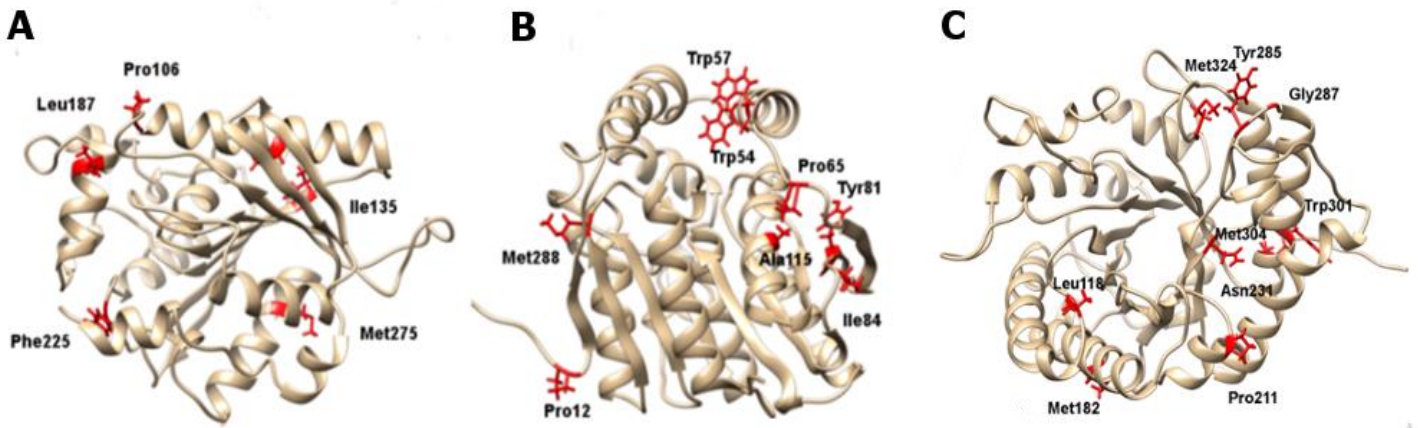
Multipurpose pluriZymes



Task 5.3: Other advanced and classical methods

- Objective
  - Engineered enzymes using synthetically based mutation and classical semi-rational engineering methods.
  - Selected targets:1 lipase (Kest3), 1 esterase (Gen0105), 1 cellulase (Gen0095),2 ancestral reconstructed sequences and 1 FireProt energy minimization mutant were generated for each sequence

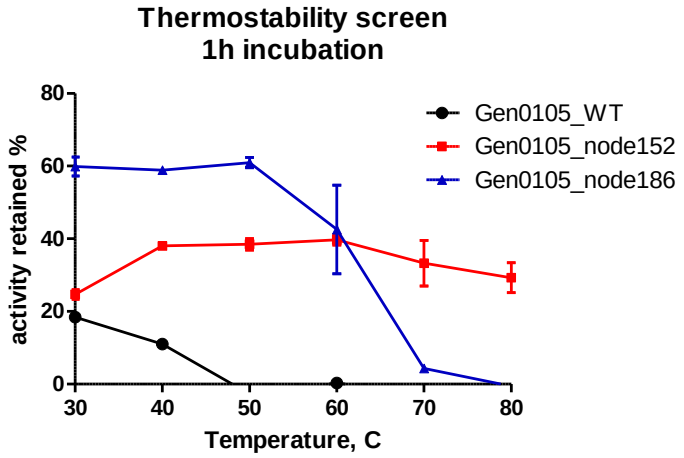
FireProt: energy minimization mutagenesis

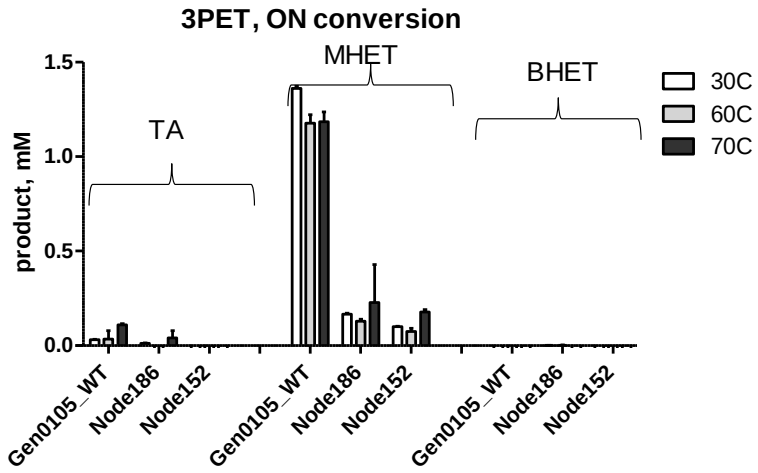


Mutant	Activity	Stabilization method	Soluble
Gen0105_EMF	PETase	Energy minimization	-
Gen0105_node152	PETase	Ancestral reconstruction	+
Gen0105_node186	PETase	Ancestral reconstruction	+
Kest3_EMF	Lipase	Energy minimization	+
Kest3_node58	Lipase	Ancestral reconstruction	-
Kest3_node88	Lipase	Ancestral reconstruction	-
Gen0095_EMF	cellulase	Energy minimization	-
Gen0095_node215	cellulase	Ancestral reconstruction	-
Gen0095_node276	cellulase	Ancestral reconstruction	+

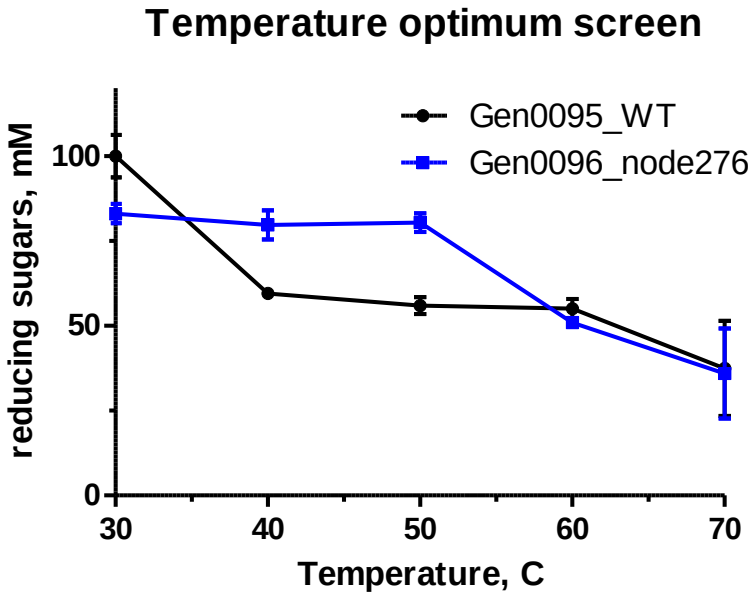
Selection of mutants in Kest3, Gen0105 and Gen0095. 3D structure of Kest3 (A) and Gen0105 (B) with mutated residues (FireProt) highlighted in red. (C) 3D structure of Gen0095. The mutated residues (Energy optimization, FireProt) are highlighted in red. Experimental validation in progress.

