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

Meeting #2 – WP5 Enhancing enzymes through innovative engineering

General Assembly – Madrid-June 2022

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

Work Package 5: Enhancing enzymes through innovative engineering

Meeting #2

General Assembly – Madrid - June 2022

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Goals

Work package number [9]	WP5	Lead beneficiary [10]	9 - FHNW
Work package title	Enhancing enzymes through innovative engineering		
Start month	3	End month	42

Objectives

WP5 starts recognizing that, only through the design of super efficient and stable enzymes, one can replace benchmark enzymes and existing products, and establish new consumer products of higher environmental quality and innovative and functional characteristics worldwide. But in most cases, this requires the application of engineering techniques to enzymes initially selected. However, if one intends to engineer natural enzymes, most current engineering efforts come short when addressing the real manufacturers' needs and requirements; this also occurs when applying current methods to newly discovered natural enzymes. For all these reasons, the main objective of WP5 is to apply both well established and novel engineering techniques to a sub-set of the 18 best enzymes from Task 4.5, namely, the best 2 enzymes per each of the 9 classes targeted. The outcome will be to generate enhanced enzyme variants with real potential for manufacturing of laundry, personal care, and textile products. This real potential will be defined on the basis of the inter-relation between high expression level and performance (activity and stability) as high as possible compared to benchmark enzymes. Five complementary techniques will be applied:

- Rational design, through disruptive in silico-directed evolution techniques and algorithms;
- Disruptive engineering computational tools to design enzymes, PluriZymes, with prominent performances and multipurpose activities;
- Synthesis-based site-directed mutagenesis;
- Rational design, through classical structural analysis; and
- Immobilization-guided supramolecular engineering in proprietary nanoparticles with tailor-made shield.

Description of work and role of partners

WP5 - Enhancing enzymes through innovative engineering [Months: 3-42]
FHNW, CSIC, BSC, BANGOR, UHAM, UDUS, INOFEA AG, EUCODIS
We propose 4 Tasks. We would like to mention that in all tasks, the variants will be expressed and characterised following conditions and scales described in Tasks 4.1 and 4.4, and their characteristics will be compared to the original proteins and benchmark enzymes. For this reason, the techniques and protocols will not be again discussed below.

Task 5.1 Disruptive engineering computational tools M3-M42
Lead partner – BSC
Participants: CSIC, BANGOR, UHAM, UDUS

Task 5.2 Developing disruptive PluriZymes with multipurpose activities M6-M42
Lead partner – BSC
Participants: CSIC, UDUS, BANGOR, FHNW, UHAM, EUC

Task 5.3 Other advanced and classical mutation methods M4-M42
Lead partner – CSIC
Participants: BSC, BANGOR, UHAM, UDUS

Task 5.4 Empowering enzymes by immobilization-guided supramolecular engineering M6-M40
Lead partner – INOFEA
Participants: FHNW, CSIC, BANGOR, UHAM, UDUS

List of deliverables

Deliverable Number[14]	Deliverable Title	Lead beneficiary	Type[15]	Dissemination level[16]	Due Date (in months)[17]
D5.1	The shortlist of at least 18 enzymes nominated for engineering	1 - CSIC	Report	Confidential, only for members of the consortium (including the Commission Services)	18
D5.2	Set of 18 mutants generated by genetic engineering	2 - BSC	Other	Confidential, only for members of the consortium (including the Commission Services)	24
D5.3	Set of 4 PluriZymes with single activites	2 - BSC	Other	Confidential, only for members of the consortium (including the Commission Services)	24
D5.4	Set of 3 multi-purpose PluriZymes	9 - FHNW	Other	Confidential, only for members of the consortium (including the Commission Services)	30
D5.5	Set of 18 improved enzymes by supramolecular engineering	11 - INOFEA AG	Other	Confidential, only for members of the consortium (including the Commission Services)	30
D5.6	Datasets of engineered variants	1 - CSIC	data sets, microdata, etc	Confidential, only for members of the consortium (including the Commission Services)	34

Goals

Main WP Goals:

- WP5 starts recognizing that, only through the design of super efficient and stable enzymes, one can replace benchmark enzymes and existing products, and establish new consumer products of higher environmental quality and innovative and functional characteristics worldwide.
- the main objective of WP5 is to apply both well established and novel engineering techniques to a sub-set of the 18 best enzymes from Task 4.5, namely, the best 2 enzymes per each of the 9 classes targeted.
- The outcome will be to generate enhanced enzyme variants with real potential for manufacturing of laundry, personal care, and textile products.

