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

2. Consortium General Assembly

Madrid, May 31st, 2022

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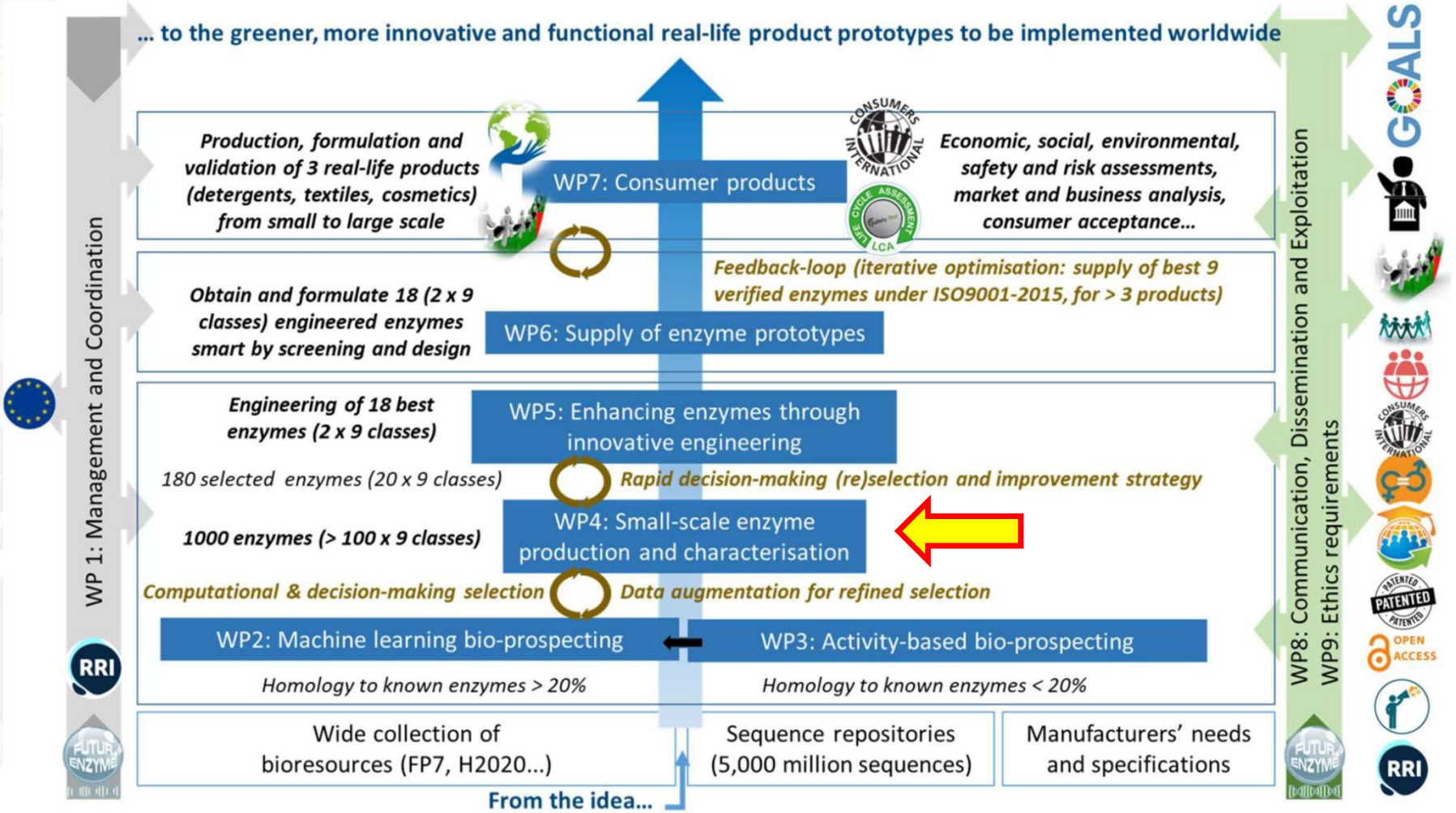
Research and Innovation Programme under grant agreement No [101000327]

WP4 Small-scale enzyme production and characterisation



WP4 Small-scale enzyme production and characterisation

Task 4.1 Ultra-fast and efficient gene cloning and synthesis: downstream enzyme production M2-M30 (UHAM)

Task 4.2 Smart design systems to produce enzymes with inherent problems of expression M2-M30 (FHNW)

Task 4.3 Production of enzymes from their natural hosts M2-M30 (CNR)

Task 4.4: Enzyme characterization for selecting those with manufacturers specification M2-M36 (UDUS)

Task 4.5: Decision-making strategy for selecting lead enzyme candidates M6-M30 (UDUS)

Task 4.6: Design of multi-enzyme blends to process complex ingredient mixtures M12-M40 (CSIC)

Work package number [9]	WP4	Lead beneficiary [10]	4 - UHAM
Work package title	Small-scale enzyme production and characterisation		
Start month	1	End month	40

Objectives

In WP4, the anticipated wealth of 1,000 enzymes pre-selected in WP2/WP3 will be expressed, purified and characterised, with the final objective to select 180 enzymes (20 x 9 enzyme classes) capable to be obtained at high yields and behaving better than benchmarks, according to their performance towards model and real-life substrate and stability under model and real-life conditions. The following sub-objectives are addressed:

- Streamlining semi-automatic, synthetic and cloning technologies compatible with more than 50 vectors and 12 hosts, cell-free expression systems, natural host production systems, and beyond state-of-the-art metamorphosis technology for the rapid and efficient expression of target and benchmark enzymes, including those with inherent problems of production;
- To efficiently generate, through fermentation and downstream processing, at least 1,000 enzymes (recombinant, native, biomimetic) for activity assessments;
- To characterise the 1,000 enzymes with model/real substrates and conditions requested by manufacturers;
- Through a decision-making strategy select ca. 180 enzymes with validated manufacturers' demands; and
- Through a combinatorial strategy to design multi-enzyme blends to process real complex substrates.

Description of work and role of partners

WP4 - Small-scale enzyme production and characterisation [Months: 1-40]
UHAM, CSIC, BANGOR, UDUS, IST ID, CNR, FHNW, Bio_Ch, EUCODIS
We propose 6 Tasks.

Task 4.1 Ultra-fast and efficient gene cloning and synthesis: downstream enzyme production M2-M30
Task Lead Partner – UHAM
Participants: CSIC, BANGOR, UDUS, CNR, EUC

Task 4.2 Smart design systems to obtain enzymes with inherent problems of expression M2-M30
Task Lead Partner – FHNW
Participants: CSIC, EUC

Task 4.3 Production of enzymes from their natural hosts M2-M30
Task Lead Partner – CNR
Participants: IST-ID, BIO_CH

Task 4.4 Enzyme characterisation for selecting those with manufacturers' specifications M2-M36
Task Lead Partner – UDUS
Participants: BANGOR, CSIC, UHAM, FHNW, IST-ID, EUC

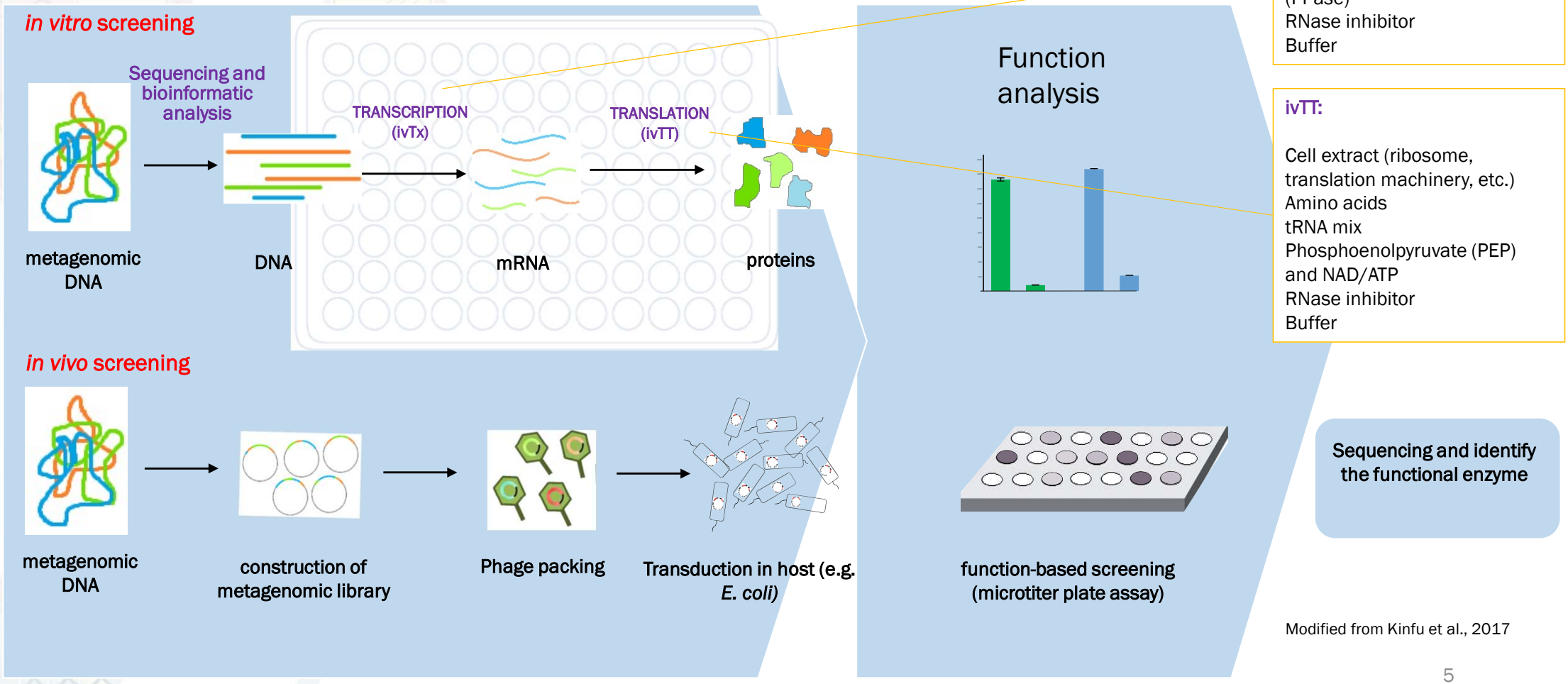
Task 4.5 Decision-making strategy for selecting lead enzyme candidates M6-M36
Lead partner – UDUS
Participants: BANGOR, UHAM, CSIC, CNR, IST-ID, FHNW, BSC, EUC

Task 4.6 Design of multi-enzyme blends to process complex ingredient mixtures M12-M40
Task Lead Partner – CSIC
Participants: BANGOR, UDUS, UHAM, IST-ID

List of deliverables

Deliverable Number[14]	Deliverable Title	Lead beneficiary	Type[15]	Dissemination level[16]	Due Date (in months)[17]
D4.1	QR barcoding system, available	1 - CSIC	Other	Confidential, only for members of the consortium (including the Commission Services)	3
D4.2	The FuturEnzyme Portfolio of 1,000 enzyme (recombinant/ native/biomimetic) material, obtained	1 - CSIC	Other	Confidential, only for members of the consortium (including the Commission Services)	16
D4.3	Cell-free expression/ reported system, developed	4 - UHAM	Other	Confidential, only for members of the consortium (including the Commission Services)	16
D4.4	Biomimetic protease production system, developed	9 - FHNW	Other	Confidential, only for members of the consortium (including the Commission Services)	16
D4.5	At least 9 enzyme crystal structures	1 - CSIC	Other	Confidential, only for members of the consortium (including the Commission Services)	30
D4.6	The metadata on expression yield, activity and stability, available	5 - UDUS	data sets, microdata, etc	Confidential, only for members of the consortium (including the Commission Services)	18
D4.7	At least 180 enzymes (recombinant, native, biomimetic) with attractive properties, available	1 - CSIC	Other	Confidential, only for members of the consortium (including the Commission Services)	18
D4.8	Set of high-performing multi-enzyme blends	1 - CSIC	Other	Confidential, only for members of the consortium (including the Commission Services)	20

Cell-free expression system



ivTx:

RNA Polymerase (RNAP)
rNTPs
inorganic pyrophosphatase (PPase)
RNase inhibitor
Buffer

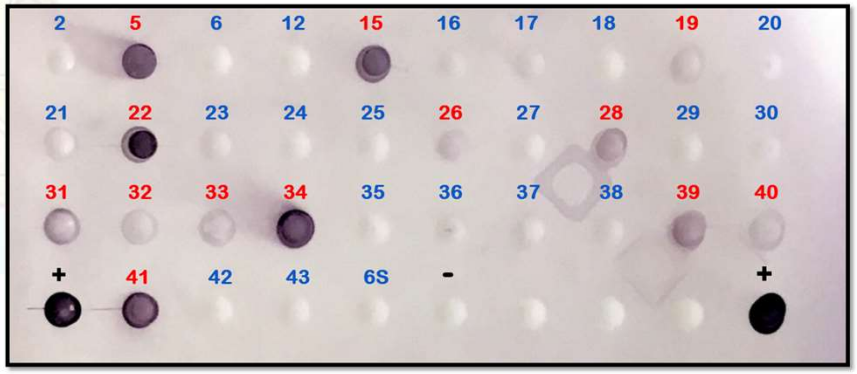
ivTT:

Cell extract (ribosome, translation machinery, etc.)
Amino acids
tRNA mix
Phosphoenolpyruvate (PEP) and NAD/ATP
RNase inhibitor
Buffer

Modified from Kinfu et al., 2017

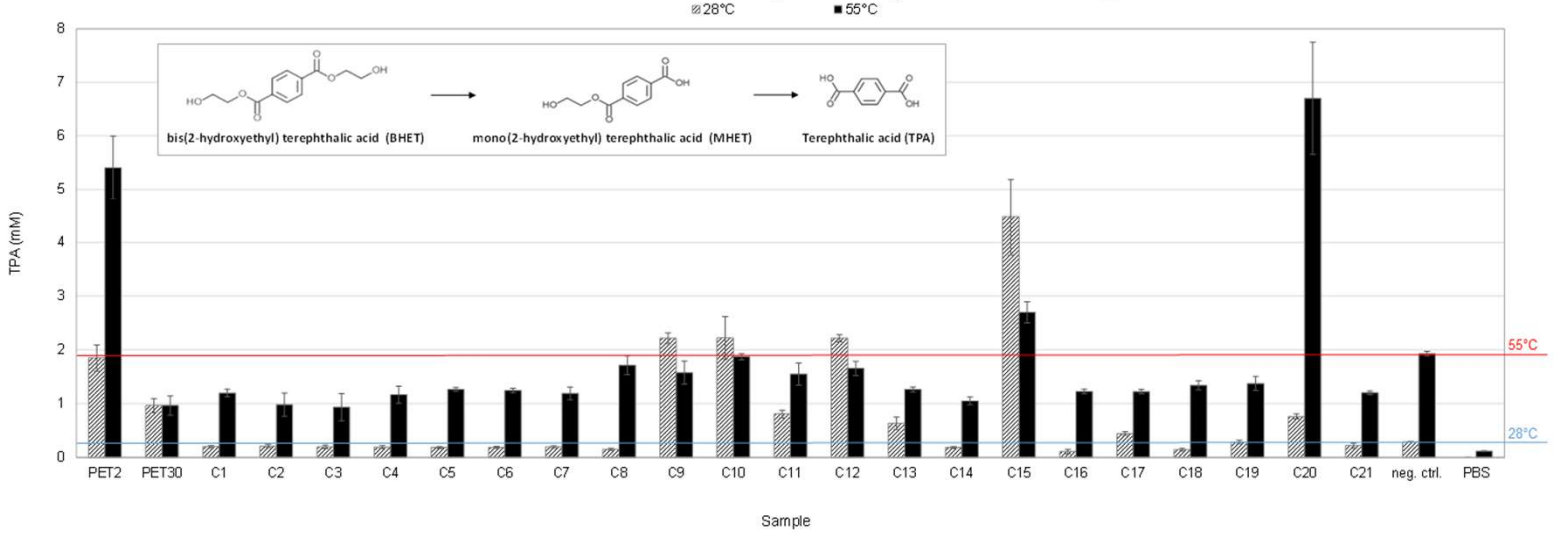
Cell-free expression of PETases

Western blot (anti-His)