Task 5.3: Other advanced and classical methods



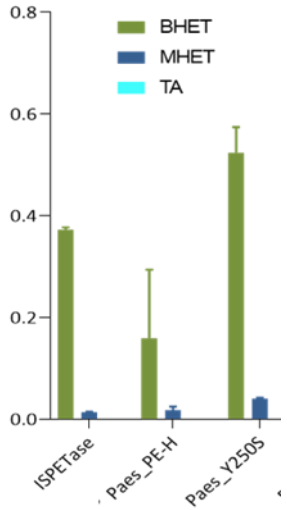


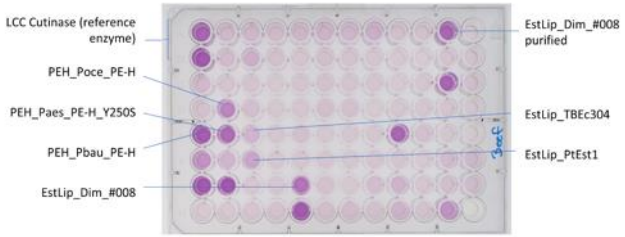
Protein	pNP -Substrate	V_m [umol/min/mg]	K_m [mM]	K_{cat} $[s^{-1}]$	K_{cat}/K_m $[s^{-1}mM^{-1}]$
Gen0105	pNP-hexanoate (C6)	11.6±1.1	0.9±0.2	6.9	7.2
	pNP-octanoate (C8)	12.8±0.6	0.1±0.02	7.7	65.0
	pNP-dodecanoate (C12)	1.6±0.2	1.3±0.4	0.9	0.7
Gen0105_Node186	pNP-hexanoate (C6)	14.8±2.8	0.55±0.28	8.8	**15.9**
	pNP-octanoate (C8)	19.8±1.9	0.51±0.13	11.7	23.0
	pNP-dodecanoate (C12)	0.6±0.1	0.08±0.06	0.4	4.4
Gen0105_Node152	pNP-hexanoate (C6)	1.4±0.3	0.7±0.4	0.8	1.1
	pNP-octanoate (C8)	0.6±0.06	0.5±0.2	0.4	0.7
	pNP-dodecanoate (C12)	N/A	N/A	N/A	N/A



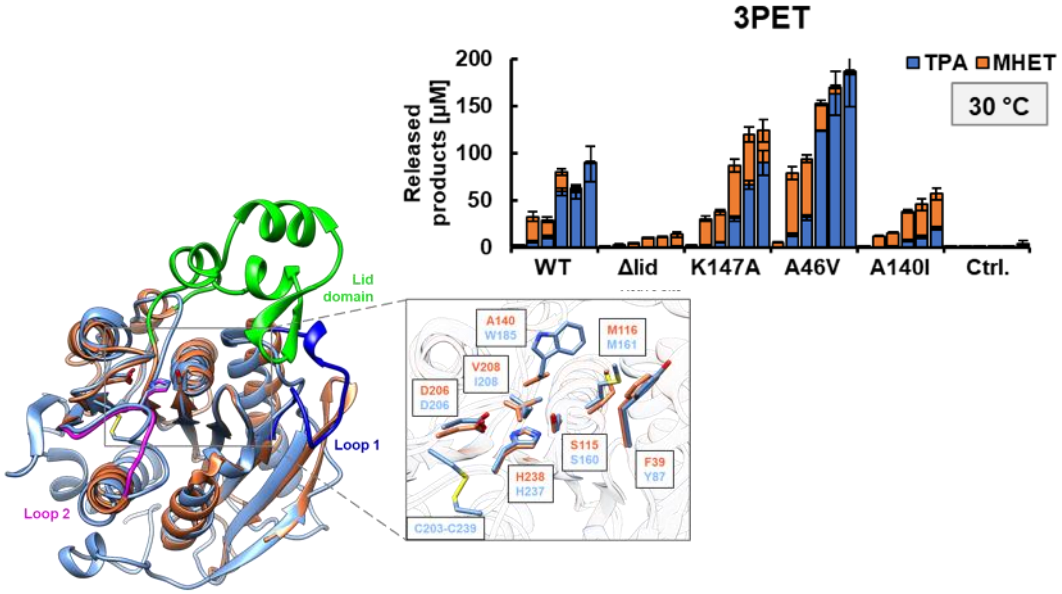
Task 5.3: Other advanced and classical methods

- Objective
  - Engineered enzymes using synthetically based mutation and classical semi-rational engineering methods.
- Progress undertaken and outputs achieved
  - A set of 2 PET hydrolases were engineered through docking and literature-informed mutagenesis





PEH_Paes_PE-H engineering. A single mutation increase by **2.5x the capacity to degrade a PET-based sample textile** 4-b 3X58 (VORB, 100% PES 100g/m^2) pretreated by alkaline boiling, and **4.4x the degradation of lipstick stain**.



PET46 uses the lid domain to effectively degrade MHET, BHET and 3PET. The A46V mutant was **3.17-fold better** than the WT enzyme to degrade 3PET.

Task 5.3: Other advanced and classical methods

EstLip_Dim_#008:
- The only true lipase in our assays.
- metagenome-derived
- An identical enzyme was isolated by others AZ –lipase
- lipase activity and thermostability

EstLip_PtEst1:
- Metagenome-derived
- identical to a database protein sequence of *Pseudonocardia thermophila.*
- high activity in washing liquor, high substrate promiscuity ,moderate thermostability.
- crystal structure published.

EstLip_Paes_TB035:
- from *Halopseudomonas aestusnigri*
- soluble expression after signal peptide exchange
- remarkable resistance towards denaturing agents, including surfactants
- highly substrate-promiscuous (but no lipase)
- structure reveals an uncommon topology with two cap domains that may be utilized for plurizyme construction.

EstLip_TBEc304´:
- Metagenome-derived
- thermostable and high activity at in washing liquor,
- high activity on fatty standard stains and therefore suggested for further exploration.
- high substrate promiscuity including the PET monomer BHET.