Five complementary techniques will be applied:

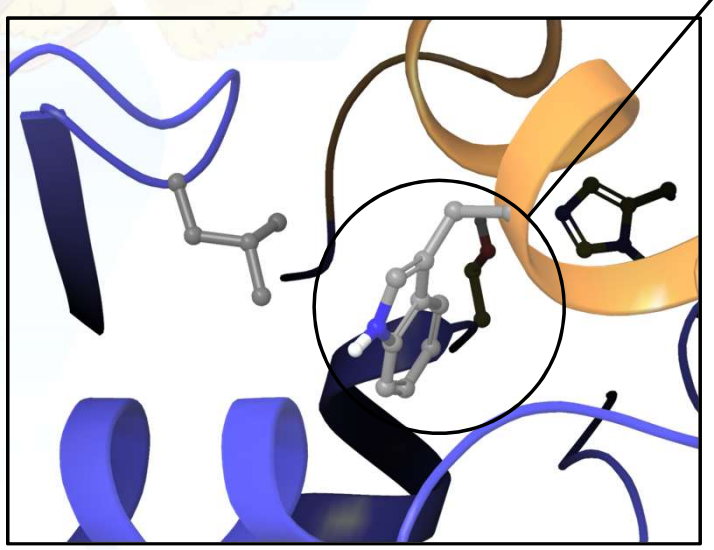
- Rational design, through disruptive in silico-directed evolution techniques and algorithms;
- Disruptive engineering computational tools to design enzymes, PluriZymes, with prominent performances and multipurpose activities;
- Synthesis-based site-directed mutagenesis;
- Rational design, through classical structural analysis; and
- Immobilization-guided supramolecular engineering in proprietary nanoparticles with tailor-made shield.

Results obtained so far

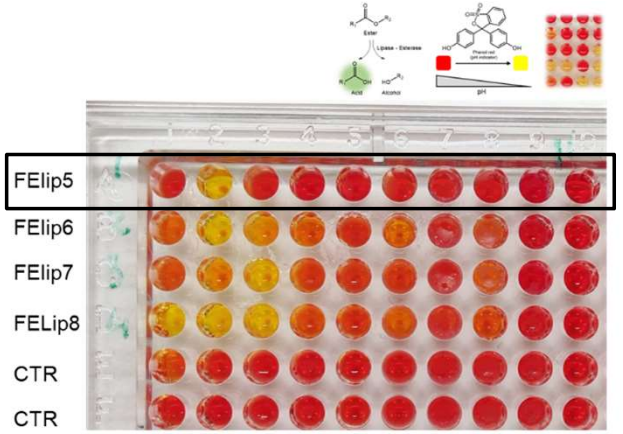
Barcelona Supercomputing Center
Centro Nacional de Supercomputación

Task 5.1. Disruptive engineering computational tools M3-M42

FELip5



W89 hinders the opening of the lid domain to bind long-chain triglycerides

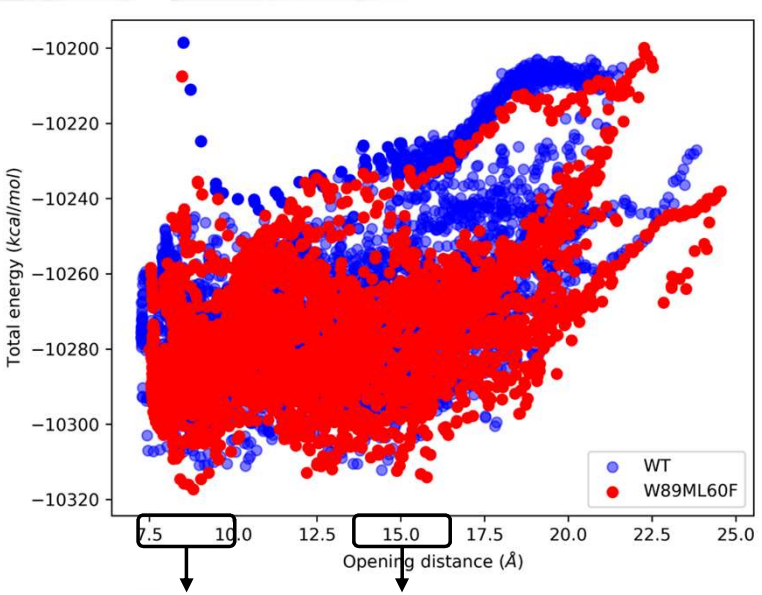


1: Glyceryl tripropionate (Tri-C3)
2: Glyceryl tributyrate (Tri-C4)
3: Glyceryl tricaproate (Tri-C8)
4: Glyceryl tridecanoate (Tri-C10)
5: Olive oil (Tri-C16:1)
6: Coconut oil (triglycerides of C8:0, C10:0, C12:0, C14:0, C-16:0, C18:0, C18:1, C18:2)
7: Glyceryl tridodecanoate (Tri-C12)
8: Palm oil (mainly C16:0, C18:0, C18:1, C18:2 and C18:3)
9: Glyceryl trimyristate (Tri-C14)
10: None