+: purified His-tagged protein
-: master mix of ivTT without RNA

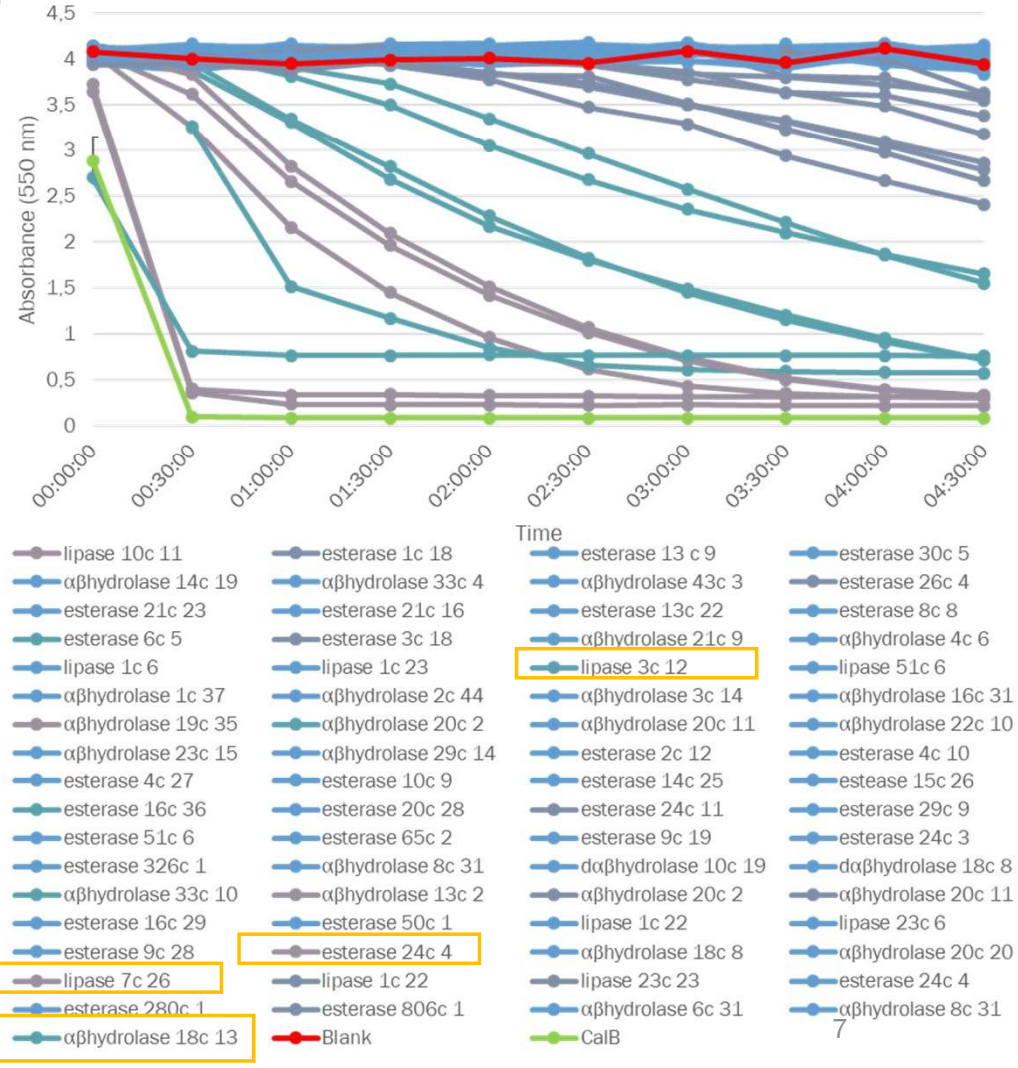
13 out of 34 PETase candidates can be expressed by ivTT with RNAP_E.



Testing the Esterase-/Lipase-collection

- Substrate tributyrin (TBT)
- pH shift assay with phenol red
- Lipolytic enzymes from UHAM expressed in small scale (2 ml) in *E. coli*
- Freeze-thaw lysis, lyophilisation

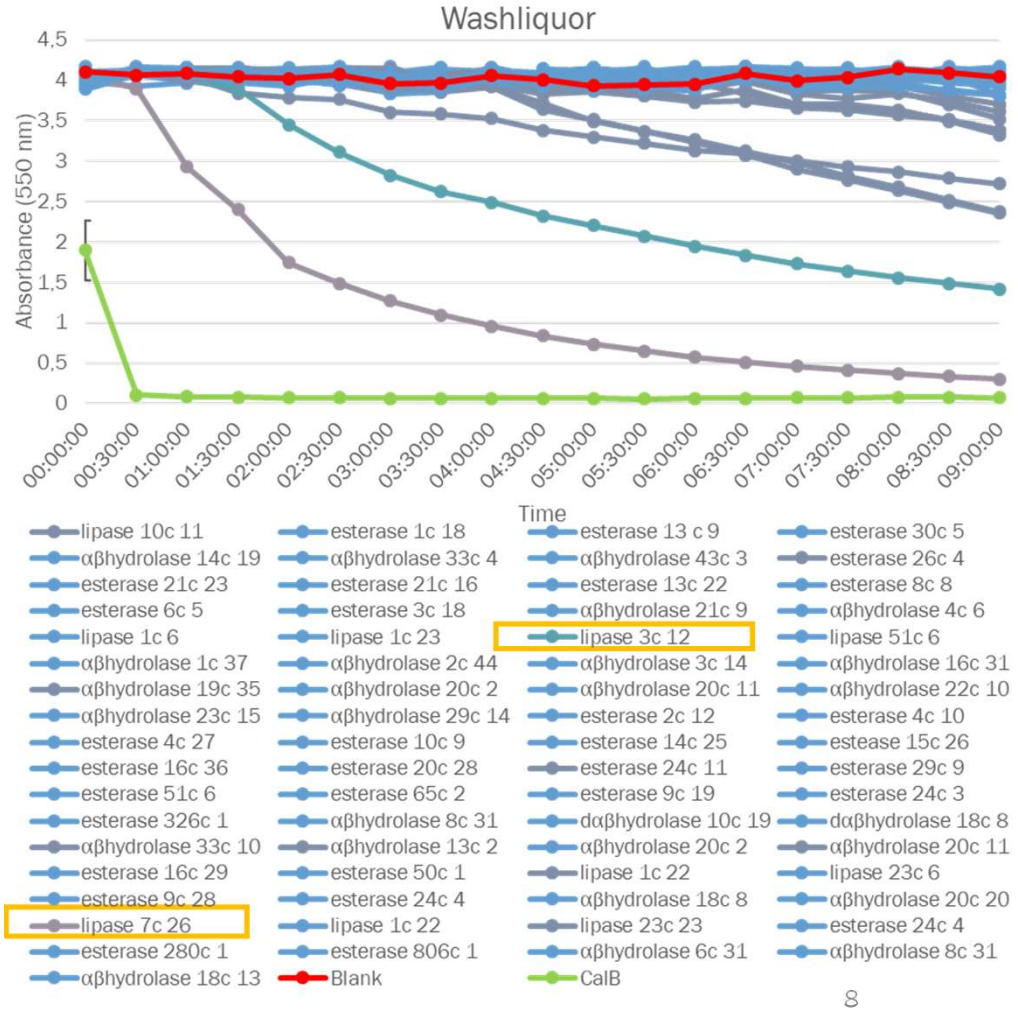
The absorbance was measured at 550 nm in 30 min intervals over 9 h. CalB was used as a positive control. The substrate was dissolved in DMSO



Testing the Esterase-/Lipase-collection

- Substrate tributyrin
- pH shift assay with phenol red
- Lipolytic enzymes from UHAM expressed in small scale in *E. coli*
- Freeze-thaw lysis, lyophilisation
- Resuspended in **laundry detergent from Henkel w/o enzymes**

- No activity on olive oil detectable…



- Lipase 3c 12
- Lipase 7c 26
- Esterase 24c 4
- α/β hydrolase 18c 13
- From fosmid library of
  - Elbe sediment Teufelsbrück, Hamburg
  - Elephant feces sample, Hamburg zoo

- Will be characterized now…

BLASTN/BLASTP

E-value 0.0 and 76% ident. Cellvibrio sp. KY-YJ-3/
E- value 0.0 and 93% hypothetical protein [Cellvibrio sp.]

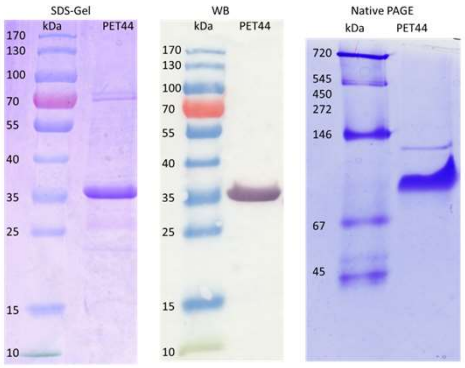
E-value 0.0 and 100% ident. uncultured bacterium /
E- value 0.0 and 100 % ATZ76728.1 Lip112 [uncultured bacterium](Martínez-Martínez et al. 2018)

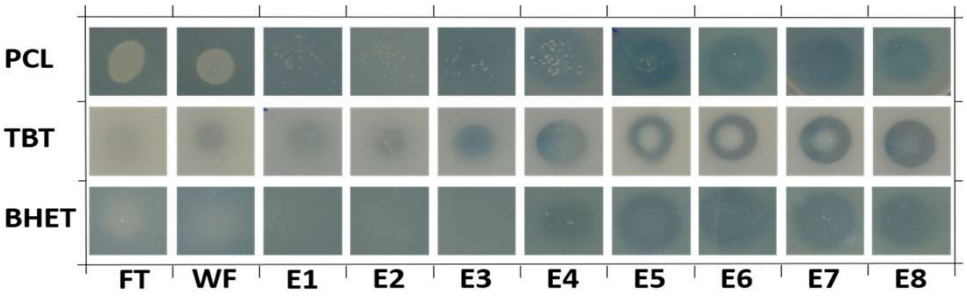
E-value 0.0 and 100% ident. uncultured bacterium /
E- value 0.0 and 100% ident LipB [uncultured bacterium] AAP76489.1 (Voget, S. et al. 2003)

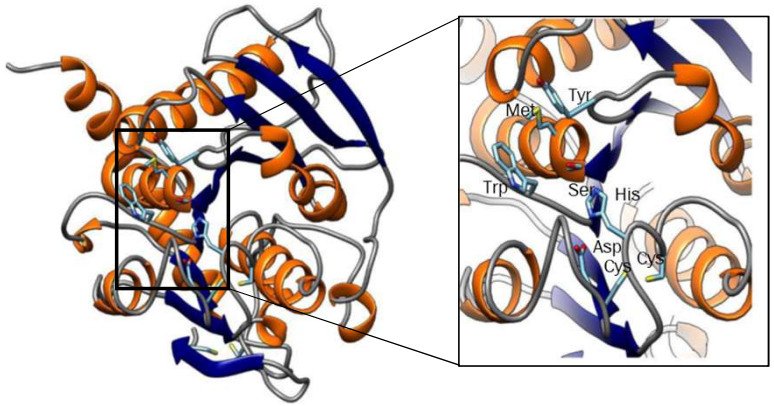
E-value 6e-18 and 82% ident. uncultured organism clone /
E- value 8e-156 71% alpha/beta hydrolase [Proteobacteria bacterium]

PET44

- Dienelactone hydrolase of species *Alkalilimnicola ehrlichii*
- PET44
  - 305 amino acids
  - 33 kDa
  - Temp. optimum 20-30 °C
- PET-degradation

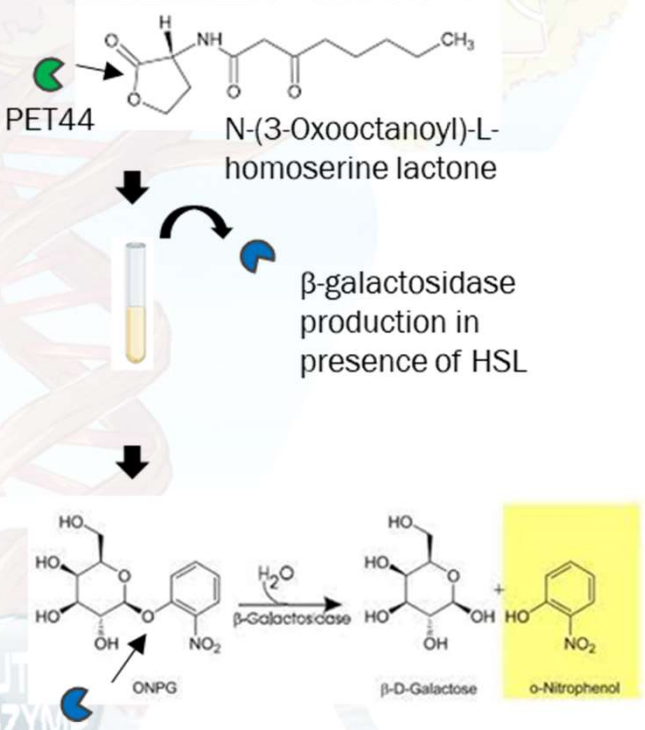


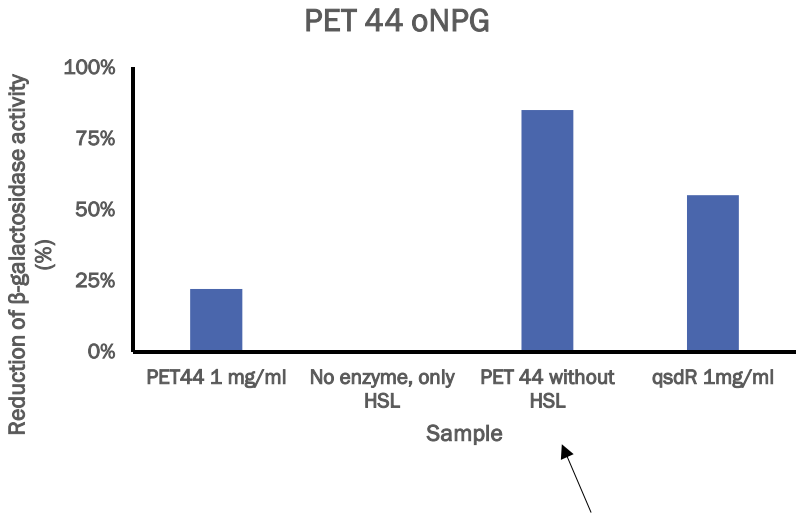




Lactonase assays

oNPG assay with reporter strain *A. tumefaciens* NTL4



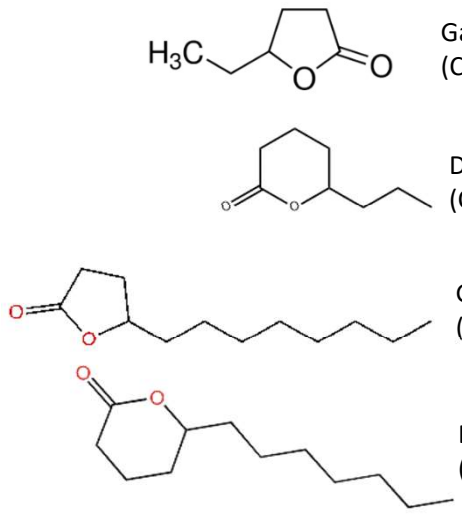


PET 44 without HSL: Shows hydrolysis of ONPG directly by PET 44 (without HSL)