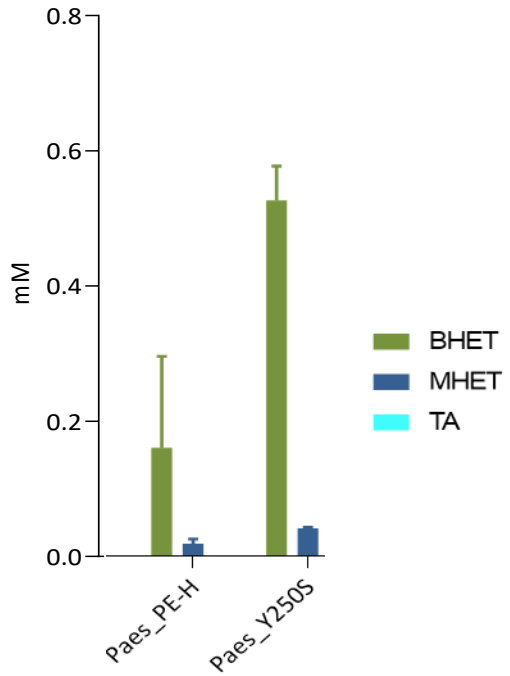
PEH_Pbau_PE-H, PEH_Poce_PE-H, PEH_Paes_PE-H_Y250S:
- homologous enzymes from *Halopseudomonas* sp. (75-90% sequence identities)
- activity towards PET fabrics (Figure 4) and with standardized stains
- activity in washing liquor
- three crystal structures available

ID	Enzyme[1]	Priority	SP[2]	Application
5	EstLip_Dim_#008 (lipase)	Yes	No	Detergent, textile
6	EstLip_Paes_TB035 (lipase)	Yes	Yes	Detergent
7	EstLip_PtEst1 (lipase)	Yes	No	Detergent
8	EstLip_TBEc304 (lipase)	No	No	Detergent
9	PEH_Paes_PE-H_Y250S (PETase)	Yes	Yes	Detergent, textile
10	PEH_Pbau_PE-H (Lipase, PETase)	No	Yes	Detergent, textile
12	PEH_Poce_PE-H (Lipase, PETase)	No	Yes	Detergent, textile

Task 5.3: Other advanced and classical methods

Literature informed mutagenesis of Paes _PE-H to improve polymer conversion:

Application: Textiles



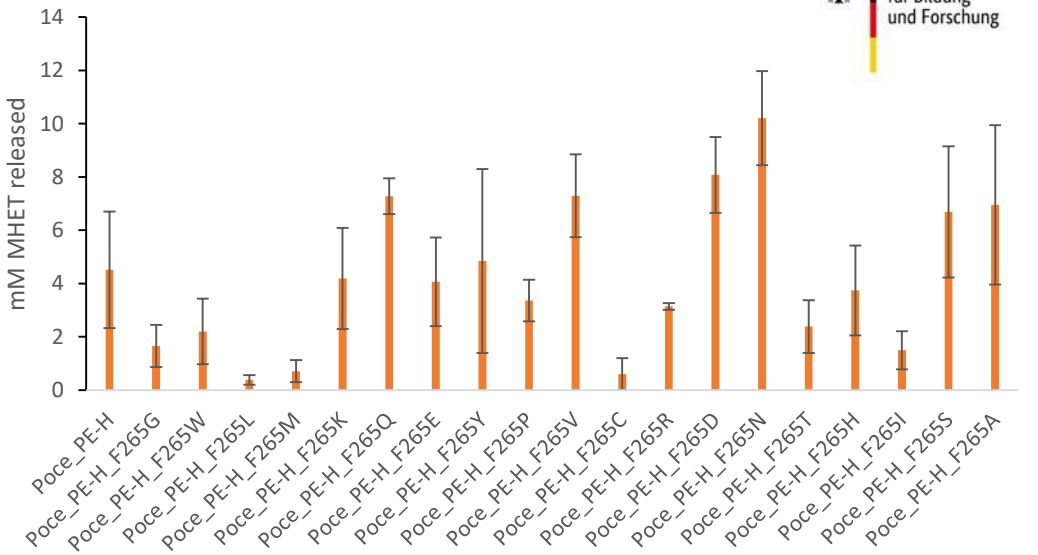
Substrate: sample textile 4-b 3X58 (VORB, 100% PES 100g/m^2) pretreated by alkaline boiling, 168h, 30°C, 100mM KPi, pH7.2

Task 5.3: Other advanced and classical methods

Literature informed mutagenesis of PE-H to improve polymer conversion:

Application: Textiles

Saturation mutagenesis His_{cat} +1

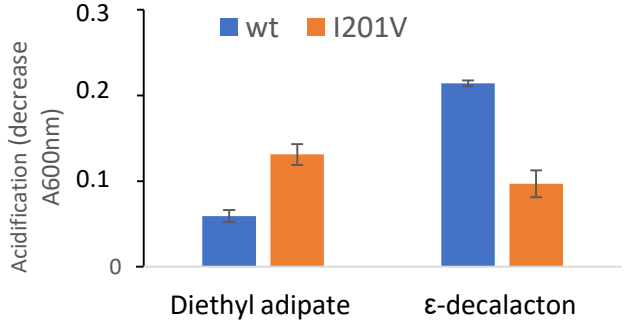


GEFÖRDERT VOM
Bundesministerium für Bildung und Forschung

- Inspired by PETase engineering

Sfre_PE-H I201V:

Improving specifity: Diethyl adipate ↑ ε-decalacton ↓



- Inspired by HiC engineering:

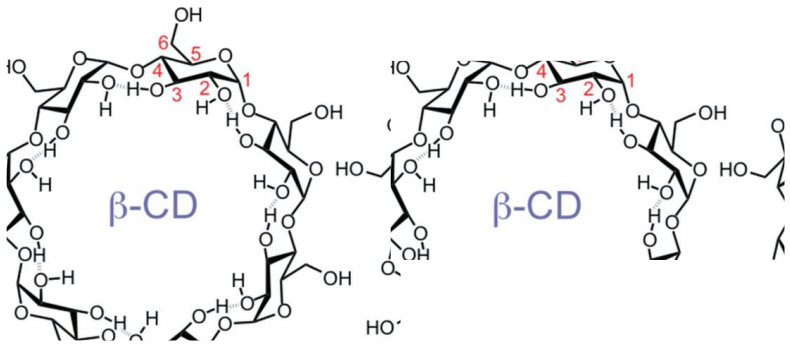
Rfa_PE-H I51S_F80A: Tm +3°C

PET amorph, 168h, 30°C, 100mM KPi, pH7.2

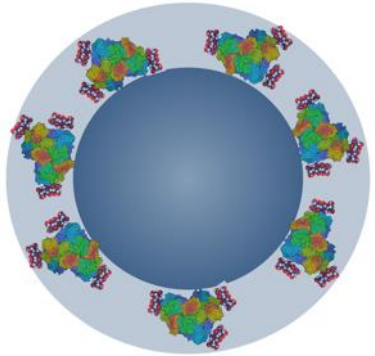
10.1021/acscatal.8b05171
10.1002/biot.201700188