Results obtained so far

Task 5.1. Disruptive engineering computational tools M3-M42

Study of FELip5's lid domain

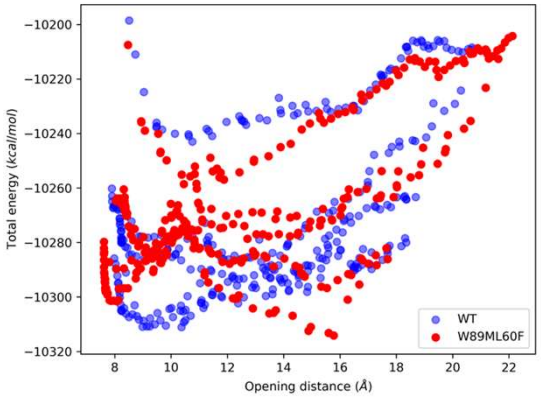


Open conformation

Closed conformation

Only showing

1 report

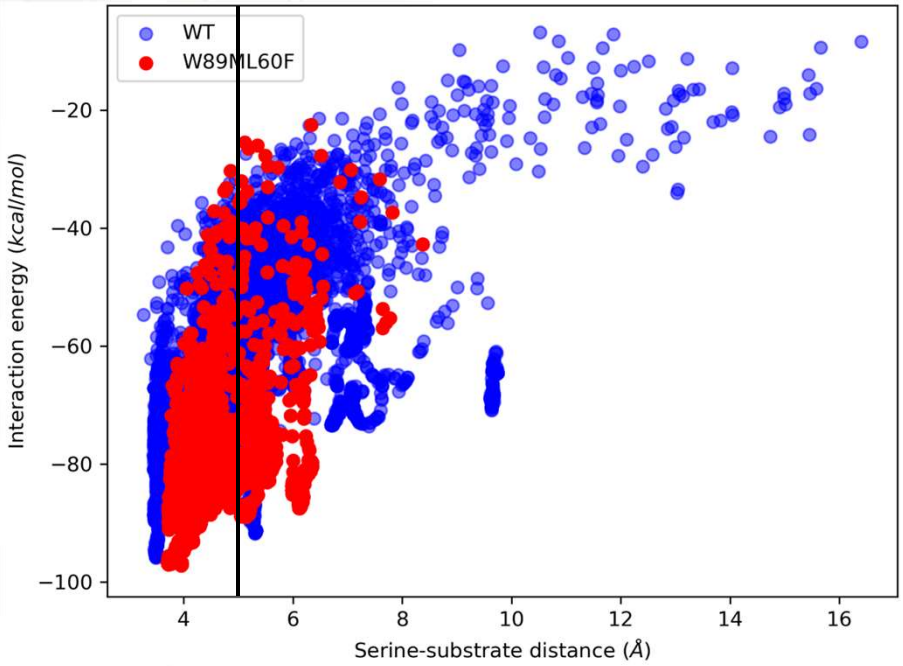


$\Delta Opening\_distance_{(W89M/L60F - WT)}$ = 1.66 Å

Results obtained so far

Task 5.1. Disruptive engineering computational tools M3-M42

Study of FELip5's lid domain



Induced fit exploration with PELE

WT → Triolein could not be docked with Glide, thus and out-in protocol from the PELE Platform was used

W89ML60F → Triolein could be directly docked with Glide. Thus, the induced fit exploration was performed without an out-in simulation.

Catalytic state →Serine-substrate distance <= 5 Å
Non-catalytic state → Serine-substrate distance > 5 Å

% Catalytic state$_{W89M/L60F}$ ≃ 82 | $\Delta E_{C\text{-}NC\ states}$ ≃ -10.11

% Catalytic state$_{WT}$ ≃ 56 | $\Delta E_{C\text{-}NC\ states}$ ≃ -6.85

Results obtained so far

Task 5.1. Disruptive engineering computational tools M3-M42

No.	Lipase	Concentration (mg/ml)	Specific activity (U/mg)		
			Tributyrin	Trioctanoin	Triolein
24	RA	0.30	990[a]	15000[d]	1167[c]

An interesting approach for the protein engineering of lipases is the exchange of lids of homologous enzymes, also referred to as "lid swapping." The amphipathic nature of the lid is very important for the substrate specificity, and it provides new insight into the structural basis of lipase substrate specificity and a way to tune the substrate preference of lipases.

Khan et al. (2017).
doi: 10.3389/fbioe.2017.00016

UniProt IDs: [I1BGQ3 , P61872]

FeLip5's lid domain:
FRGTEITQIKDWLTDA

RA's lid domain:
FRGTNSFRSAITDIVF

Lid domains do not contain a W in their sequence

Results obtained so far

Barcelona Supercomputing Center
Centro Nacional de Supercomputación

Task 5.1. Disruptive engineering computational tools M3-M42

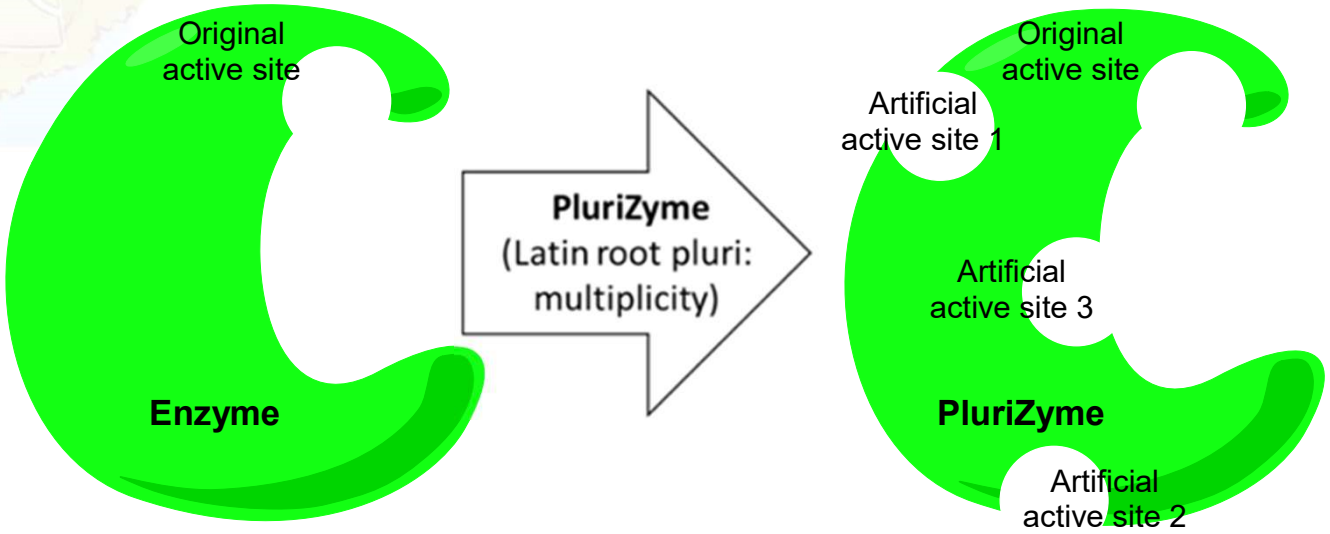
Two variants of the Lip5 hydrolase were designed by BSC and their activity tested