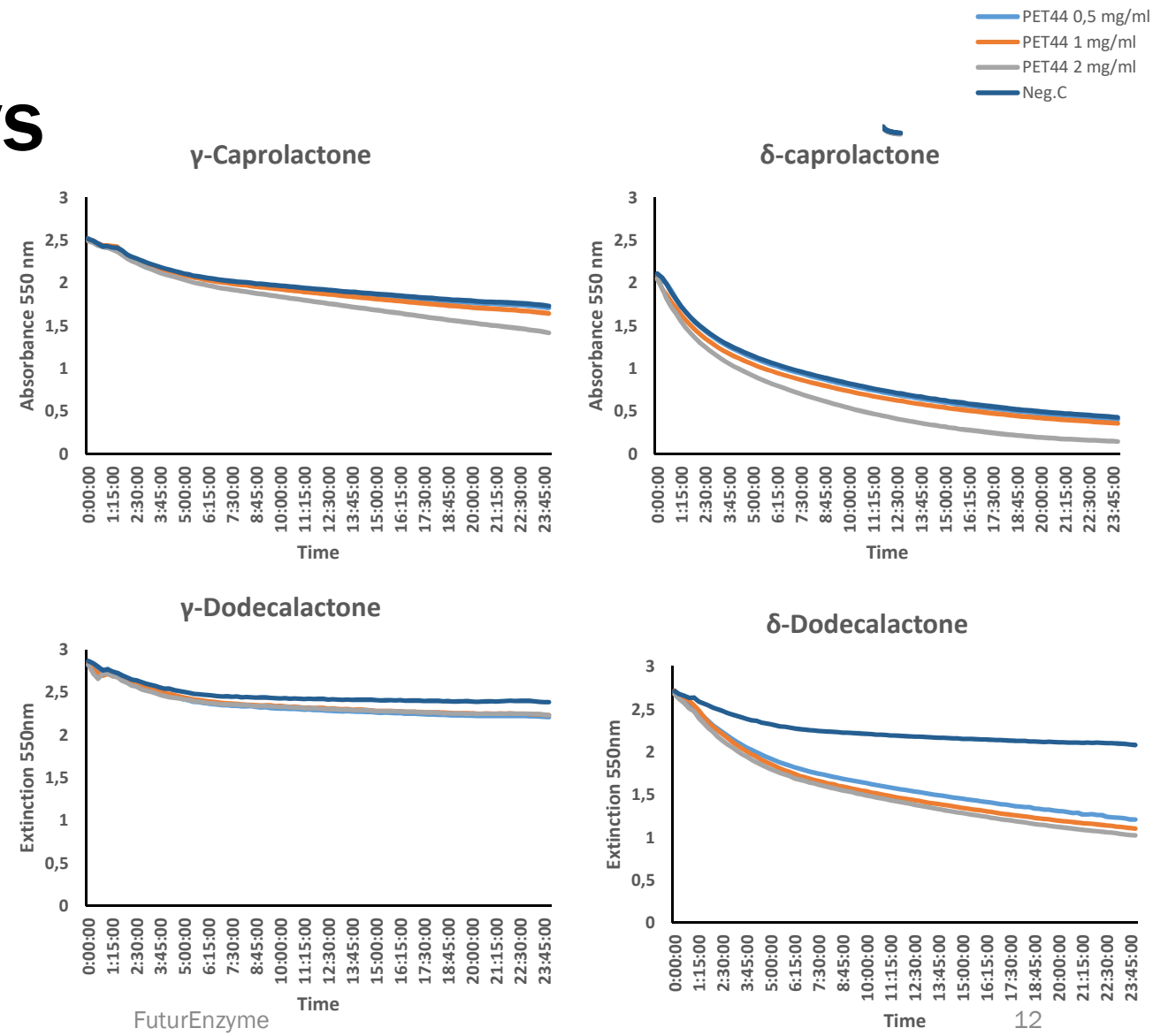
→ HSL hydrolysis by PET44 is detected
→ oNPG is directly hydrolysed by PET44

Lactonase assays

Phenol red assay with lactones:



-> works also for some PET-esterases tested so far



Next…

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Task 4.1 Ultra-fast and efficient gene cloning and synthesis: downstream enzyme production M2-M30

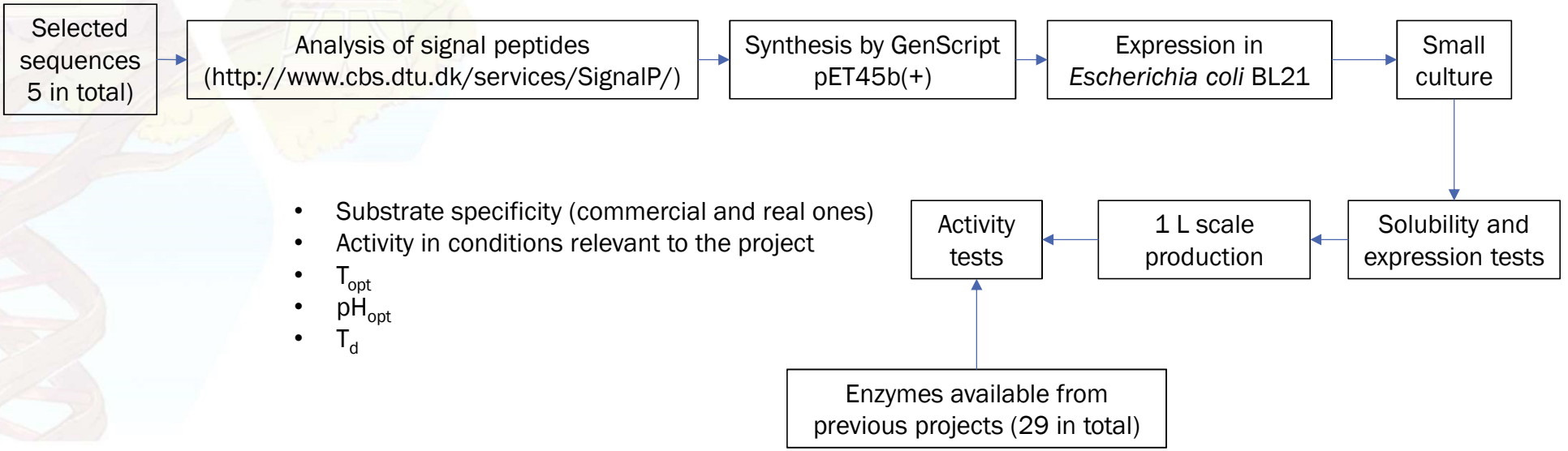
- Actually, 52 genes have been subjected to gene synthesis by CSIC.
  - These genes (identified by *in silico* and naïve screens) are relevant to detergents, textiles and cosmetics

CSIC used the pET-45b(+) expression vector and *E. coli* BL21(DE3), that supports the expression of N-terminal histidine (his) fusion proteins

- **22 esterases-lipases** (**FELip5***, FELip6, FELip7, **FELip8***, **FELip9***, **FEPolur1*, sid 180273*, EstA8***, XP_031855304.1, XP_018987368.1, NODE_14548, NODE_14220, **NODE_494**, **NEAIFBCB_56221***, NEAIFBCB_30006, NEAIFBCB_21793, **NEAIFBCB_14161***, NEAIFBCB_13373, **NEAIFBCB_72253, NEAIFBCB_16077*, NEAIFBCB_14633*, NEAIFBCB_100972***, NEAIFBCB_59123
- **7 hydrolases related to polymer degradation** (FEmeth3, FEmeth4, FEmeth5, FEmeth6, FEpolym1, **FEpolym2*** and FEpolym3), plus EH0, EH1, EH3, EH7, EH11, EH21, EH26, EH37, **FELip5***, **FELip9*** and **FEPolur1***
- **1 peptidase** (**FEm04_1***)
- **6 amidases** (FEami1, FEami2, FEami3, FEami4, FEami5 and FEami6)
- **4 amylases** (FEamy1, **FEamy2***, FEamy3 and FEamy4)
- **11 hyaluronidases** (FEhyal1, FEhyal2, FEhyal3, FEhyal4, FEhyal5, FEhyal6, FEhyal8, FEhyal9, FEhyal10, FEhyal14 and **HRDSV_2334***)

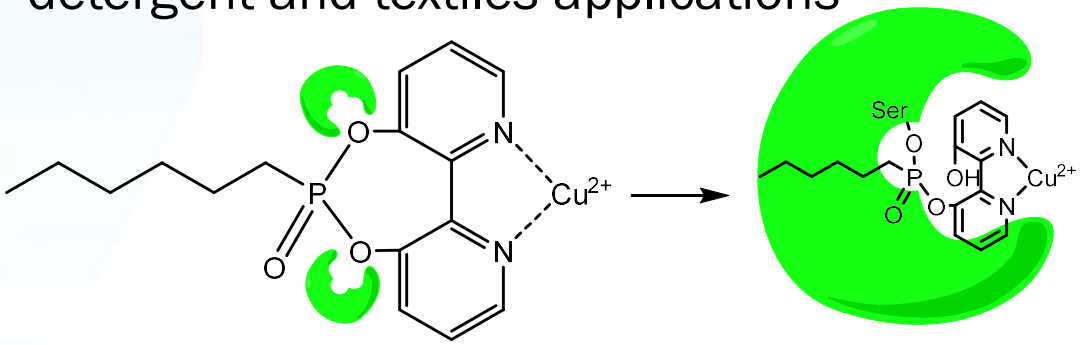
**Enzymes found to be produced with high expression level, in soluble form and active*

Task 4.1 Ultra-fast and efficient gene cloning and synthesis: downstream enzyme production M2-M30

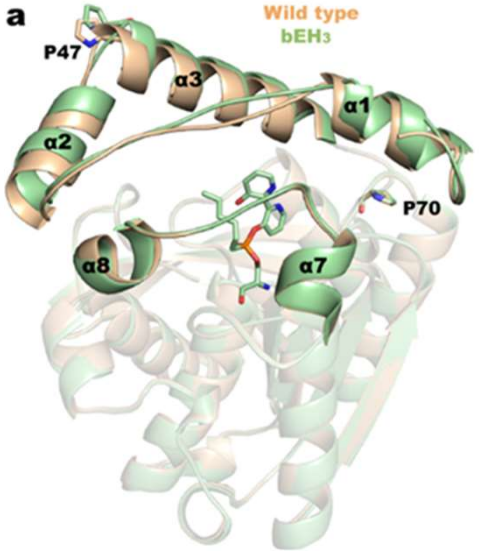


Task 4.2 Smart design systems to obtain sneymzes with inherent problems of expression M2-M30

- CSIC, EUCODIS and FHNW designed a versatile metal-chelating inhibitor to metamorphose esterase into proteases or oxidases
  - Esterase (EH3) easy to produce into a biomimetic protease relevant for detergent and textiles applications



6-hexyl-[1,3,2]dioxaphosphepino[5,4-b:6,7-b']dipyridine 6-oxide



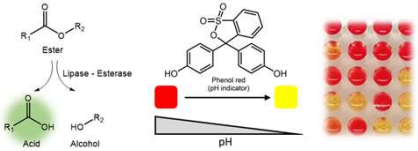
Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications M2-M36

- Lipase tests:

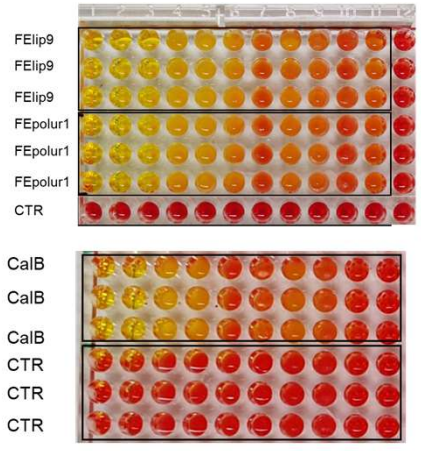
Reaction conditions for 384 plates:

- 40 µL 5 mM EPPS buffer pH 8.0 with 0.45 mM Phenol Red are added to each well
- Then, 2 µL substrate suspension* is added
- Finally, 2 µL enzyme solution (1.5-3.0 mg/ml) are added

Measure the decrease of absorbance at 550 nm in a spectrophotometer at 30ºC every 3 min.