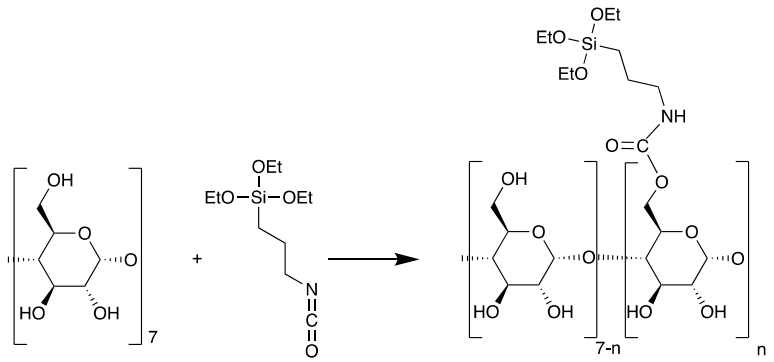
Task 5.4: Empowering enzymes by immobilization-guided supramolecular engineering

- Supramolecular engineering using artificial chaperones, as building blocks of the protective layer.

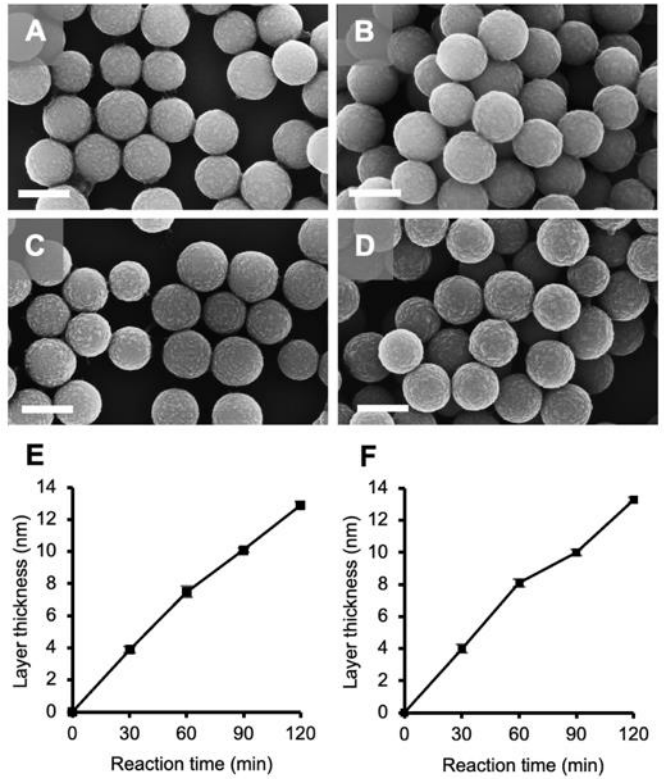


Crystal structure of β-cyclodextrin





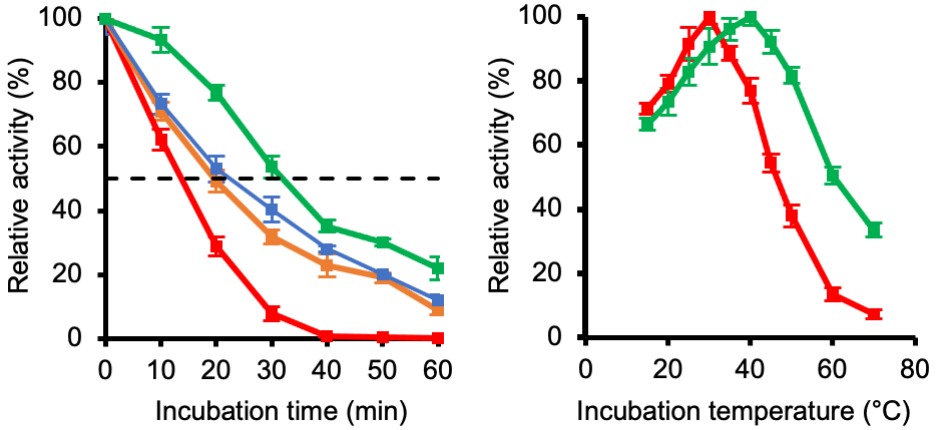
Chemical synthesis of a β-cyclodextrin derivative bearing a chemical moiety allowing integration within protective shields



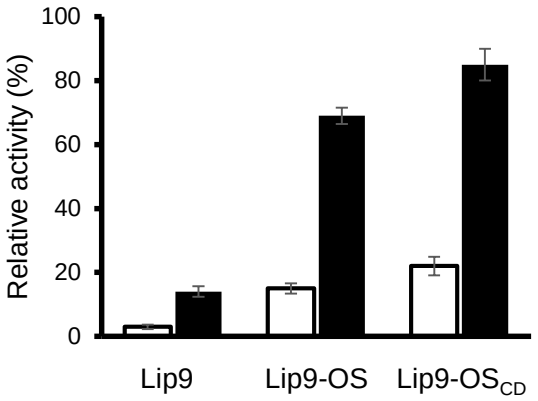
Scanning electron micrographs of SP-Lip9-OS_{CD} measured after 30 (**A**), 60 (**B**), 90 (**C**) and 120 (**D**) min of layer polycondensation reaction. Scale bars represent 200 nm.

Task 5.4: Empowering enzymes by immobilization-guided supramolecular engineering

- Supramolecular engineering using artificial chaperones, as building blocks of the protective layer.



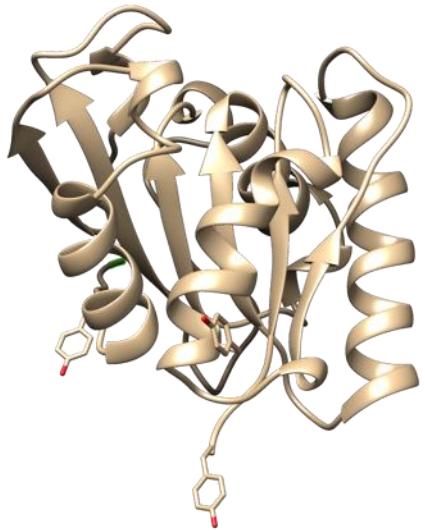
Enzymatic activity measured (left) after treatment at 50 °C for increasing durations (left) and (right) at increasing reaction temperatures. Red: soluble enzyme, orange: immobilized enzyme, blue: shielded enzyme in the absence of CD and green: SP-Lip9-OS_{CD}