FELip5_Lid was confirmed as a variant with lipase activity

Substrate	U/g			
	6	CalB	FELip5_W89ML60F	FELip5_Lid
Glyceryl tripropionate (Tri_{C3})	985.8	461.18	935,0	778,6
Glyceryl tributyrate (Tri_{C4})	1084.2	445.61	154,3	262,5
Glyceryl trioctanoate (Tri_{C8})	3.228	63.30	1323,2	87,5
Glyceryl tridecanoate (Tri_{C10})	0	40.92	1224,9	58,6
Glyceryl tridodecanoate (Tri_{C12})	0	31.23	111,0	49,6
Glyceryl trimyristate (Tri_{C14})	0	19.29	1,8	7,4
Coconut oil	0	11.15	0,0	61,0
Palm oil	0	16.09	0,0	28,6
Olive oil	0	3.26	0,0	0,0

Results obtained so far

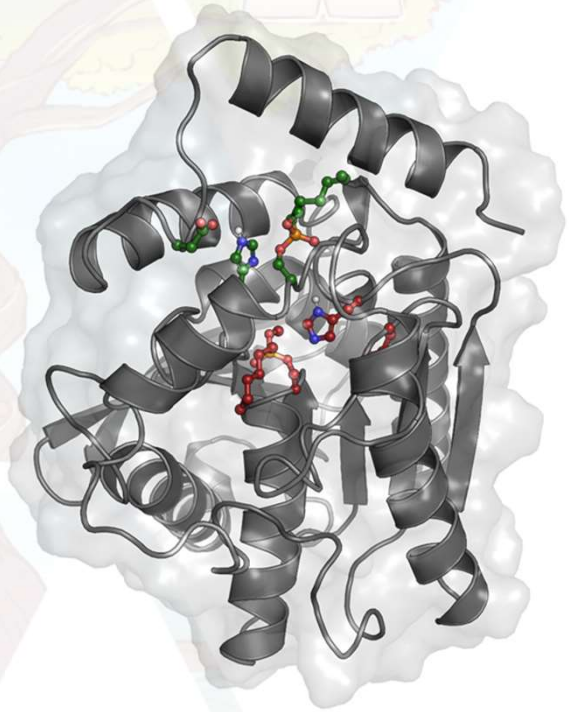
Task 5.2 Developing disruptive PluriZymes with multipurpose activities M6-M42



Results obtained so far

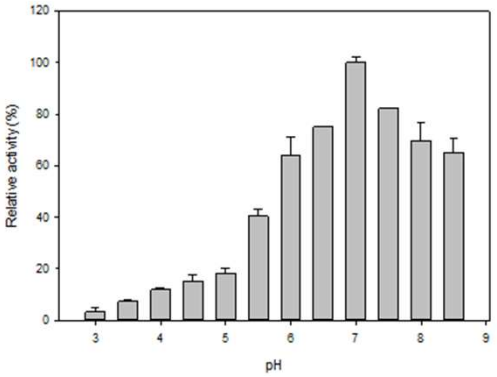
Task 5.2 Developing disruptive PluriZymes with multipurpose activities M6-M42