Substrates
Glyceryl tripropionate (Tri_{C3})
Glyceryl tributyrate (Tri_{C4})
Glyceryl trioctanoate (Tri_{C8})
Glyceryl tridecanoate (Tri_{C10})
Glyceryl tridodecanoate (Tri_{C12})
Glyceryl trimyristate (Tri_{C14})
Coconut oil
Palm oil
Olive oil



1: Glyceryl triacetate (Tri-C2) (under assay conditions the substrates hydrolyzed alone)
2: Glyceryl tripropionate (Tri-C3)
3: Glyceryl tributyrate (Tri-C4)
4: Glyceryl trihexanoate (Tri-C6) (under assay conditions the substrates hydrolyzed alone)
5: Glyceryl tricaproate (Tri-C8)
6: Glyceryl tridecanoate (Tri-C10)
7: Olive oil (Tri-C16:1)
8: Coconut oil (triglycerides of C8:0, C10:0, C12:0, C14:0, C-16:0, C18:0, C18:1, C18:2)
9: Glyceryl tridodecanoate (Tri-C12)
10: Palm oil (mainly C16:0, C18:0, C18:1, C18:2 and C18:3)
11: Glyceryl trimyristate (Tri-C14)
12: None

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

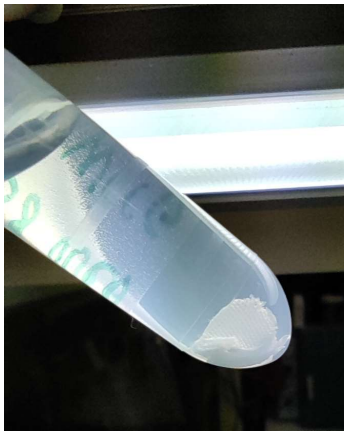
Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications M2-M36

- Lipase tests:

Substrates
Glyceryl tripropionate (Tri_{C3})
Glyceryl tributyrate (Tri_{C4})
Glyceryl trioctanoate (Tri_{C8})
Glyceryl tridecanoate (Tri_{C10})
Glyceryl tridodecanoate (Tri_{C12})
Glyceryl trimyristate (Tri_{C14})
Coconut oil
Palm oil
Olive oil

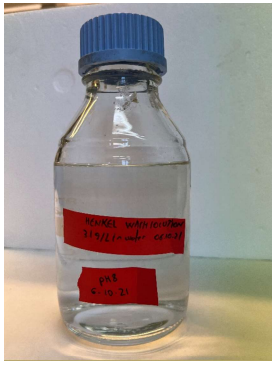
Triglyceride tests

pH-stat with stained swatches from CFT



Tests with textiles (modification)

PET/Fabrics degradation tests



Activity and stability in washing liquour

Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications M2-M36

- 0.1 mg butterfat cotton fabric in 20 ml of EPPS 5mM pH8
- 2% Lip9
- 2000 U/ml Lip9
- 2% CalB Novozyme
- 22.17 U/ml CalB Novozyme
- Room temperature 25°C

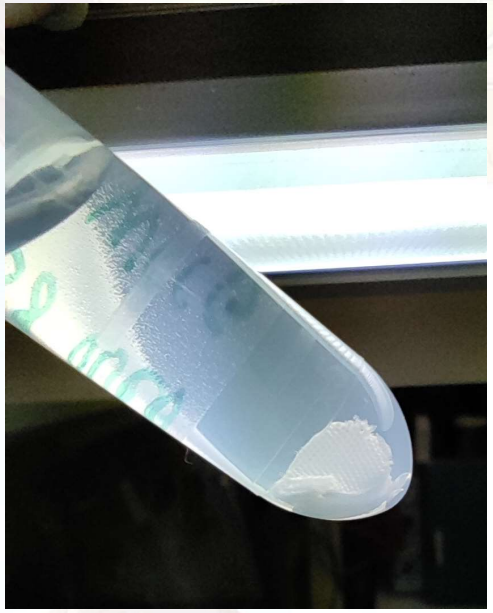
C-S-10 Butterfat with colourant

Dehydrated butter coloured with yellow colourant. Suitable to evaluate performance of active systems and lipase enzymes.

Not aged at elevated temperature.

Order code:	Substrate:	Width:	Yu:	Order code:	Substrate:	Width:	Yu:
C-S-10	Cotton (CN-11)	45 cm	89,1	PA-S-10	PolyAcryl (PAN-01)	45 cm	89,3
KC-S-10	Knitted Cotton (CN-42)	35 cm	94,5	S-S-10	Silk (T-601)	45 cm	83,9
PC-S-10	Polyester/Cotton (PCN-01)	45 cm	87,8	W-S-10	Wool (T-541)	45 cm	67,2
P-S-10	Polyester (PN-01)	45 cm	86,5	N-S-10	Nylon (T-365)	45 cm	87,4

Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications M2-M36



Fabrics:

Sample	Sample quality	Finished goods artikel Nr.	Schoeller's Description of the quality ROH=Raw and VORB= Pre-treated	Components / Weight	Pre-treatment steps	Comments
3	3-a	67007	67007 ROH	88% PA,12% EL 135g/m2		2m piece
	3-b		67007 VORB		Washing	2m piece
4	4-a	3X58	2X34G ROH	100% PES 100g/m2		2m piece
	4-b		3X58 VORB		Alkaline boiling	2m piece

Reaction conditions:

- Fabrics: 67007 (both) around 10 mg, 3X58(both) around 15mg
- 1.5 ml HEPES 40mM pH7
- Enzymes: 7-50 µg
- 40 °C, 1000 RPM, 48 hours

Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications M2-M36

Enzyme ID	Determination of pHopt															
	pH															
	3	3,5	4	4,5	5	5,5	6	6,5	7	7,5	8	8,5	9	9,5	10	11
EH0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	12,8	34,0	56,1	61,4	71,3	77,6	100,0	61,5	17,3
EH1	0,0	0,0	0,0	4,9	37,7	67,5	71,0	86,3	98,3	96,5	100,0	86,0	65,6	24,9	12,0	3,4
EH2	0,0	0,0	15,3	15,9	13,2	18,2	26,3	34,6	38,0	41,6	44,9	45,4	51,4	85,9	100,0	0,0
EH3	0,0	0,0	3,1	3,5	5,7	25,0	36,5	47,5	68,5	84,1	95,2	100,0	85,8	55,4	24,2	9,7
EH4	0,0	0,0	0,0	0,0	7,4	49,1	65,2	86,8	92,6	95,7	100,0	74,8	59,0	0,5	0,0	0,0
EH5	0,0	0,0	0,0	0,0	0,0	0,0	0,0	21,8	86,0	90,0	100,0	82,8	53,3	26,0	5,2	4,5
EH7	2,3	2,4	2,5	12,3	21,7	45,6	64,8	83,5	95,0	96,2	95,7	93,7	100,0	92,1	88,0	21,3
EH8	0,0	0,0	0,0	15,7	15,4	24,7	31,3	38,3	48,3	64,6	91,0	109,0	146,6	148,6	139,5	51,2
EH9	7,0	1,5	1,4	2,2	7,5	21,8	39,7	49,8	60,3	66,7	71,8	76,5	86,5	94,8	100,0	71,4
EH11 - EstA4	0,0	0,0	0,0	2,7	41,2	68,8	83,5	95,3	100,0	97,7	96,0	94,0	87,9	56,8	25,9	8,0
EH12	0,0	0,0	0,0	0,0	0,3	0,6	1,2	1,3	32,2	47,1	70,8	71,8	100,0	71,5	27,8	7,8
EH15 - EstA1	0,0	0,0	0,0	0,0	15,7	57,1	77,3	83,9	93,1	100,0	96,4	91,9	70,7	28,6	7,5	0,0
EH17	0,0	0,0	1,6	4,0	6,0	27,6	38,3	52,7	69,2	79,1	85,4	98,2	100,0	92,7	71,8	23,5
EH20 - EstA2	0,0	0,0	0,0	3,0	18,3	36,1	65,2	79,0	92,2	94,1	99,9	100,0	97,6	82,6	70,5	0,0
EH21	0,0	6,2	25,3	11,2	9,1	12,3	19,7	30,9	28,9	39,5	41,3	43,6	73,1	71,2	100,0	0,0
EH22 - EstA6	0,0	0,0	0,0	5,5	15,2	39,9	28,5	27,0	40,7	54,2	73,0	99,4	100,0	61,3	50,8	19,3
EH26	0,0	0,0	0,0	0,0	10,2	11,3	17,2	31,6	50,6	64,2	71,4	70,5	85,6	84,9	100,0	75,7
EH29 - EstA5	0,0	0,0	3,6	10,9	28,3	35,7	45,6	48,0	53,3	63,4	85,8	90,9	100,0	61,1	57,1	16,4
EH30 - EstB2	0,0	0,0	2,1	12,9	20,1	49,0	71,5	80,5	93,6	99,3	100,0	95,8	86,5	60,7	41,2	7,8
EH32	32,7	26,0	11,0	14,7	19,0	30,6	42,1	49,1	57,9	63,2	64,9	68,3	66,3	74,9	100,0	0,0
EH33	0,0	0,0	0,0	0,0	5,7	19,4	42,3	63,7	77,7	92,3	93,5	100,0	96,8	87,1	86,9	37,5
EH36	2,2	2,4	2,9	1,9	4,8	24,5	47,0	69,2	81,9	93,8	94,4	100,0	98,0	96,1	95,7	62,1
EH37	0,0	0,0	0,0	0,2	13,4	29,6	53,2	76,8	95,0	100,0	95,2	91,2	78,8	48,4	0,0	0,0
EH43	0,0	0,0	0,0	25,9	20,1	7,2	8,2	18,4	32,1	53,0	62,2	70,3	70,9	76,9	100,0	0,0
EH45 - EstB1	0,0	0,0	0,0	6,0	20,4	55,7	63,1	78,2	83,1	100,0	82,4	77,5	79,2	69,5	24,3	1,5
EH59 - EstA7	0,0	0,0	0,0	9,5	28,6	44,5	51,6	69,7	78,7	91,8	100,0	98,9	96,4	63,2	17,3	0,0
EH63	2,3	1,2	1,3	5,0	14,3	36,2	62,7	72,8	86,5	86,9	100,0	81,6	75,1	58,6	49,3	38,6
EH73 - EstA3	0,0	0,0	0,0	9,0	15,2	33,1	59,1	86,7	99,4	100,0	99,0	89,5	83,5	65,3	45,1	17,0
EH108	0,0	3,0	2,3	4,7	9,1	26,7	44,8	73,8	81,5	90,2	99,5	98,9	100,0	85,9	73,6	19,5
Lip5	0,0	0,0	0,0	15,0	31,3	40,0	50,6	100,0	69,9	66,4	67,7	64,1	57,3	64,0	35,5	76,1
Lip9	0,0	0,0	0,0	0,0	12,9	32,1	46,1	75,3	84,1	92,1	91,3	92,9	100,0	82,2	76,9	48,8
MetH3	0,0	0,0	0,0	0,0	0,0	0,0	49,7	72,8	80,6	88,5	100,0	89,4	84,4	76,6	74,3	38,1
Polur1	0,0	0,0	0,0	32,1	47,9	50,2	55,8	72,2	83,9	100,0	84,4	70,0	70,6	72,6	51,0	12,8
EstA8 - KY010301	0,0	0,0	0,0	2,0	25,3	59,9	66,4	68,5	81,7	84,5	85,5	100,0	64,1	51,2	25,2	0,0
FELip5_W89ML60F	0,0	0,0	0,0	15,0	31,3	40,0	50,6	100,0	69,9	66,4	67,7	64,1	57,3	64,0	35,5	76,1
FELip5_Lid	0,0	0,0	0,0	15,0	31,3	40,0	50,6	100,0	69,9	66,4	67,7	64,1	57,3	64,0	35,5	76,1
HRDSV_2334 (ID 3)	0,0	0,0	1,5	0,2	17,4	32,9	57,1	77,7	90,4	94,3	100,0	99,7	85,8	82,2	74,9	0,0
sid|180273 (ID 4)	0,0	0,0	0,0	1,1	11,0	25,4	47,9	69,3	79,4	85,4	87,7	91,0	100,0	88,3	74,3	18,1
NODE_494 (ID 9)	0,0	0,0	0,0	0,0	0,0	0,0	2,9	2,9	2,9	20,0	17,1	28,6	28,6	100,0	0,0	0,0
NEAIFBCB_56221 (ID 10)	0,0	0,0	0,0	1,1	18,7	40,7	50,5	50,5	41,8	54,9	89,0	90,1	98,9	82,4	100,0	63,7
NEAIFBCB_14161 (ID 21)	0,0	0,0	0,0	5,2	6,1	21,7	41,4	53,8	60,6	65,6	68,9	70,1	100,0	70,8	37,7	0,0
NEAIFBCB_72253 (ID 14)	0,0	0,0	2,7	5,3	9,0	19,5	38,5	58,5	74,5	80,4	89,0	97,2	100,0	92,5	96,9	53,7
NEAIFBCB_16077 (ID 15)	0,0	0,0	9,1	0,0	38,0	45,6	50,2	55,2	70,9	85,0	69,7	76,5	100,0	71,2	44,3	0,0
NEAIFBCB_14633 (ID 16)	0,0	0,0	8,3	32,7	51,8	47,2	63,8	95,4	83,1	81,3	78,9	100,0	35,3	50,8	49,1	33,8
NEAIFBCB_100972 (ID 17)	0,0	0,0	0,0	1,7	4,2	26,8	28,7	70,6	88,8	91,4	100,0	98,0	86,1	88,6	93,5	37,1

Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications M2-M36

Enzyme ID	Determination of Topt Temperature (°C)											
	10	20	25	30	35	40	45	50	55	60	65	70
EH0	25,9	53,6	54,2	73,8	85,5	89,4	100,0	46,0	25,9	27,7	21,9	10,0
EH1	41,1	50,7	61,9	79,3	94,7	100,0	97,0	84,7	53,0	18,5	3,6	0,0
EH2	56,2	52,2	71,5	74,0	88,6	88,3	100,0	72,8	53,9	39,0	36,5	29,1
EH3	40,3	43,4	47,4	57,5	68,3	86,2	99,3	100,0	89,1	71,4	65,5	29,8
EH4	51,1	80,5	71,9	86,5	100,0	83,4	56,6	57,9	33,9	41,3	40,2	31,9
EH5	77,6	100,0	85,2	59,4	39,9	11,7	32,6	20,1	21,5	31,6	20,7	18,9
EH7	23,3	49,0	59,3	60,0	71,3	82,4	82,9	96,9	94,3	100,0	98,2	77,8
EH8	82,0	100,0	83,3	64,5	34,8	22,3	20,3	27,4	14,7	0,0	0,0	0,0
EH9	45,0	91,8	100,0	89,3	69,8	52,5	36,3	40,3	42,5	28,8	34,9	25,6
EH11 - EstA4	32,6	60,6	66,6	73,8	75,3	84,2	85,5	89,1	100,0	91,3	49,2	11,6
EH12	82,5	100,0	87,1	79,0	40,5	44,3	32,7	39,1	21,1	29,5	22,9	22,8
EH15 - EstA1	42,0	59,0	67,0	78,1	81,7	100,0	99,5	93,1	88,2	82,6	20,3	2,5
EH17	43,0	48,6	55,3	72,4	90,0	100,0	96,9	95,6	88,0	63,6	34,7	32,5
EH20 - EstA2	44,6	67,4	72,4	91,5	91,8	96,4	100,0	95,8	89,0	78,2	73,1	31,5
EH21	87,7	91,1	100,0	68,5	86,0	47,2	65,1	24,7	0,0	0,0	0,0	0,0
EH22 - EstA6	54,5	67,1	76,1	79,6	82,4	95,9	100,0	90,6	82,4	82,5	53,7	11,0
EH29 - EstA5	60,7	82,1	85,9	100,0	95,4	93,3	83,0	78,6	77,4	69,3	60,7	20,2
EH30 - EstB2	17,6	34,9	47,4	49,5	58,7	72,1	81,8	87,1	100,0	97,8	95,6	65,4
EH32	82,9	97,4	100,0	92,5	34,0	38,8	26,8	28,8	4,6	9,4	0,0	0,0
EH33	44,5	58,6	59,1	100,0	97,8	87,4	56,5	26,0	30,5	33,0	35,2	25,4
EH36	27,8	50,1	72,7	100,0	76,3	46,0	19,8	30,3	19,7	16,4	21,5	7,9
EH37	46,9	76,5	100,0	79,3	53,9	30,2	22,7	19,8	13,3	24,8	17,9	24,4
EH43	63,8	70,7	81,5	88,8	100,0	61,4	59,3	57,0	42,1	44,3	42,7	29,8
EH45 - EstB1	56,1	64,6	74,3	80,1	81,3	90,3	92,3	100,0	97,3	88,8	80,1	30,1
EH59 - EstA7	13,7	26,5	43,3	53,0	61,4	64,1	81,0	88,0	91,3	100,0	85,9	35,2
EH63	20,7	24,8	38,5	63,8	60,0	69,6	84,9	91,1	100,0	87,9	27,5	19,1
EH73 - EstA3	23,2	40,0	43,1	49,1	55,9	62,0	67,6	79,3	83,9	100,0	91,4	41,2
EH108	30,7	40,1	50,7	73,3	79,3	100,0	73,2	59,9	32,4	25,5	16,5	22,7
Lip5	7,3	17,8	25,1	34,0	46,3	61,9	100,0	83,0	39,6	3,4	0,4	0,0
Lip9	12,0	53,2	57,4	100,0	90,7	88,2	69,8	36,1	24,5	17,8	8,9	6,7
MetH3	10,1	28,73	33,48	49,2	60,08	68,67	79,33	86,99	91,09	100	73,01	52,2
Polur1	5,3	31,0	36,5	54,1	68,7	83,0	99,6	100,0	94,9	66,1	21,4	4,7
EstA8 - KY010301	13,9	69,9	81,8	97,0	100,0	93,5	68,7	46,7	34,2	20,5	3,2	0,0
NODE_494 (ID 9)	20,1	54,1	58,0	60,1	85,2	100,0	55,6	10,8	10,0	8,9	1,1	0,0

Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications M2-M36

Enzyme ID	Specific activity (units(mg)								
	TRIC3	TRIC4	TRIC8	TRIC10	TRIC12	TRIC14	COCONUT OIL	PALM OIL	OLIVE OIL
EH0	8396,5	29433,2	6613,0	0,0	0,0	0,0	0,0	0,0	0,0
EH1	1116,0	2247,0	440,8	0,0	0,0	0,0	0,0	0,0	0,0
EH2	421,8	284,6	867,6	30,1	0,0	0,0	0,0	0,0	0,0
EH3	1475,9	1730,3	1504,1	0,0	0,0	0,0	0,0	0,0	0,0
EH4	427,0	58136,3	469,9	44,5	0,0	0,0	0,0	0,0	0,0
EH5	1298,0	416,7	991,1	0,0	0,0	0,0	0,0	0,0	0,0
EH7	760,4	1683,8	1640,2	0,0	0,0	0,0	0,0	0,0	0,0
EH8	38,2	2511,9	235,2	4,7	0,0	0,0	0,0	0,0	0,0
EH9	2453,2	2041,3	731,2	0,0	0,0	0,0	0,0	0,0	0,0
EH11 - EstA4	11346,3	53174,7	168,1	0,0	0,0	0,0	0,0	0,0	0,0
EH12	1555,2	3360,0	8341,0	0,0	0,0	0,0	0,0	0,0	0,0
EH15 - EstA1	218,8	850,3	0,0	0,0	0,0	0,0	0,0	0,0	0,0
EH17	0,2	23,5	6,8	0,0	0,0	0,0	0,0	0,0	0,0
EH20 - EstA2	16,7	26,6	0,4	0,0	0,0	0,0	0,0	0,0	0,0
EH21	157,3	963,5	28,7	1,5	0,0	0,0	0,0	0,0	0,0
EH22 - EstA6	218,5	665,5	159,3	0,0	0,0	0,0	0,0	0,0	0,0
EH26	0,0	3,8	0,0	0,0	0,0	0,0	0,0	0,0	0,0
EH29 - EstA5	1086,0	2938,4	352,0	0,0	0,0	0,0	0,0	0,0	0,0
EH30 - EstB2	5,3	23,1	6,8	0,0	0,0	0,0	0,0	0,0	0,0
EH32	25,4	242,6	27,0	0,0	0,0	0,0	0,0	0,0	0,0
EH33	16,5	256,9	61,6	19,3	0,0	0,0	0,0	0,0	0,0
EH36	7,4	264,8	4,3	0,8	0,0	0,0	0,0	0,0	0,0
EH37	2,0	59,8	30,8	0,0	0,0	0,0	0,0	0,0	0,0
EH43	278,6	12,8	14,8	0,0	0,0	0,0	0,0	0,0	0,0
EH45 - EstB1	2,1	22,8	0,4	0,0	0,0	0,0	0,0	0,0	0,0
EH59 - EstA7	2,2	18,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
EH63	10,1	38,2	41,5	0,0	0,0	0,0	0,0	0,0	0,0
EH73 - EstA3	678,0	110,1	0,3	0,0	0,0	0,0	0,0	0,0	0,0
EH108	0,2	14,8	20,2	0,0	0,0	0,0	0,0	0,0	0,0
Lip5	985,8	1084,2	3228,0	0,0	0,0	0,0	0,0	0,0	0,0
MetH3	59,5	114,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0
EstA8 - KY010301	1011,2	4999,6	734,9	5,6	0,0	0,0	0,0	0,0	0,0
HRDSV_2334 (ID 3)	186,9	72,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0
NEAIFBCB_56221 (ID 10)	785,3	306,3	9,8	2,0	1,0	0,0	0,0	0,0	0,0
NEAIFBCB_14161 (ID 21)	11,3	125,4	78,6	0,0	0,0	0,0	0,0	0,0	0,0
XP_031855304.1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
XP_018987368.1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
NODE_14548_length_1646_cov_1.690368_2	40,6	72,7	0,0	0,0	0,0	0,0	0,0	0,0	0,0
NODE_14220_length_5773_cov_2.183645_2	91,5	86,8	0,0	0,0	0,0	0,0	0,0	0,0	0,0
NEAIFBCB_30006	173,1	147,5	0,0	0,0	0,0	0,0	0,0	0,0	0,0
NEAIFBCB_21793	38,3	39,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0
NEAIFBCB_59123	0,7	1,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0
NEAIFBCB_13373	12,2	18,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0
IS12	1071,7	2950,1	1558,0	0,0	0,0	0,0	0,0	0,0	0,0
IS10	979,1	3429,9	1100,0	0,0	0,0	0,0	0,0	0,0	0,0
IS11	9,0	32,2	30,9	0,0	0,0	0,0	0,0	0,0	0,0
Lip6	88,9	93,2	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Lip7	129,6	137,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0

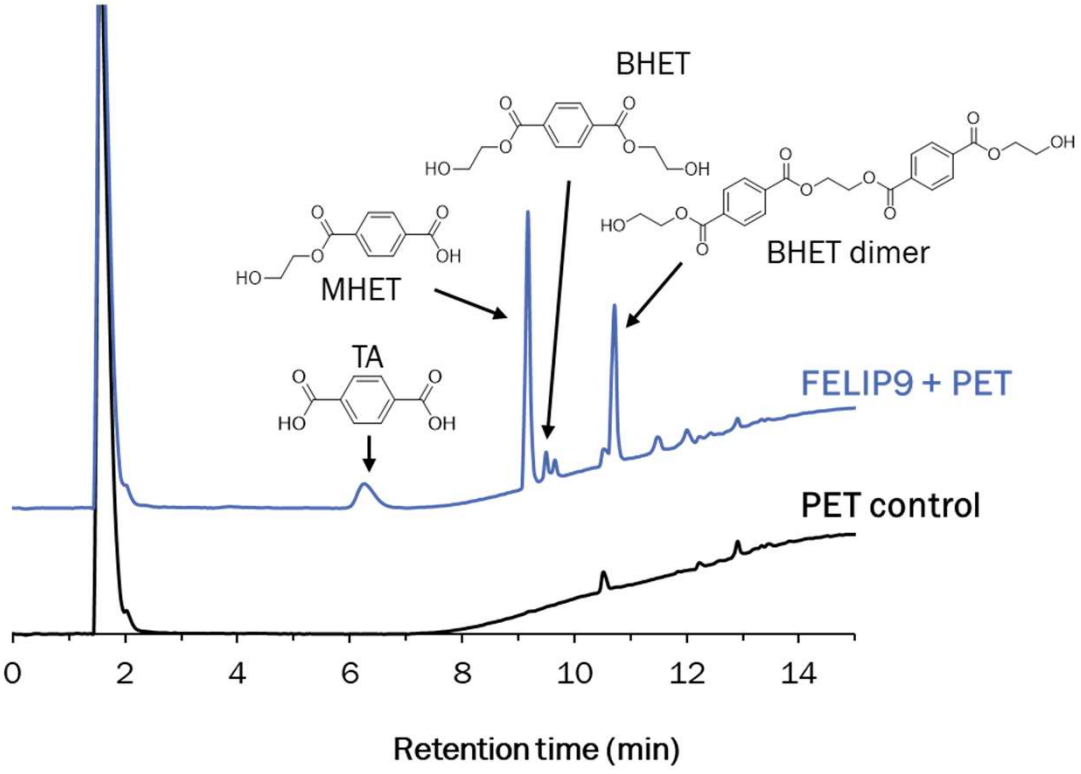
Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications M2-M36

Enzyme ID	Specific activity (units(mg)											
	TRIC3	TRIC4	TRIC8	TRIC10	TRIC12	TRIC14	COCONUT OIL	PALM OIL	OLIVE OIL	Stable in washing liquour	Topt	pHopt
Lip9	471,0	965,1	1720,1	886,8	314,2	111,4	582,7	144,5	73,8	YES	25-30°C	8,5-9,5
Polur1	169,6	348,3	392,1	156,0	117,9	33,7	254,0	30,5	27,3	YES	45-55°C	7,0-7,0
FELip5_W89ML60F	935,0	154,3	1323,2	1224,9	111758,0	1,8	0,0	0,0	0,0	YES	35-40°C	6,5-7,0
FELip5_Lid	778,6	262,5	87,5	58,6	49,6	7,4	61,0	28,6	0,0	YES	35-40°C	6,5-7,0
sid|180273 (ID 4)	82,1	241,9	229,2	18,7	8,7	4,3	16,4	1,1	1397,3	NO	ND	7,0-9,5
NODE_494 (ID 9)	12,0	100,4	1604,1	174,7	4,0	0,4	6,0	4,0	0,5	YES	35-40°C	9,0-9,5
NEAIFBCB_72253 (ID 14)	145,8	607,2	138,2	15,5	6,4	7,7	12,8	1,5	1,4	YES	ND	7,5-10
NEAIFBCB_16077 (ID 15)	475,1	133,1	122,9	23,7	4,3	2,1	9,8	1,8	1,8	YES	ND	8,5-9,5
NEAIFBCB_14633 (ID 16)	240,8	41,8	12,3	9,7	8,4	3,2	8,6	4,4	0,0	YES	ND	6,5-8,5
NEAIFBCB_100972 (ID 17)	2866,9	1356,6	1258,9	893,5	67,0	34,4	421,8	16,9	11,4	YES	ND	7,0-10,0
Lip8	172,9	177,6	203,8	23,8	22,7	7,2	4,4	2,7	0,0	YES	ND	ND
CalB	461,18	445,61	63,3	40,92	31,23	19,29	11,15	16,09	3,26	YES	ND	ND

Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications M2-M36

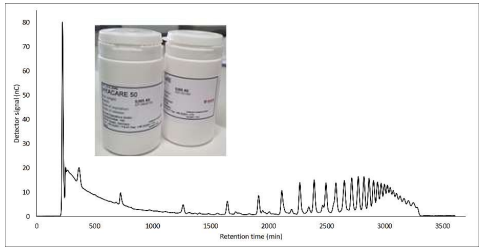
	Degradation tests with BHET					
Enzyme ID	U/g	SD	TA	MHET	BHET	Conversion (%)
EH0	163,63	6,15	0,00	2,47	1,53	61,8
EH1	7,68	0,02	0,00	0,13	3,87	3,3
EH3	134,60	11,27	0,02	3,38	0,61	84,8
EH7	241,61	8,50	0,35	3,06	0,59	85,2
EH11 - EstA4	98,02	0,07	0,07	1,97	1,96	50,9
EH21	0,10	0,01	0,00	0,37	3,63	9,3
EH26	76,54	0,18	0,00	3,29	0,71	82,3
EH37	4,43	0,26	0,00	0,09	3,91	2,2
Lip5	3,91	0,01	0,00	0,61	3,39	15,3
Lip9	0,78	0,00	0,18	1,91	1,90	52,4
Polur1	4,43	0,01	0,00	0,87	3,13	21,8

	Degradation tests with PET			
Enzyme ID	TA	MHET	BHET	Conversion (%)
EH0	0	0	0	0
EH1	0	0	0	0
EH3	0	0	0	0
EH7	0	0	0	0
EH11 - EstA4	0	0	0	0
EH21	0	0	0	0
EH26	0	0	0	0
EH37	0	0	0	0
Lip5	0	0	0	0
Lip9	0,039	0,170	0,015	20,4
Polur1	0	0	0	0

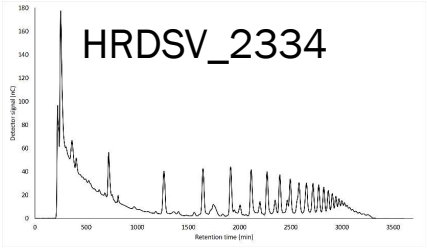


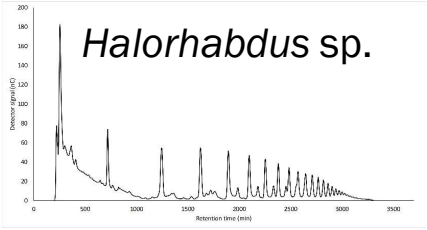
Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications M2-M36

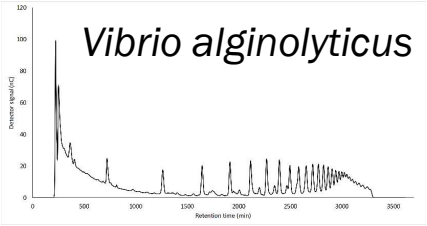
- Hyluronidase tests:
  - HRDSV_2334
  - *Halorhabdus* sp. SivX81 from CNR
  - *Vibrio alginolyticus from CNR*
  - *Other 5 strains from CNR*