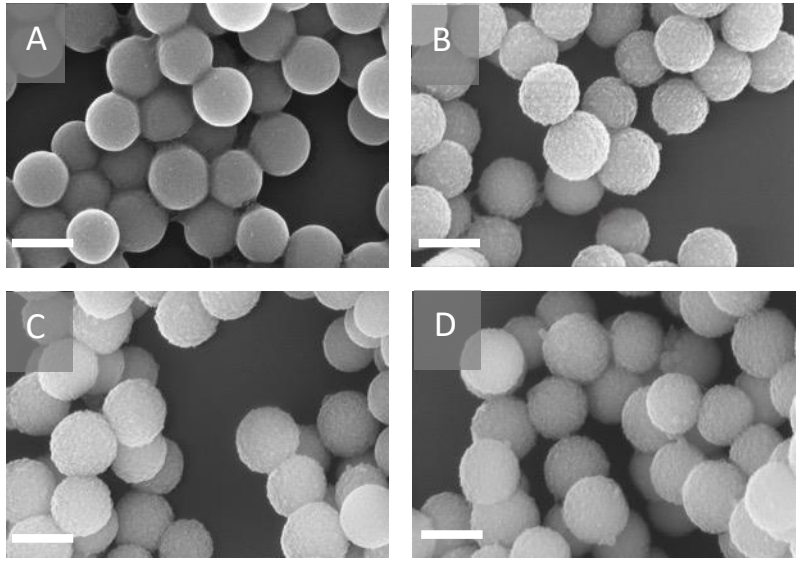
Activity retention of the free (Lip9), shielded without CD (Lip9-OS), and shielded with CD (Lip9-OS_{CD}) enzyme after treatment with 1% SDS for 20 min (white bars) and after further dialysis (black bars).

Task 5.4: Empowering enzymes by immobilization-guided supramolecular engineering

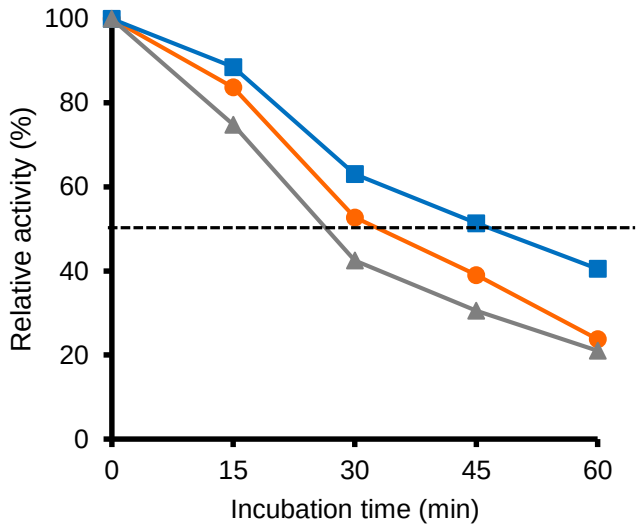
- Supramolecular engineering using artificial chaperones, as building blocks of the protective layer. Engineering Lip9 to increase interaction points with CD



Enghineered Lip9 with substitution of 2 Ala and 1 Gly by 3 Tyr at the enzyme surface, as follows: MYEHNPVVMVHGIGGASYNFFSIKSYLYTQGWDRNQLYAI DFIDKTGNNRNNGPRLSRFVKDVLDKTGAKKVDIVAHSMG GANTLYYIKNLDGGDKIENVVTIGGANGLVSSRALPGTDPN QKILYTSVYSSADLIVVNSLSRLIGARNVLIHGVGHIGLLTSSQ VKGYIKEGLNGYGQNTN



SEM micrographs of shielded lip9 mutant with α-CD after (A) 30 min, (B) 60 min, (C) 90 min and (D) 120 min of layer growth reaction duration yielding 3.8, 8.2, 11.8 and 15 nm layer thickness,



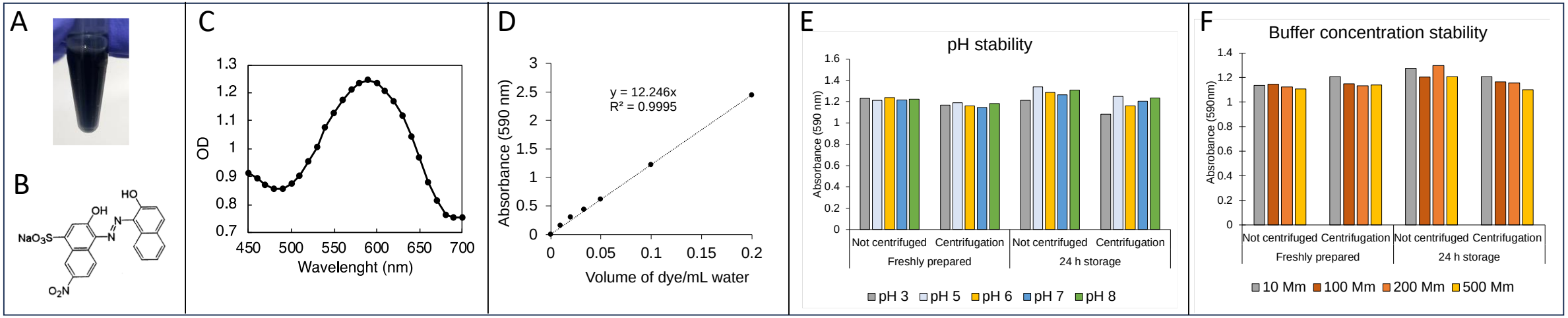
Thermostability of Lip9 Tyr mutant shielded with α-CD (blue), Lip9 with α-CD (orange), Mutant shielded without α-CD (grey) at 50 °C.

Task 5.4: Empowering enzymes by immobilization-guided supramolecular engineering

- Physicochemical characterization of Bemaplex Black (dye from Schoeller Textiles)

The development of an enzymatic systems to promote the dye removal during fabrics processing is of interest in the frame of the FuturEnzyme project.

In order to set the optimal conditions for the enzymatic degradation of the dye, a physicochemical characterization by UV-VIS spectroscopy of Bemaplex Black was carried out. The water miscibility of the dye was studied by preparing volumetric dilutions in water, a standard curve was plotted. Also, the stabilities (absence of dye precipitation after centrifugation) at different pH values and at different buffer concentrations were studied.



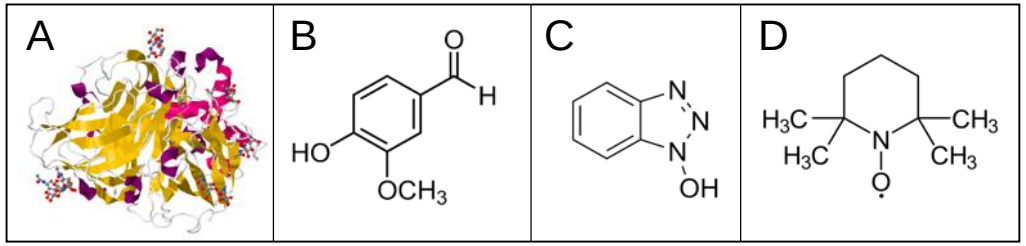
Bemaplex Blak characterization: A) dye appearance; B) chemical structure; C) UV-VIS spectrum (absorbance peak at 590 nm); D) linearity study at different volumetric dilutions of the dye in water; E-F) stability at different pH values and buffer concentrations after 24 hours storage and centrifugation.