***PluriZyme* with two-esterase and one-protease active sites**

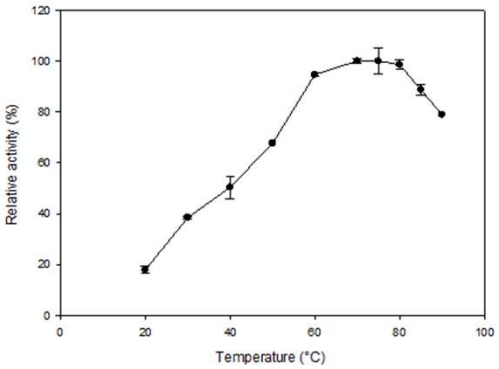


Casein	Neutrase 0.8 L Novozymes	EH1ABC
Spec. act. (U/mg)	1,86±0,10	2,63±0,06

Azocasein as substrate



Azocasein as substrate

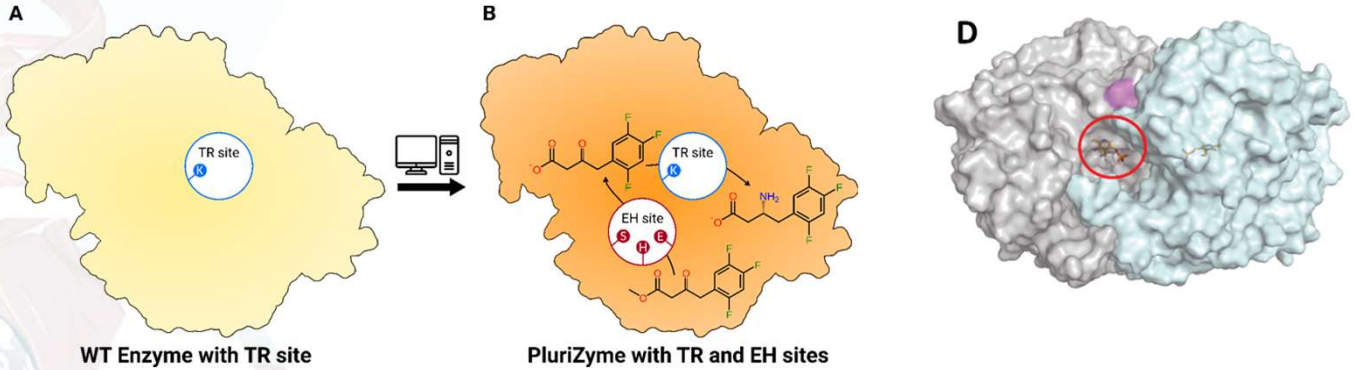


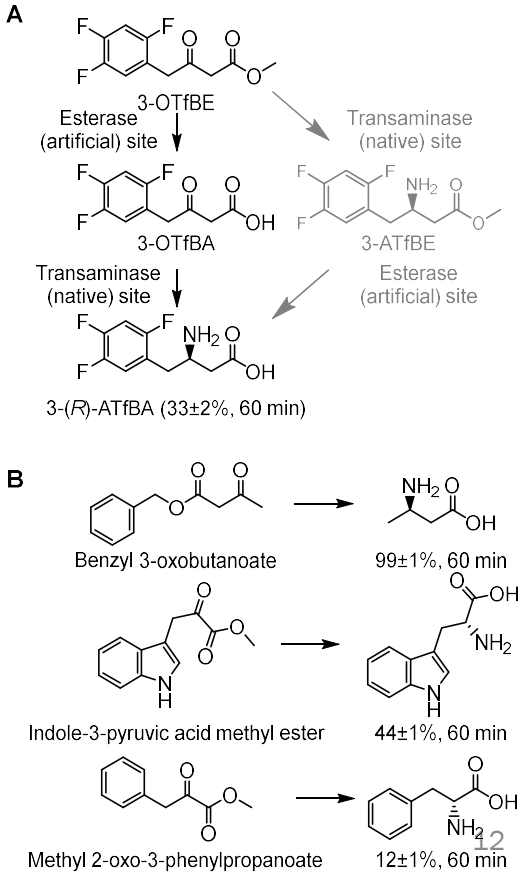
Results obtained so far

Task 5.2 Developing disruptive PluriZymes with multipurpose activities M6-M42

A *PluriZyme* with transaminase and hydrolase was designed

- The enzyme was functional and was capable of converting multiple oxo-esters into bet-amino acids
- activity catalyzes cascade reactions
- This will all the development of future enzyme designs integrating two biotic-catalytic centers, which, being independent, can also work in synergy for cascade reactions

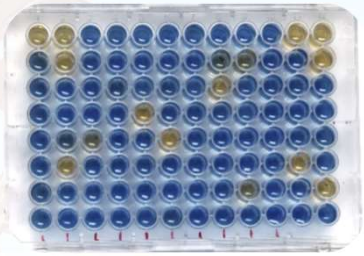




Results obtained so far

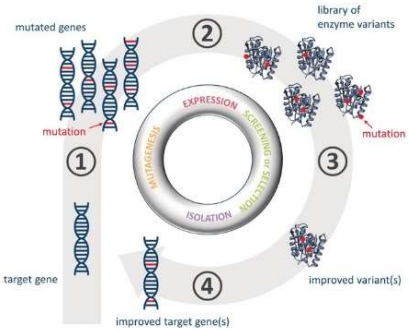
Task 5.3 Other advanced and classical mutation methods M4-M42

Characterization of selected candidate enzymes

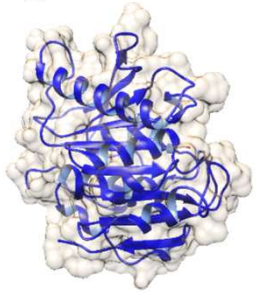


- activity against substrate derivatives
- stability (temperature, pH, solvents, additives)
- enzymatic properties (K_m, K_{cat}, K_{cat}/K_m)

Engineering approaches to increase the catalytic capabilities and enzyme performance



- random mutagenesis (epPCR)
- site directed mutagenesis
- site saturation mutagenesis
- crystallization



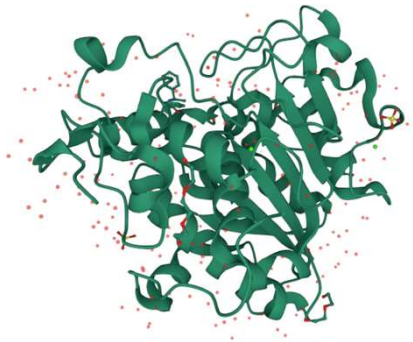
Results obtained so far

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

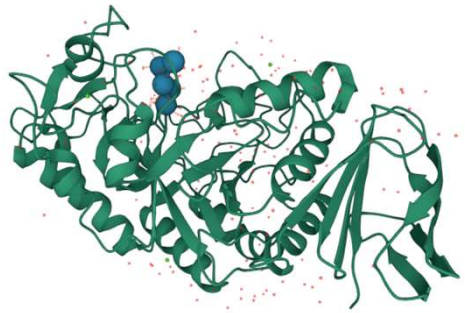
Pilot experiments: immobilization and shielding of benchmark enzymes to be used for comparative purposes.