Gel Filtration Chromatography (GFC):
Shodex OHPak SB804
HQ 300x 7.8 mm



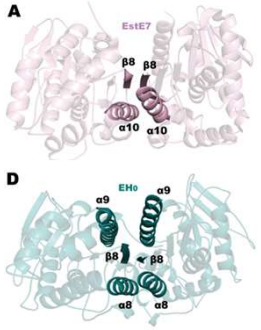




Task 4.5 Decision-making strategy for selecting enzyme candidates M6-M36

BASED ON SUMMARY TASK 4.4 CSIC SUGGEST TO FOCUS ON THE FOLLOWING ENZYMES:

- 11 lipases (Lip9, Polur1, FELip5_W89ML60F, FELip5_Lid, sid|180273, NODE_494, NEAIFBCB_72253, NEAIFBCB_16077, NEAIFBCB_14633, NEAIFBCB_100972 and Lip8) showed properties of interest to detergents and textiles: they are active towards long-fatty acid triglycerides and active and stable in washing liquour and 20-45ºC.
- 11 hydrolases are degrading BHET (EH0, EH1, EH3, EH7, EH11, EH21, EH26, EH37, **FELip5**, **FELip9,** and **FEPolur1**) – the X-Ray structure solved for 2 of them
  - From 2,2 to 84,8% when using 0.1 mg protein/ml, 2 mg material/ml at 30ºC and 24 h
- 1 hydrolase (**FELip9**) is degrading PET and polyester textiles
  - 20,4% conversion when using 0.1 mg protein/ml, 2 mg/ml material at 30ºC and 24 h
- 1 hyaluronidase (HRDSV_2334) and two strains showed interestign features for HA degradation
  - 50% conversion when using 1 mg protein/ml, 100 mg/ml material at 30ºC and 24 h



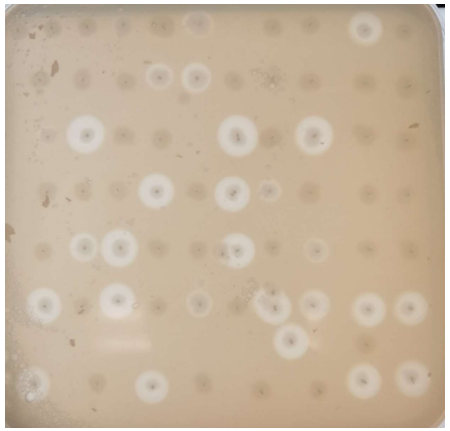
Next…

UDUS

- WP4
  - Task 4.1 Ultra-fast and efficient gene cloning and synthesis: downstream enzyme production

✓60 enzymes out of 88 lipolytic enzymes expressed in active form using *E. coli* in deep well plates for HTS with cell extracts

✓Cutinase-like enzymes productions studies in *B. subtilis* as alternative host to enhance soluble protein yields



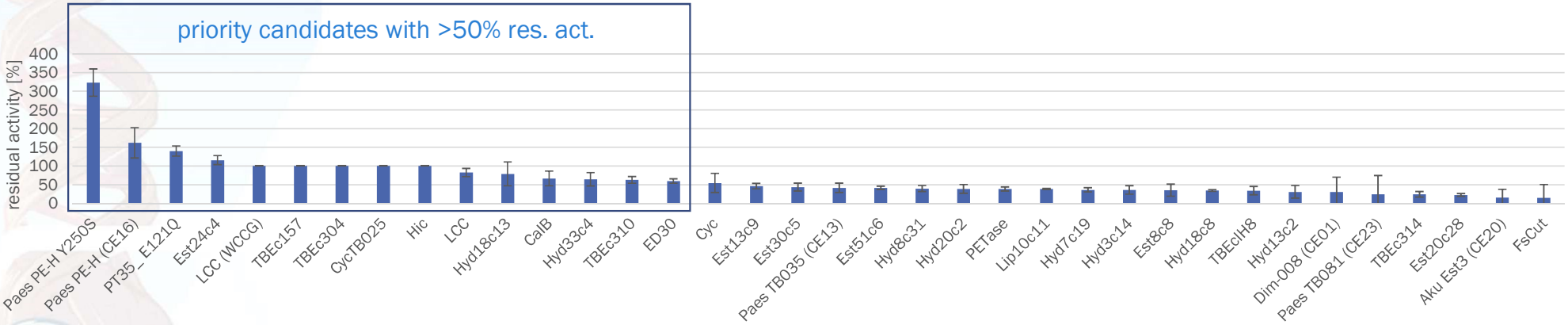
B. subtilis producing the cutinase-like enzyme Paes_PE-H from a signal peptide library on polyester indicator plates

UDUS

- WP4
  - ➢ Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications

✓Esterase & lipase set: first tests with cell extracts for detergent compatibility (Henkel)

✓Residual enzyme activity (*p*-NPB) after 1h under washing liquor conditions



➢Next: choose priority candidates to test real substrates & shelf life

UDUS

- WP4
  - ➢ Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications

✓Activity screening of esterase/lipase collection with industry-oriented substrates using the NEFA kit

➢ Detection of **N**on-**E**sterified **F**atty **A**cid (NEFA) in a two-step colorimetric assay

➢ Substrates: standardized stains of:

1) beef fat on polyester/cotton mix
2) lipstick on polyester/cotton mix
3) collar stain on polyester/cotton mix

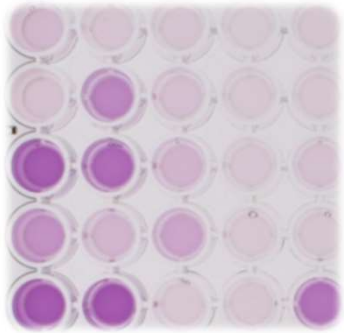
Expression in BL21(DE3) and subsequent lysis

Incubation with ca. 0.1 cm^2 of stained fabric in KPi + 1% Triton

Transfer of reaction mix to new plate for NEFA assay

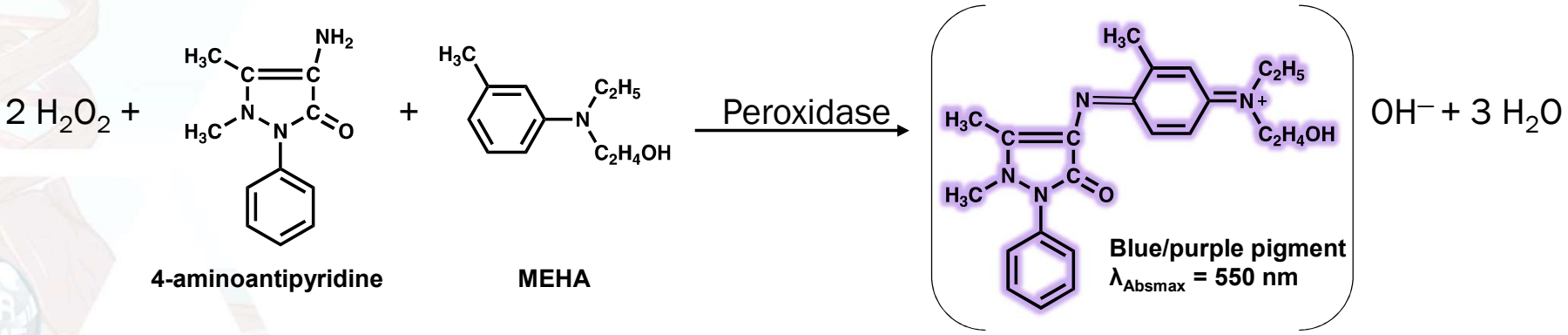
UDUS

- NEFA Kit: Detection of Non-Esterified Fatty Acids
- Two-step colorimetric assay



1) RCOOH + ATP + CoA $\xrightarrow{\text{Acyl-CoA synthase}}$ Acyl-CoA + AMP + PPi

2) Acyl-CoA+ O_2 $\xrightarrow{\text{Acyl-CoA oxidase}}$ 2,3-trans-Enoyl-CoA + H_2O_2

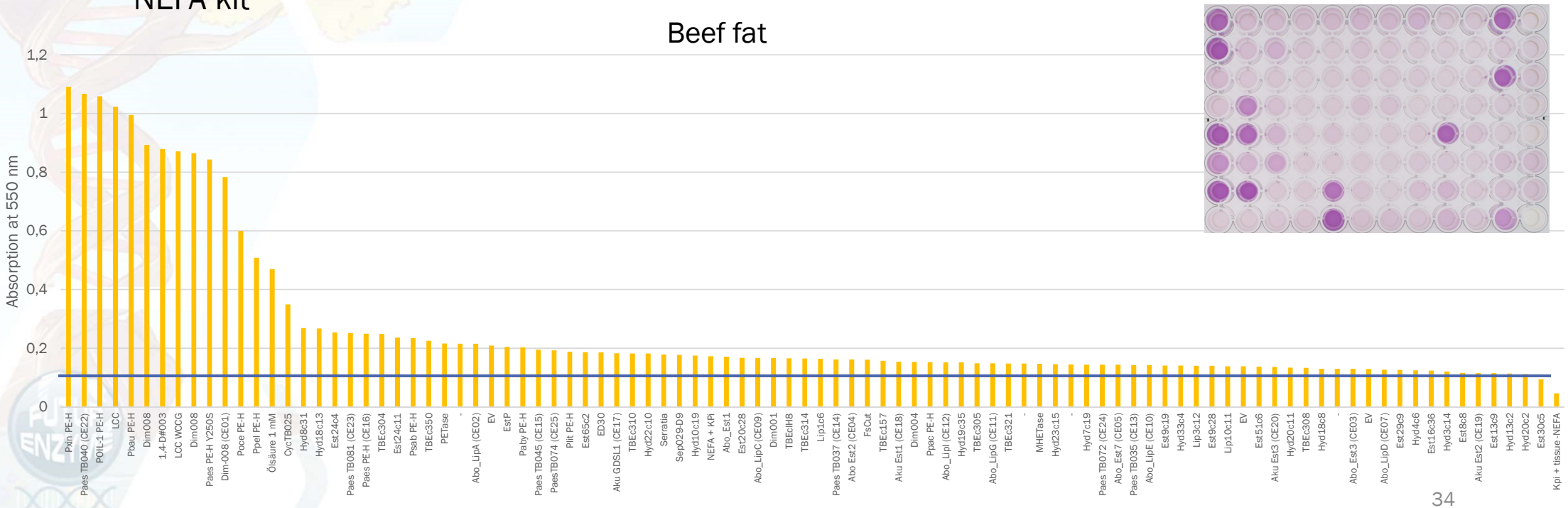


4-aminoantipyridine **MEHA**

UDUS

- WP4
  - ➢ Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications

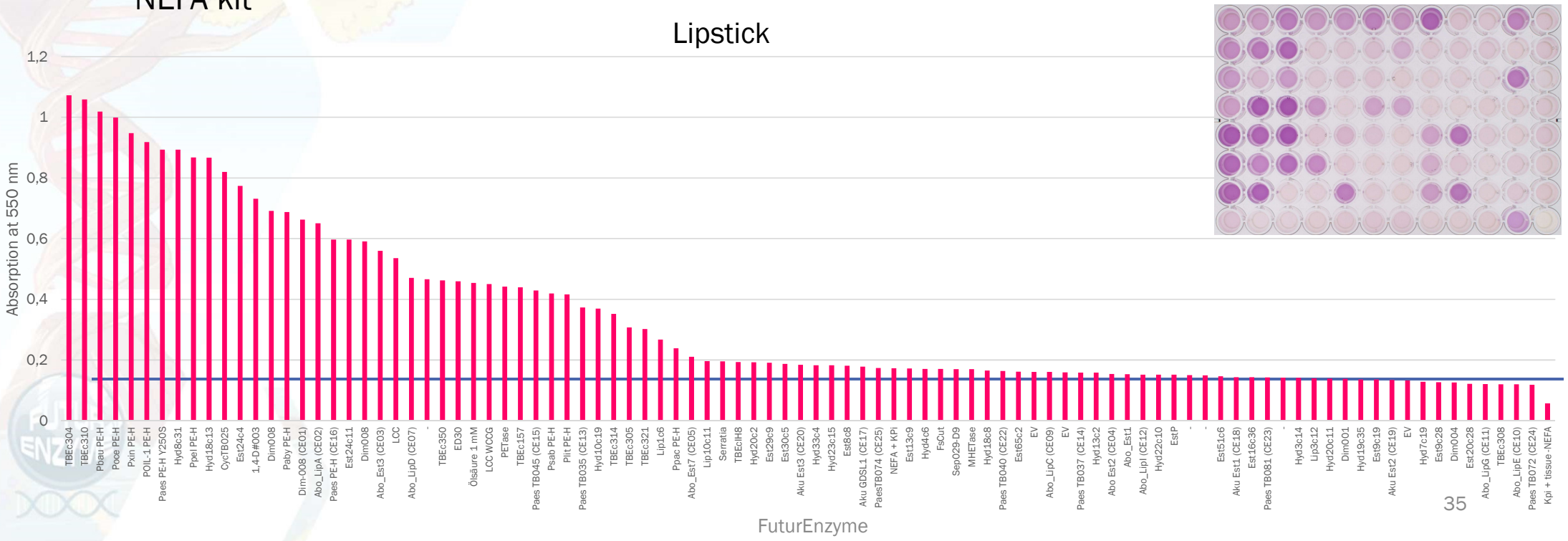
✓ Activity screening of esterase/lipase collection with industry-oriented substrates using the NEFA kit



UDUS

- WP4
  - ➢ Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications

✓ Activity screening of esterase/lipase collection with industry-oriented substrates using the NEFA kit



UDUS

- WP4
  - ➢ Task 4.4 Enzyme characterization for selecting those with manufacturers' specifications

✓ Activity screening of esterase/lipase collection with industry-oriented substrates using the NEFA kit

➢ Substrates: standardized stains of beef fat, lipstick, collar stain on polyester/cotton mix

- Beef fat with violet pigment: 17 enzymes with lipolytic activity
- Pink lipstick: 40 enzymes with lipolytic acitvity
- Collar: no specific activity detectable since stain might already be composed of free fatty acids

➢ 12 lipolytic enzymes (PE hydrolases, hydrolases and put. lipases) with detectable activity on both lipstick and beef stains

Next...