Task 5.4: Empowering enzymes by immobilization-guided supramolecular engineering

- Discoloration of Bemaplex Black by soluble Laccase

Laccase from *T. versicolor* was selected as candidate enzyme.

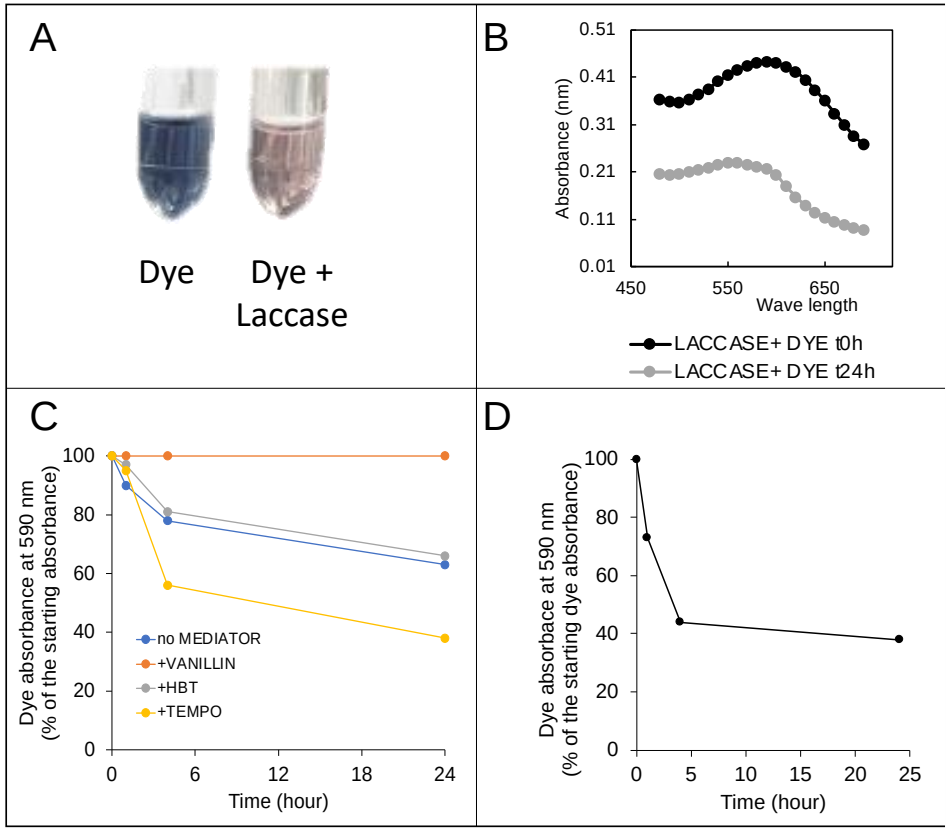
Three enzymes' mediators, at a concentration of 1 mM respectively, were tested in the selected reaction conditions (citrate buffer pH 5, presence of dye, 30°C, 24 hours reaction).



The laccase and its mediators. A) laccase from *T. versicolor.* Molecular weight: 70 KDa. PDB no.: 1GYC; B) Vanillin; C) 1-hydroxybenzotriazole or HBT; D) (2,2,6,6- tetramethylpiperidin-1-yl)oxyl, or TEMPO.

The reaction was started by the addition of 1 unit of soluble laccase. Samples were collected and measured in a microwell plate over time at 590 nm.

TEMPO is the best enzyme mediator. After 30 min and 4 hours, 27% and 56% respectively of dye were discolored. After 24 hours, 62% of the dye was discolored.



Enzymatic dye discoloration. A) Bemaplex Black before and after 24 h incubation with soluble Laccase. B) spectrum of absorbance of the dye at the beginning and after 24 hours incubation with soluble Laccase. C) effect of different mediators in the laccase mediated dye discoloration (shown as decrease of the absorbance at 590 nm). D) laccase mediated dye discoloration.

Task 5.4: Empowering enzymes by immobilization-guided supramolecular engineering

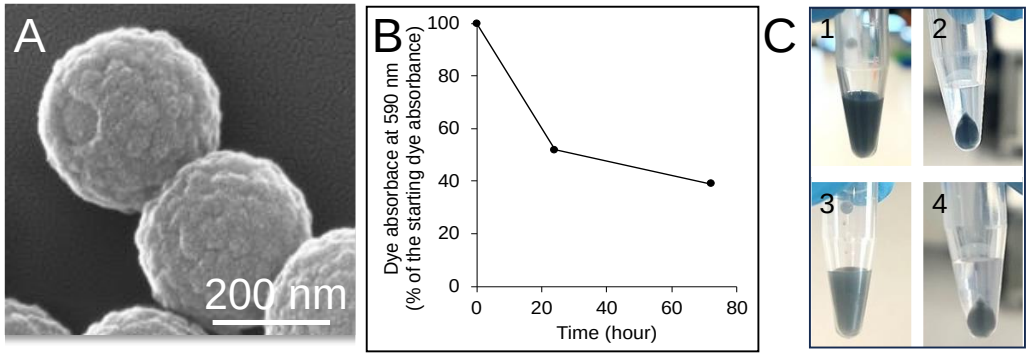
INOFEA
empowering enzymes

Discoloration of Bemaplex Black by supramolecular engineered laccase

Dye discoloration catalyzed by immobilized and shielded Laccase

Laccase was covalently immobilized on the surface of silica particles and shielded with an organosilica layer. Particles carrying 2 Units of laccase were resuspended (final concentration 10 mg/mL) in a solution containing Bemaplex Black, (dilution 1:20 in citrate buffer at pH 5) and 1 mM TEMPO. The particles suspension was incubated for 72 hours at 30 °C under shaking. After 24 and 72 hours, 48% and 61% respectively of dye was discolored.

The discoloration catalyzed by the immobilized and shielded laccase was longer compared to the one catalyzed by the soluble enzyme (62% after 24 hours).