The following benchmark enzymes were selected for immobilization on silica particles and 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



CalB with product analogue in the catalytic site. Molecular weight: 33 kDa. PDB no.: 6J1T



α-amylase with substrate analogue (G5) in the catalytic site. Molecular weight: 58 kDa. PDB no.: 1BAG

Results obtained so far

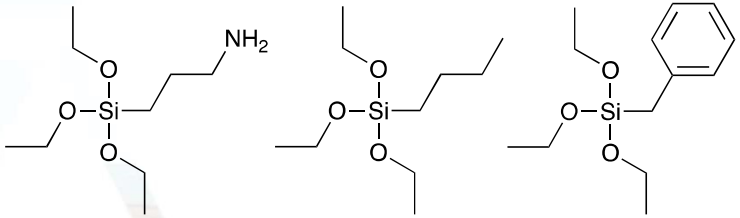
Task 5.4 Empowering enzymes by immobilization-guided supramolecular engineering M6-M40

Pilot experiments: immobilization and shielding of benchmark enzymes to be used for comparative purposes: CalB.

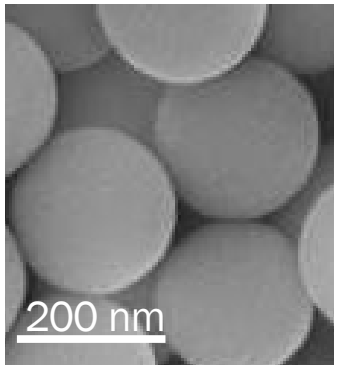
To optimize the bioconjugation of CalB on silica particles (SP, diameter 230 nm), the surface of the carrier was modified with mixtures of organosilanes owning different chemical functionalities. The applied organosilanes mixtures are:

- (3–aminopropyl)triethoxysilane (APTES)
- n-Butyltriethoxysilane (Butyl) + APTES
- benzyltriethoxysilane (Benzyl) + APTES

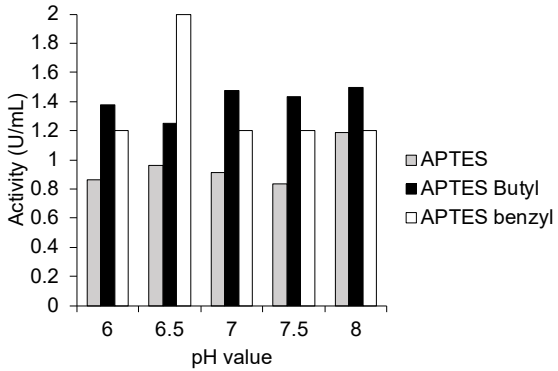
Enzyme absorption on the carrier was performed by incubating the particles suspension with solutions of soluble CalB for 1 h at 20 °C in buffer at different pH.



APTES Butyl Benzyl



SEM micrograph of silica particles



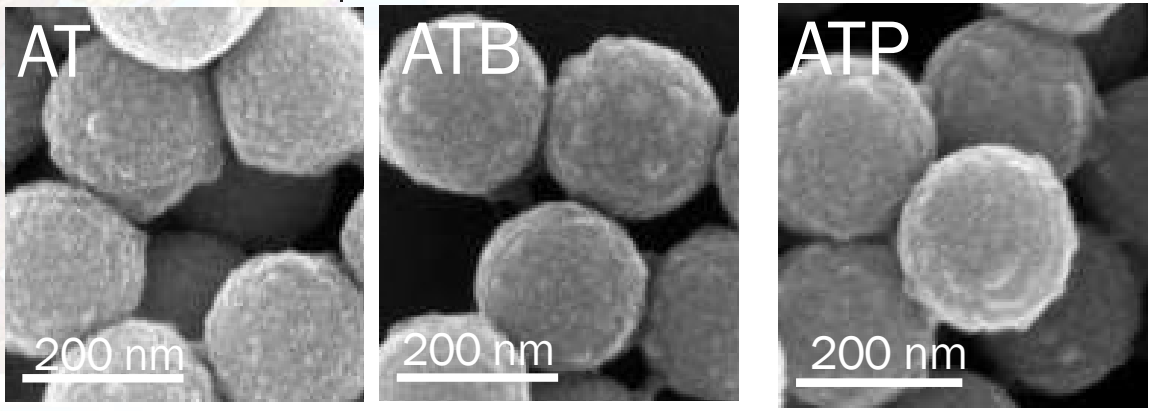
Activity of CalB absorbed on silica particles with different surface modification