Heinrich Heine Universität Düsseldorf

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Task 4.3 Production of enzymes from their natural hosts

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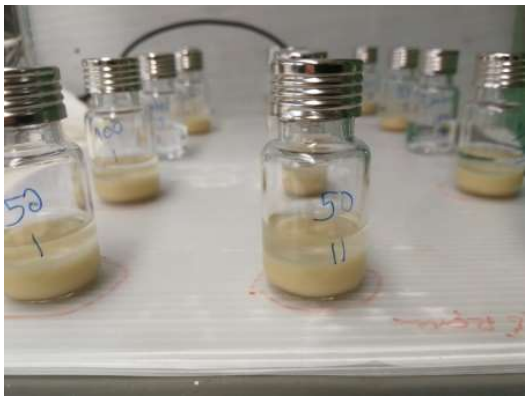
- conditions for cultivation of microbes for highest enzyme activities; assessment of enzyme performance under industrial relevant conditions; testing enzymes in small scale bioreactors



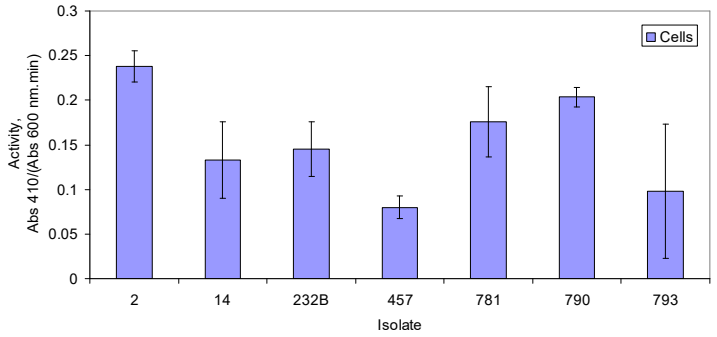



Task 4.4 Enzyme characterisation for selecting those with manufacturers' specifications

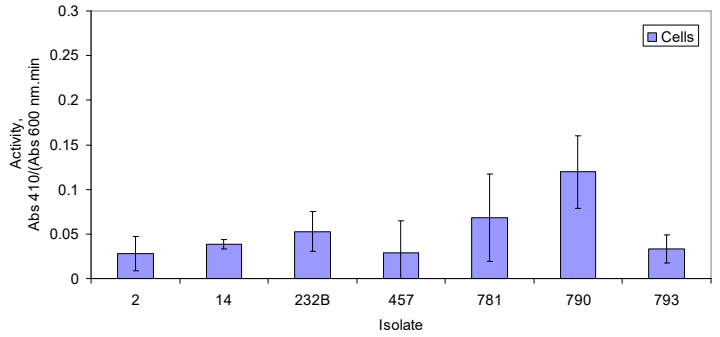
- evaluation of the performance of the enzymes

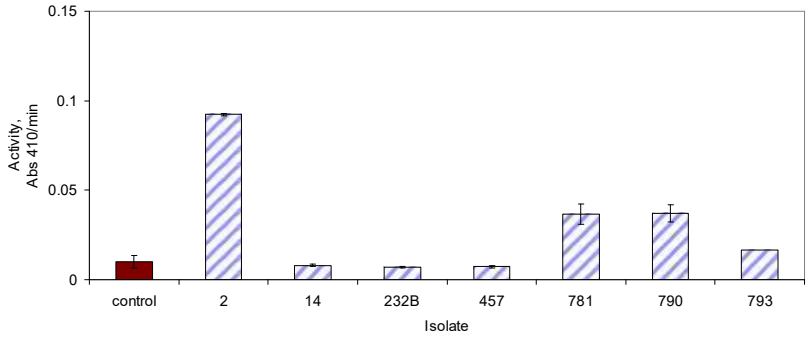
Lipase/Esterase activity

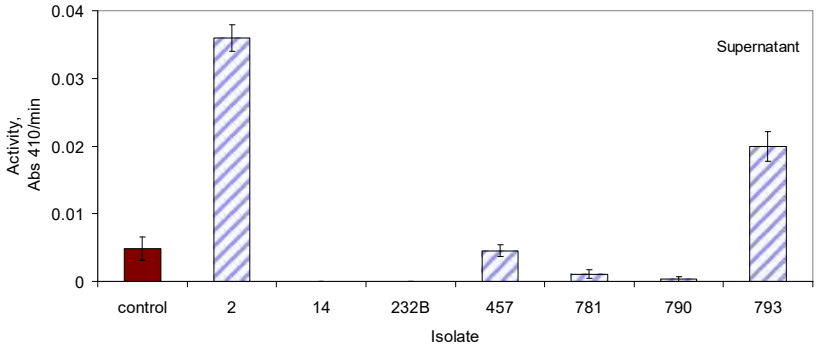
pNP-butyrate



pNP-laurate





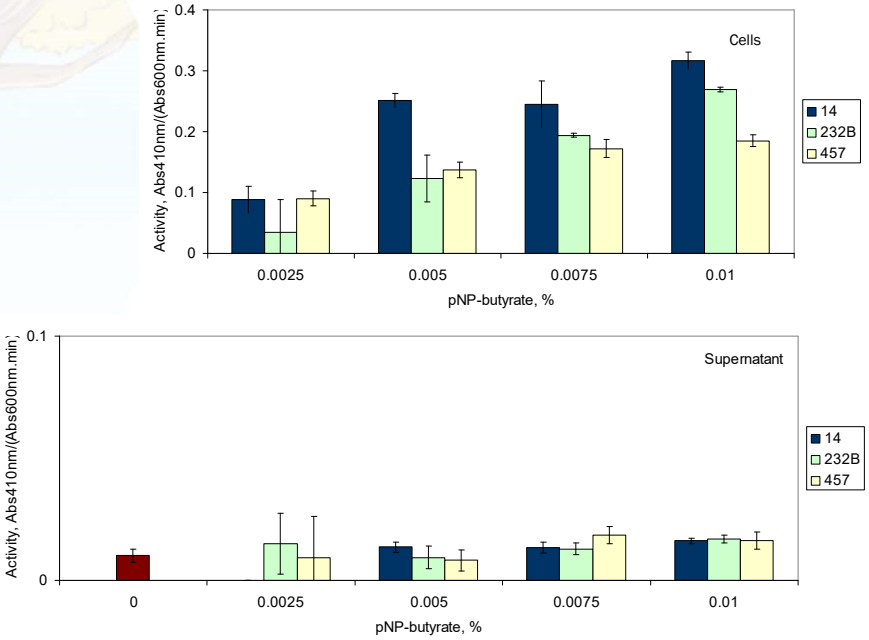


Task 4.4 Enzyme characterisation for selecting those with manufacturers' specifications

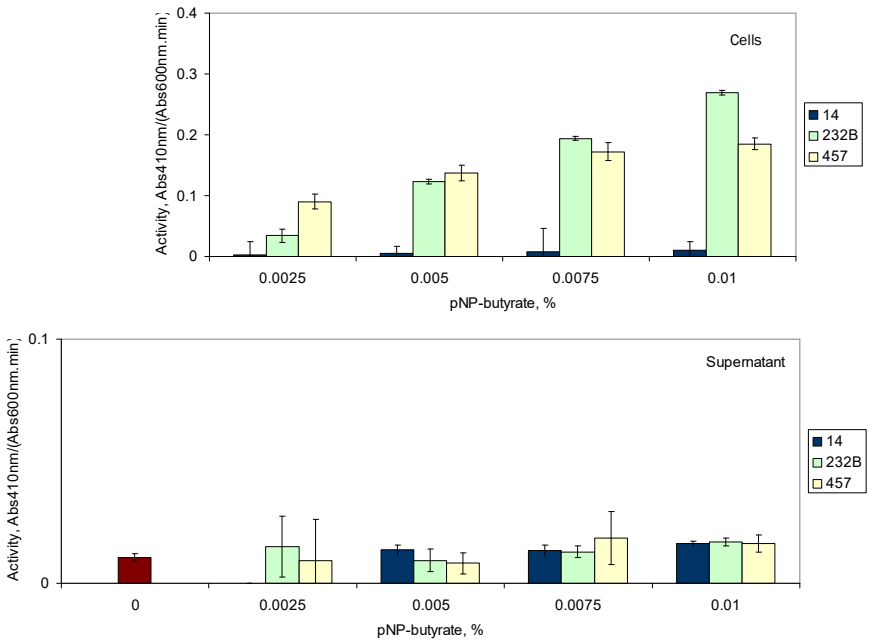
- evaluation of the performance of the enzymes

Lipase/Esterase activity

pNP-butyrate



pNP-laurate



Task 4.4 Enzyme characterisation for selecting those with manufacturers' specifications

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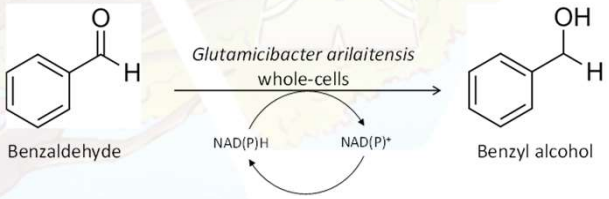
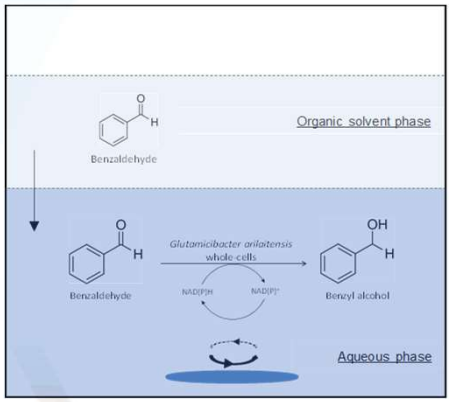
- evaluation of the performance of the enzymes

Production of benzyl alcohol from benzaldehyde

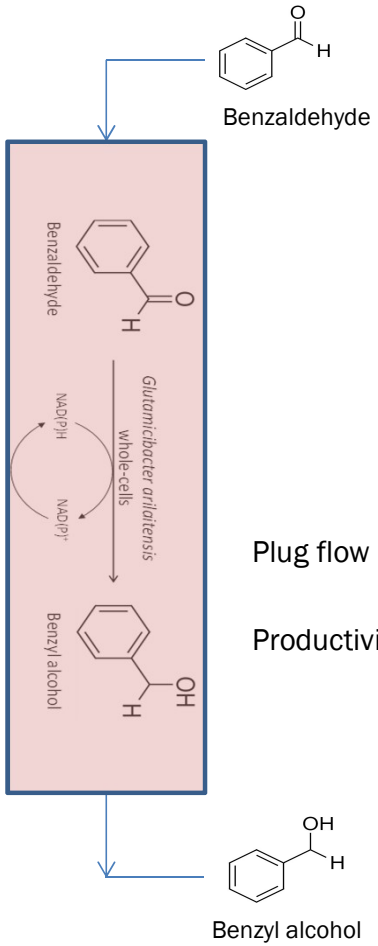


Stirred tank reactor



Benzyl alcohol inhibited the biotransformation.

Productivity = 0.122 $g_{benzyl\ alcohol}/g_{DCW}Lh$



G. arilaitensis isolated from samples collected in the Azores

Plug flow reactor with immobilized cells

Productivity = 1.16 $g_{benzyl\ alcohol}/(g_{DCW}Lh)$

Rodrigues and de Carvalho (2022) *Microorganisms* 10, 966.

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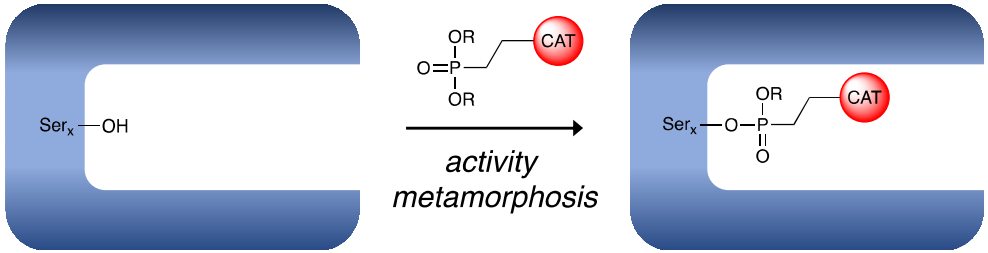
Northwestern Switzerland

University of Applied Sciences and Arts Northwestern Switzerland

FHNW

Task 4.2. Smart design systems to obtain enzymes with inherent problems of expression M2-M30

The foremost objective of Task 4.2 is to develop a novel class of artificial proteolytic enzymes exploiting the large capacity to produce esterases/lipases within the consortium to construct biomimetic proteolytic systems that can be produced in large quantities. It is based on a meticulous biochemical modification of the catalytic site of an esterase/lipase by a synthetic catalytic suicide inhibitor.



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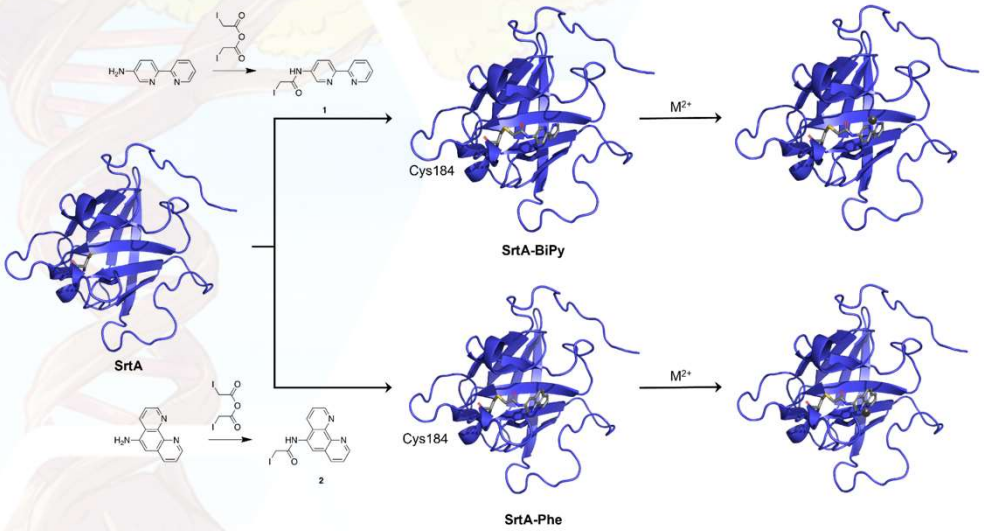
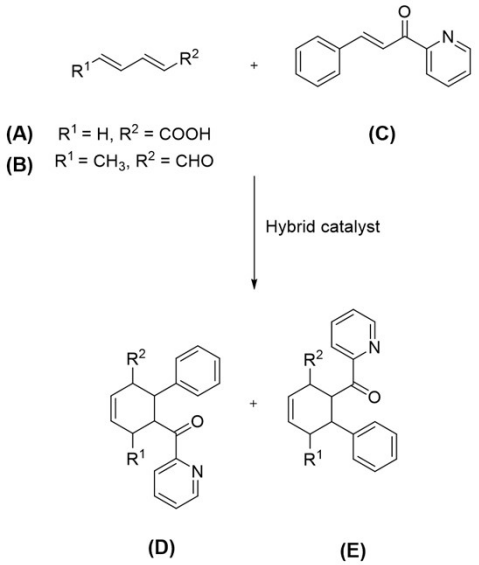
Task 4.2. Smart design systems to obtain enzymes with inherent problems of expression M2-M30

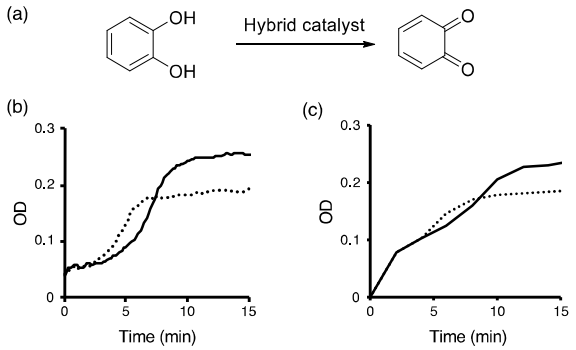
Using a bacterial transpeptidase as scaffold



Production of SrtA-based hybrid catalysts. SrtA inhibition reaction using *N*-([2,2'-bipyridin]-5-yl)-2-iodoacetamide (**1**) and 2-iodo-*N*-(1,10-phenanthrolin-5-yl)acetamide (**2**) followed by metal complexation (M^{2+} : Cu^{2+} or Fe^{2+}).