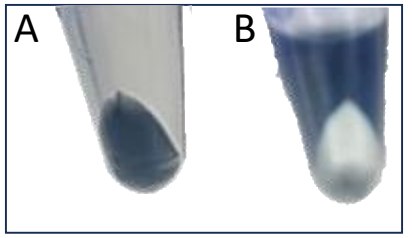


Discoloration of Bemaplex Black by supramolecular engineered laccase. A) scanning electron micrographs of shielded silica particles. B) dye discoloration (shown as decrease of the absorbance at 590 nm) catalyzed by the shielded laccase. C) suspension of the shielded silica particle at the time zero of the reaction (C1) and after centrifugation (C2). suspension of the shielded silica particle after 24 hours of reaction (C3) and after centrifugation (C4).

Interaction of Bemaplex Black with the enzyme's carrier

When particles were spinned-down, the obtained pellet was blue indicating that the dye was adsorbed into the carrier.

When the particles were washed in buffer containing 1% of Triton-100X, the dye was released.
This result suggests that the enzyme's carrier may be used as an absorbent of dyes used in textiles manufacturing.



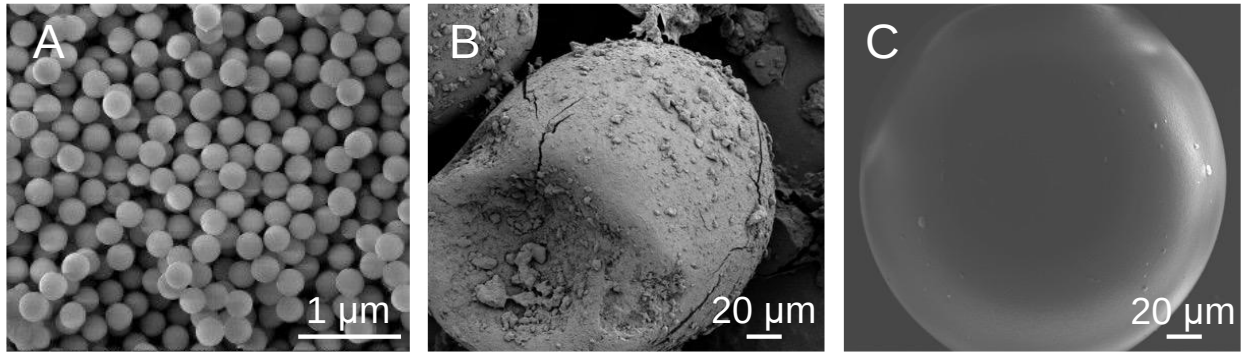
Interaction of Bemaplex Black with silica particles.
A: the dye is adsorbed into the particles (blue pellet). B: the dye is released from the particles (white pellet).

Task 5.4: Empowering enzymes by immobilization-guided supramolecular engineering

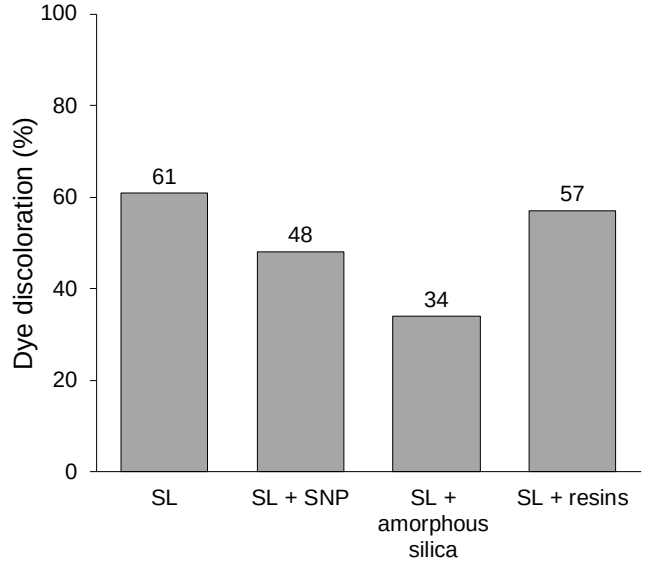
- Laccase mediated discoloration of Bemaplex Black using different types of particles as adsorbent

Diluted dye (dilution V/V, 1:20) in citrate buffer was incubated for 24 hours with soluble laccase (1 U) in presence of different enzyme carriers (colloidal silica particles, amorphous silica, methacrylate resins) in citrate buffer (pH 5) at 30°C.

After 24 hours, the dye discoloration was measured. The best activity was found when the dye was incubated in the presence of methacrylate resins (57% of dye discoloration).