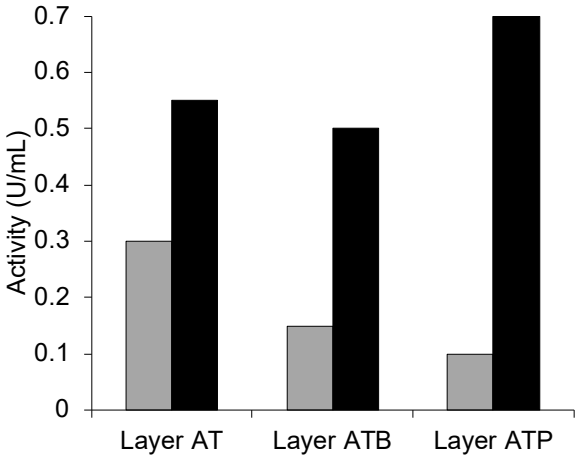
Results obtained so far

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

Pilot experiments: immobilization and shielding of benchmark enzymes to be used for comparative purposes: CalB

Effect of curing conditions on the activity of the shielded enzymes





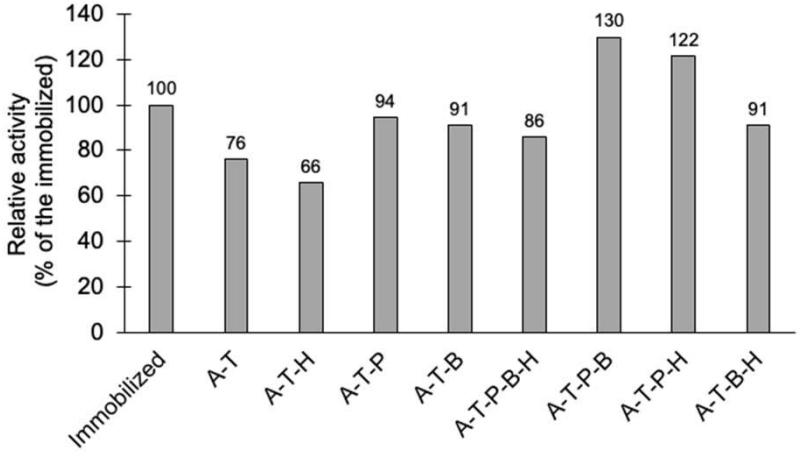
Activity of CalB covalently immobilized on silica particles and shielded with layers of different compositions (cured in buffer (grey) or in solvent (black). This set of results shows that both shield composition and curing conditions (solvent vs. buffer) have a direct influence on the activity of the nanobiocatalysts produced.

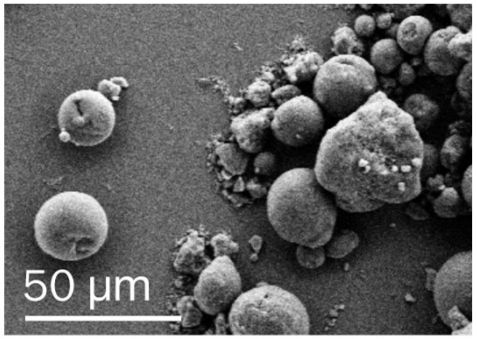
Results obtained so far

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

Pilot experiments: immobilization and shielding of benchmark enzymes to be used for comparative purposes: α-amlyase

α-Amylase was immobilized on amino modified porous, large silica particles (Sipernat®, diameter 13 µm), using glutaraldehyde as homo-bifunctional crosslinker. As the substrate of α-Amylase is a large polysaccharide (starch) a partial shielding was applied (target thickness 4 nm). Different layer compositions were tested.





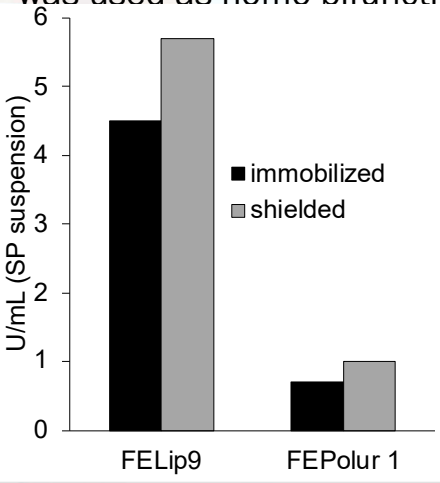
The immobilized enzyme had an activity of 90 U/mL of particles suspension at a concentration of 10 mg/mL. The particles with a partial layer made of ATPB showed the best activity (130% of the immobilized-only α-Amylase) corresponding to 117 U/mL of particles suspension.

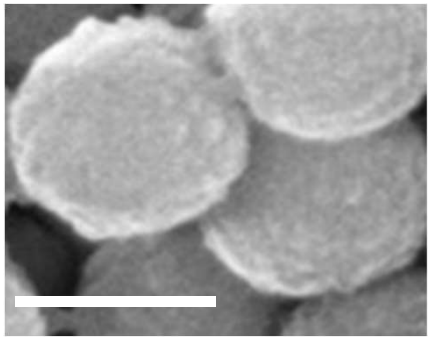
Results obtained so far

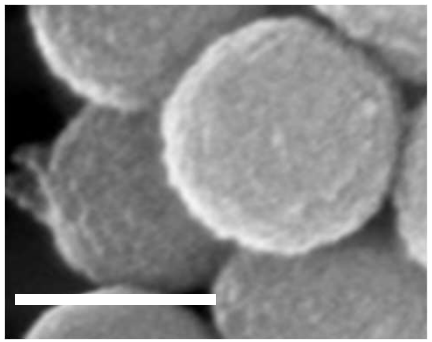
Task 5.4 Empowering enzymes by immobilization-guided supramolecular engineering M6-M40