Schematic representation of the Diels-Alder reactions catalysed by the produced hybrid catalysts.



Pyrocatechol oxidation. (a) Schematic representation of the pyrocatechol oxidation reaction, (b) 1,2-benzoquinone formation followed at 400 nm, (c) Fe(II)-semiquinone formation followed at 530 nm catalysed by SrtA-BiPy-Fe (solid line) and SrtA-Phen-Fe (dashed line).

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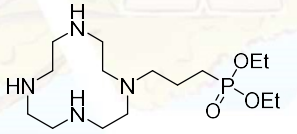
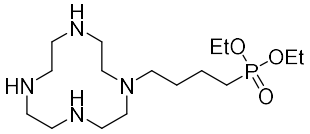
Task 4.2. Smart design systems to obtain enzymes with inherent problems of expression M2-M30

Synthesis of esterase inhibtors



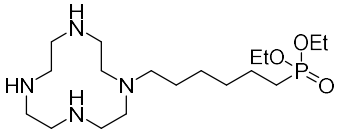
Chemical Formula: $C_{15}H_{35}N_4O_3P$
Molecular Weight: 350.44

SHA-MB-42 (S42 / S4)



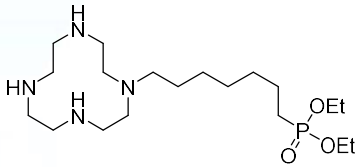
Chemical Formula: $C_{16}H_{37}N_4O_3P$
Molecular Weight: 364.47

SHA-MB-43 (S43)



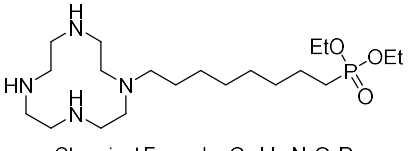
Chemical Formula: $C_{18}H_{41}N_4O_3P$
Molecular Weight: 392.52

SHA-MB-44 (S44)



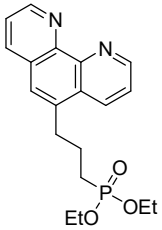
Chemical Formula: $C_{19}H_{43}N_4O_3P$
Molecular Weight: 406.55

SHA-MB-45 (S45)



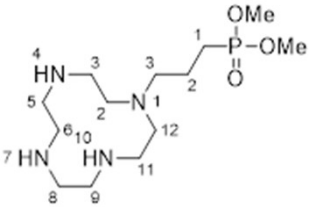
Chemical Formula: $C_{20}H_{45}N_4O_3P$
Molecular Weight: 420.58

SHA-MB-46 (S46)



Chemical Formula: $C_{19}H_{23}N_2O_3P$
Molecular Weight: 358.38

Phenanthroline chelator (S3)



SHA-MB-58

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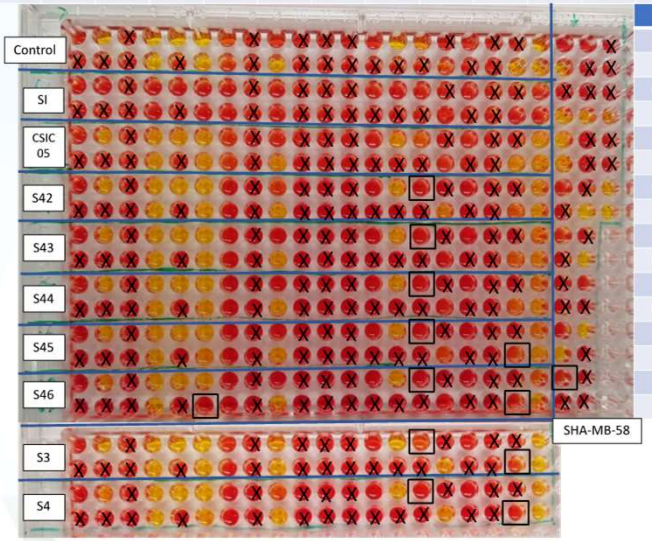
Task 4.2. Smart design systems to obtain enzymes with inherent problems of expression M2-M30

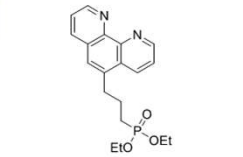
Esterase inhibition (tested by CSIC)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A	EH17	EH21	EH115	IS12	EH0	EH3	EH108	EH12	EH37	EH124	EH92	EH127	EH26	EH7	EH63	EH5	EH36	EH69	EH60	EH33
B	-	EH38	EH1A1	Lip9	Lip8	Polur1	Meth3	EH37 P88G	EH37 36F	EH37 36E	EH37 294S	EH37 E87G	EH37 P242G	EH37 N214I	EH37 36M	EH37 36H	EH37 K62I	EH37 I41W	Lip5	IS10



21	22	23
EH17	EH36	EH37 P242G
EH21	EH69	EH37 N214I
EH115	EH60	EH37 36M
IS12	EH33	EH37 36H
EH0	-	EH37 K62I
EH3	EH38	EH37 I41W
EH108	EH1A1	Lip5
EH12	Lip9	IS10
EH37	Lip8	
EH124	Polur1	
EH92	Meth3	
EH127	EH37 P88G	
EH26	EH37 36F	
EH7	EH37 36E	
EH63	EH37 294S	
EH5	EH37 E87G	

1,5 Hours



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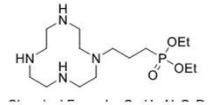

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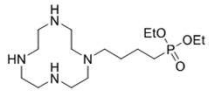

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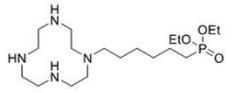

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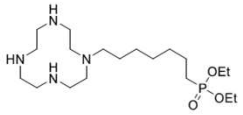

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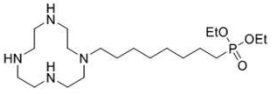

S46

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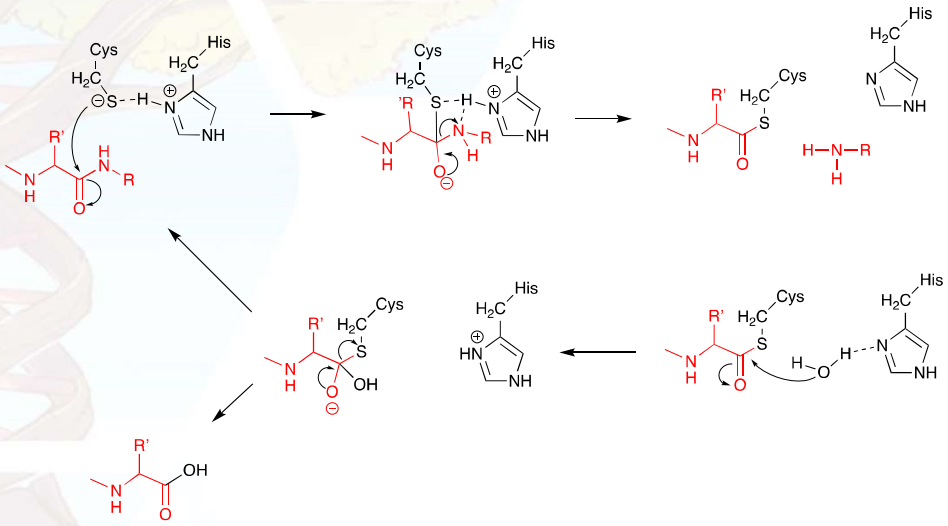
Task 4.2. Smart design systems to obtain enzymes with inherent problems of expression M2-M30

Esterase inhibition: mimicking Cys-protease (ongoing)



- **Catalytic site**
  - **Cys** that acts as a nucleophile, which attacks the carbonyl of the target peptide bond (more nucleophilic than Ser in Ser-proteases) with pKa = 8-9
  - **His** increases nucleophilicity of **Cys**
- **Objective: produce an anologue, which can be accomodated in the active site of esterases (to be tested), via:**
  - designer peptides
  - Designer organocatalysts (to be started Q2 2022)
  - Protein engineering
- **Characterization:**
  - MALDI-TOF MS
  - Protease activity
  - Universal protease assay and small artificial substrate
  - Single crystal X-ray diffraction

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Next…

WP4 Results obtained by Eucodis

Task 4.1. Ultra-fast and efficient gene cloning and synthesis: downstream enzyme production (M2-30)

Goal: Improvement of current expression systems in WP4 to be able to produce enzymes in WP6 in the desired quantities for the downstream partners

Approach:

- Expression in *Pichia pastoris* (ongoing):
  - Design of integration plasmids for faster cloning, establishment of secretion signal/pro-peptide library, fermentation optimization with improved plasmids, new promoters to be tested
- Expression in *Corynebacterium glutamicum* (planned):
  - Design of integration plasmids for stable integration into genome, Design and test of secretion signal peptide library, antibiotics-free expression for food/cosmetics grade enzymes

WP4 Results obtained by Eucodis

Task 4.1. Ultra-fast and efficient gene cloning and synthesis: downstream enzyme production (M2-30)

- Expression in *Pichia pastoris*: Signal peptide library
  - Plasmids for four different signal peptides, integration into AOX locus, for directed, scarless cloning or recombinatorial cloning of enzyme genes Optimization of secretion signal/pro-peptide for higher yields,

  Results:
  - SPs optmized and clooned: aMF, Ost1, aMFd5770, Sp-short
  - Tested with to lipases as model enzymes:
    - CalB wildtype: 25 % more yield with aMFd5770 compared to standard aMF
    - EL032 (Eucodis lipase 032): 62 % more yield with Ost1 compared to standard aMF

➢Screening of SP library necessary to identify best SP for each Consortium enzyme

WP4 Results obtained by Eucodis

Task 4.1. Ultra-fast and efficient gene cloning and synthesis: downstream enzyme production (M2-30)

- Expression in *Pichia pastoris*: Fermentation protocol optimization
  - Fermentation optimization using CalB wildtype as model enzyme for reduced methanol use:
    - Feeding strategy (methanol/glycerol co-feeding)
    - Induction strategy (spiked versus continuous, amounts, time)

Results:

- Several fermentations performed to optimize protocols for mixed feed (15%/85%),
- Yield for CalB wildtype increased by 50%-75% compared to old Pichia strains/protocols,
- Yield for EL032 doubled compared to E. coli protocols

➢ Improved Pichia protocols ready for Consortium enzymes

Work package number [9]	WP4	Lead beneficiary [10]	4 - UHAM
Work package title	Small-scale enzyme production and characterisation		
Start month	1	End month	40

Objectives

In WP4, the anticipated wealth of 1,000 enzymes pre-selected in WP2/WP3 will be expressed, purified and characterised, with the final objective to select 180 enzymes (20 x 9 enzyme classes) capable to be obtained at high yields and behaving better than benchmarks, according to their performance towards model and real-life substrate and stability under model and real-life conditions. The following sub-objectives are addressed:

- Streamlining semi-automatic, synthetic and cloning technologies compatible with more than 50 vectors and 12 hosts, cell-free expression systems, natural host production systems, and beyond state-of-the-art metamorphosis technology for the rapid and efficient expression of target and benchmark enzymes, including those with inherent problems of production;
- To efficiently generate, through fermentation and downstream processing, at least 1,000 enzymes (recombinant, native, biomimetic) for activity assessments;
- To characterise the 1,000 enzymes with model/real substrates and conditions requested by manufacturers;
- Through a decision-making strategy select ca. 180 enzymes with validated manufacturers' demands; and
- Through a combinatorial strategy to design multi-enzyme blends to process real complex substrates.

Description of work and role of partners

WP4 - Small-scale enzyme production and characterisation [Months: 1-40]
UHAM, CSIC, BANGOR, UDUS, IST ID, CNR, FHNW, Bio_Ch, EUCODIS
We propose 6 Tasks.

Task 4.1 Ultra-fast and efficient gene cloning and synthesis: downstream enzyme production M2-M30
Task Lead Partner – UHAM
Participants: CSIC, BANGOR, UDUS, CNR, EUC

Task 4.2 Smart design systems to obtain enzymes with inherent problems of expression M2-M30
Task Lead Partner – FHNW
Participants: CSIC, EUC

Task 4.3 Production of enzymes from their natural hosts M2-M30
Task Lead Partner – CNR
Participants: IST-ID, BIO_CH

Task 4.4 Enzyme characterisation for selecting those with manufacturers' specifications M2-M36
Task Lead Partner – UDUS
Participants: BANGOR, CSIC, UHAM, FHNW, IST-ID, EUC

Task 4.5 Decision-making strategy for selecting lead enzyme candidates M6-M36
Lead partner – UDUS
Participants: BANGOR, UHAM, CSIC, CNR, IST-ID, FHNW, BSC, EUC

Task 4.6 Design of multi-enzyme blends to process complex ingredient mixtures M12-M40
Task Lead Partner – CSIC
Participants: BANGOR, UDUS, UHAM, IST-ID

List of deliverables

Deliverable Number[14]	Deliverable Title	Lead beneficiary	Type[15]	Dissemination level[16]	Due Date (in months)[17]
D4.1	QR barcoding system, available	1 - CSIC	Other	Confidential, only for members of the consortium (including the Commission Services)	3
D4.2	The FuturEnzyme Portfolio of 1,000 enzyme (recombinant/ native/biomimetic) material, obtained	1 - CSIC	Other	Confidential, only for members of the consortium (including the Commission Services)	16
D4.3	Cell-free expression/ reported system, developed	4 - UHAM	Other	Confidential, only for members of the consortium (including the Commission Services)	16
D4.4	Biomimetic protease production system, developed	9 - FHNW	Other	Confidential, only for members of the consortium (including the Commission Services)	16
D4.5	At least 9 enzyme crystal structures	1 - CSIC	Other	Confidential, only for members of the consortium (including the Commission Services)	30
D4.6	The metadata on expression yield, activity and stability, available	5 - UDUS	data sets, microdata, etc	Confidential, only for members of the consortium (including the Commission Services)	18
D4.7	At least 180 enzymes (recombinant, native, biomimetic) with attractive properties, available	1 - CSIC	Other	Confidential, only for members of the consortium (including the Commission Services)	18
D4.8	Set of high-performing multi-enzyme blends	1 - CSIC	Other	Confidential, only for members of the consortium (including the Commission Services)	20