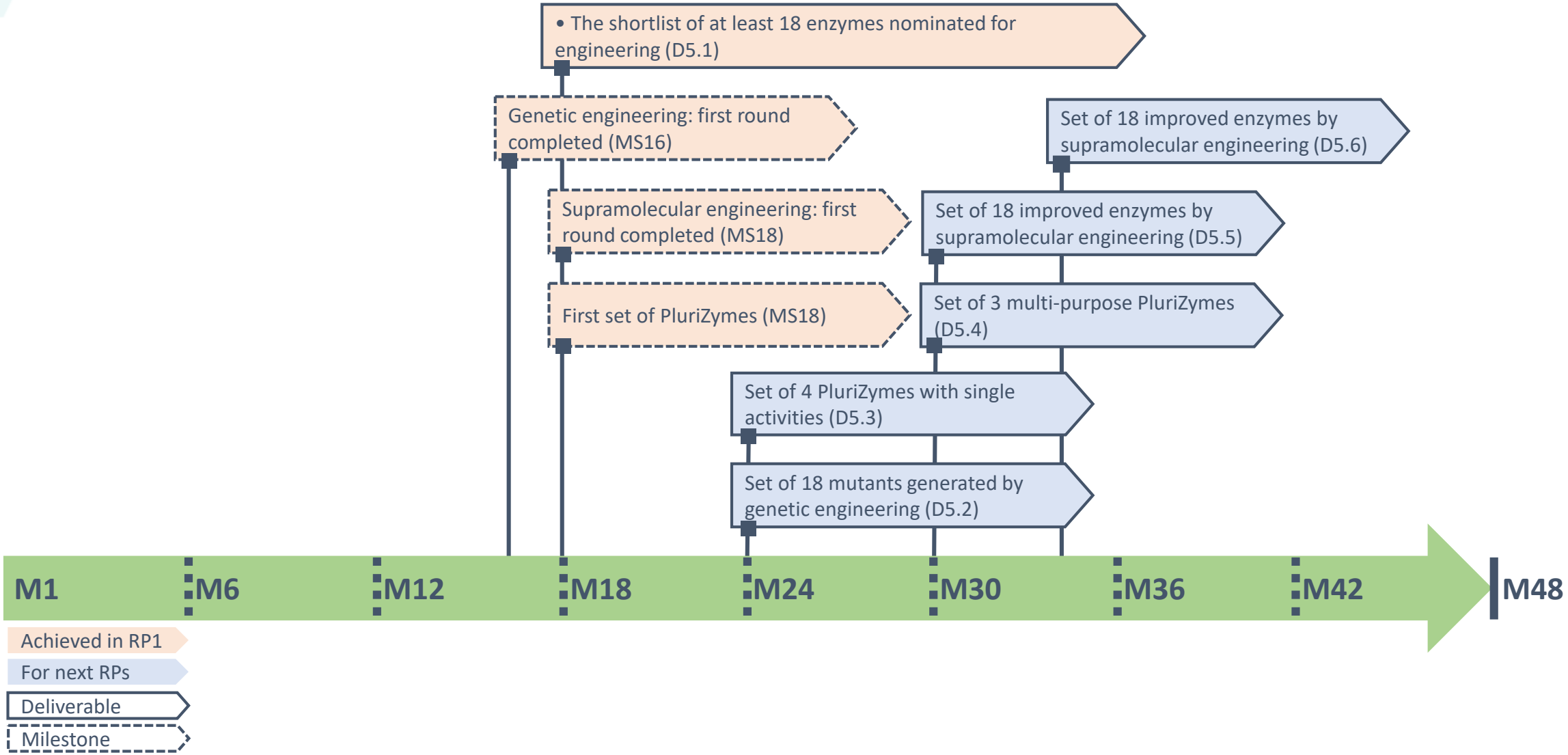


Scanning electron micrographs of particulate carriers used as dye adsorbent. A) colloidal silica particles, diameter: 300 nm. B) amorphous silica particles, average diameter: 250 µm. C) methacrylate resin, average diameter: 300 µm.



SL: soluble laccase
SL + SNP: soluble laccase + colloidal silica particles
SL + amorphous silica: soluble laccase + amorphous silica
SL + resins: soluble laccase + methacrylate resins

WP5 – Deliverables and milestones



WP5 – Future actions

- Future actions (six months ahead)
  - BSC: To apply the mutagenesis algorithms developed in BSC, AsiteDesign and MultiOptimization Design, and test mutants with PELE, to find variants with higher activities and stabilities for enzymes selected in WPs 2 and 3, and the design of both uni- and multi-functional pluriZymes of interest in the detergent and textile sectors.
  - UDUS, inputs needed:
    - Plurizyme contributions (construction and tests?)
    - Targets to improve detergent stability?
    - Targets to improve activity to specific substrates?
  - INOFEA: to optimize the synthesis of immobilized and shielded laccase (and other enzymes produced by the consortium partners) on different carriers (e.g., resins, silica particles) for dye removal during fabrics processing.
  - FHNW:
    - Finalize the work with CD-engineering and to apply to other consortium enzymes.
    - Test partial layer for testing PET films degradation (with CSIC)

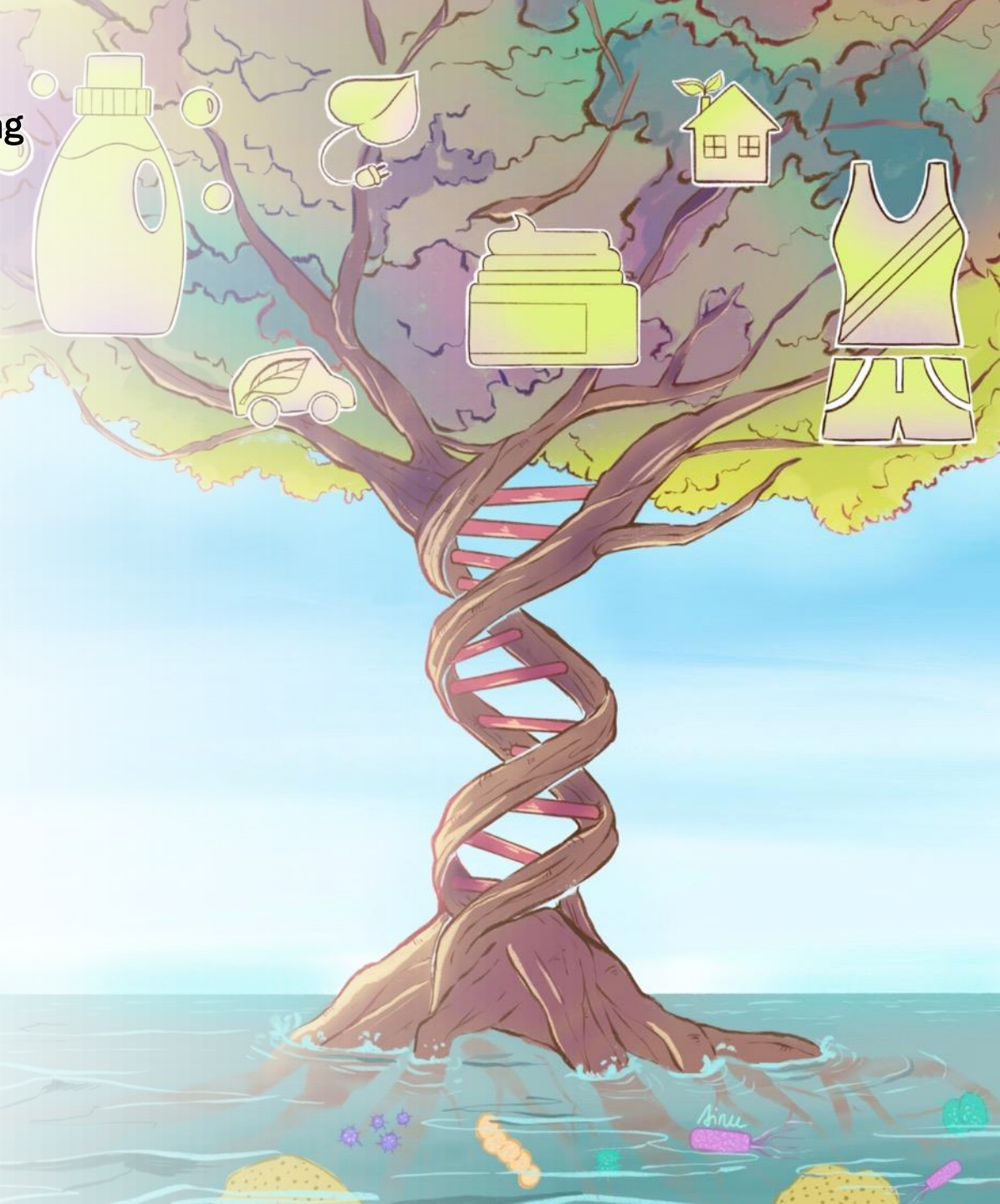
WP5 – Deviations

- Deviations
  - No deviations found in the activities planned in the GA, and no mitigation actions are required.

WP5 – Enhancing enzymes through innovative engineering

FuturEnzyme

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



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