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*

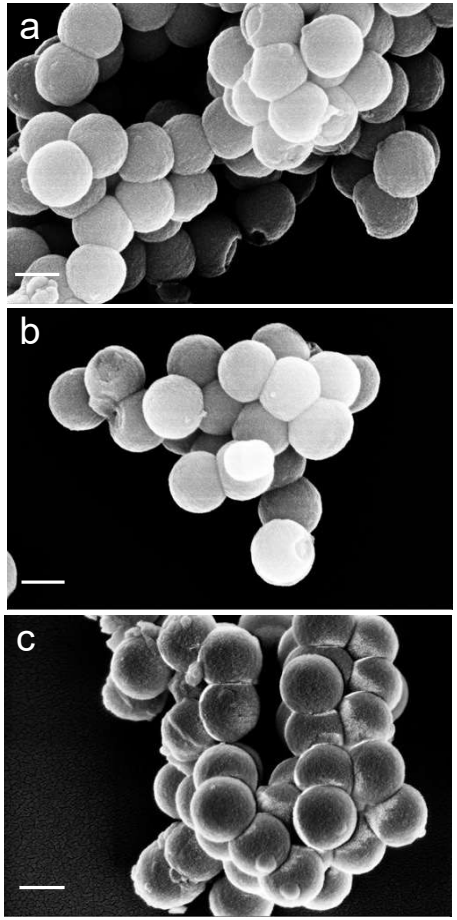


Figure: SEM micrographs of lipase-based nanobiocatalysts shielded with an organosilica layer of controlled thickness. All scale bars represent 200 nm

Results obtained so far

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

FuturEnzyme for a greener textile-manufacture and treatment

The challenge: to set-up enzymatic treatments of textiles to avoid the use of harsh and hazardous chemicals.

Pilot work: to set-up and validate a method to measure/quantify the action of enzymes in the pre-treatment of raw textiles.

Chemical Cleaning

Washing at 80°C with or without defoamers; Drying/fixing

Alkaline Boiling

Pretreatment by boiling at 132°C for 45 min in 2g/L soda; Rinsing

Washing

Washing at 80°C with surfactant and defoamers; Drying

Desizing, Washing, Bleaching

Impregnation with dispersing agents, defoamers and starch removal for 24 h; Washing at 80°C with surfactant and defoamers; Drying; Impregnation with dispersing agents, defoamers, soda 50%, hydrogen peroxide 35% for 24h. Washing at 90 °C, with defoamers; drying, fixing, brushing.

Current practice for pre-treatment steps of raw textiles; After pre-treatments, ether-soluble and hot water-soluble substances are quantified by IR-spectroscopy.

Results obtained so far

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

FuturEnzyme for a greener textile-manufacture and treatment

Selection of textiles with different yarn composition.

1. 92 % CO[1], 8 % EL[2] (Raw/chemically-treated)
2. 88 % PA[3], 12 % EL (Raw/chemically-treated)
3. 100 % PES[4] (Raw/chemically-treated)

[1]: Cotton
[2]: Elastane
[3]: Polyamide
[4]: Polyester

Treatment of raw textile with enzymes of different classes: lipases, amylases, proteases.
Evaluation of different parameter (conditions, duration, sequence, ..).

The wettability before and after treatment will be tested by contact angle.

The general procedure for measuring a textile pretreatment will be applied.

The results will confirm that the enzymatic treatment is a suitable alternative to the chemical process.

Results obtained so far

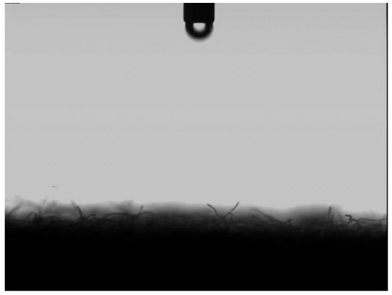
Task 5.4 Empowering enzymes by immobilization-guided supramolecular engineering M6-M40

FuturEnzyme for a greener textile-manufacture and treatment

Raw textile

Chemically treated textile

Enzymatically treated textile with soluble lipases



Setting up an analytical method to assess the effects of the treatment of selected textiles (Cotton 92 %, Elastane 8 % (240 g/m^2) with commercially available enzymes, *i.e.* lipase from *Aspergillus niger* and *Mucor javanicus*. Reaction conditions: phosphate buffer pH 7.2, 37 °C, 72 h.

Deliverables

Deliverable			Due	Status
D5.1	The shortlist of at least 18 enzymes nominated for engineering	CSIC	M18	-
D5.2	Set of 18 mutants generated by genetic engineering	BSC	M24	-
D5.3	Set of 4 PluriZymes with single activites	BSC	M24	-
D5.4	Set of 3 multi-purpose PluriZymes	FHNW	M30	-
D5.5	Set of 18 improved enzymes by supramolecular engineering	INOFEA	M30	-
D5.6	Set of 18 improved enzymes by supramolecular engineering	CSIC	M34	-