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

FuturEnzyme M24 Meeting WP6

06/07 July 2023

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

WP6 – Development and supply of best enzyme prototypes

- OBJECTIVE

  To **develop and scale-up fermentation and DSP methods** for the production, adapted to requirements of WP7 partners, formulation and quality assessments of best selected enzyme candidates. WP6 will, together with WP7, evaluate the **Performance** of the candidates in application tests, their **Stability** during application and storage (technical feasibility), **Producibility** of the respective enzyme at cost level acceptable for application (economic feasibility), as well as the **Environmental feasibility.**

  - TASKS
    - Fermentation and DSP of best 18 project enzymes (M16 – M42) (TASK 6.1)
    - Upscaling (non-GMP) fermentation and DSP of 9 best enzymes (M20 – M48) (TASK 6.2)
    - Development of optimized formulations of 9 lead enzyme candidates (M20 – M48) (TASK 6.3)
    - Safety, risk and environmental impact assessments of enzyme supply (M20 – M48) (TASK 6.4)

WP6 – Development and supply of best enzyme prototypes

- WORK DONE/STARTED
  - Task 6.1: Fermentation and DSP of best 18 project enzymes (M16 – M42):
    1) Selection of first round of enzyme candidates with WP4, WP5 and WP7 partners
    2) Sequences transferred to WP6; gene optimization started for secreted expression
    3) Strains transferred for expression from native sources
    4) Fermentation and isolation of two lead candidates in *E. coli*
  - Task 6.2: Upscaling (non-GMP) fermentation and DSP of 9 best enzymes (M20 – M48):
    1) Not yet started
  - Task 6.3: Development of optimized formulations of 9 lead enzyme candidates (M20 – M48):
    1) Not yet started
  - Task 6.4: Safety, risk and environmental impact assessments of enzyme supply (M20 – M48):
    1) Not yet started

WP6 - Partners involved

- 2 universities/research institutions
- 2 clusters
- 3 SMEs

CSIC, Agencia Estatal Consejo Superior de Investigaciones Científicas

IST-ID, The Association of Instituto Superior Técnico For Research and Development

CLIB, Cluster Industrielle Biotechnologie e.V.

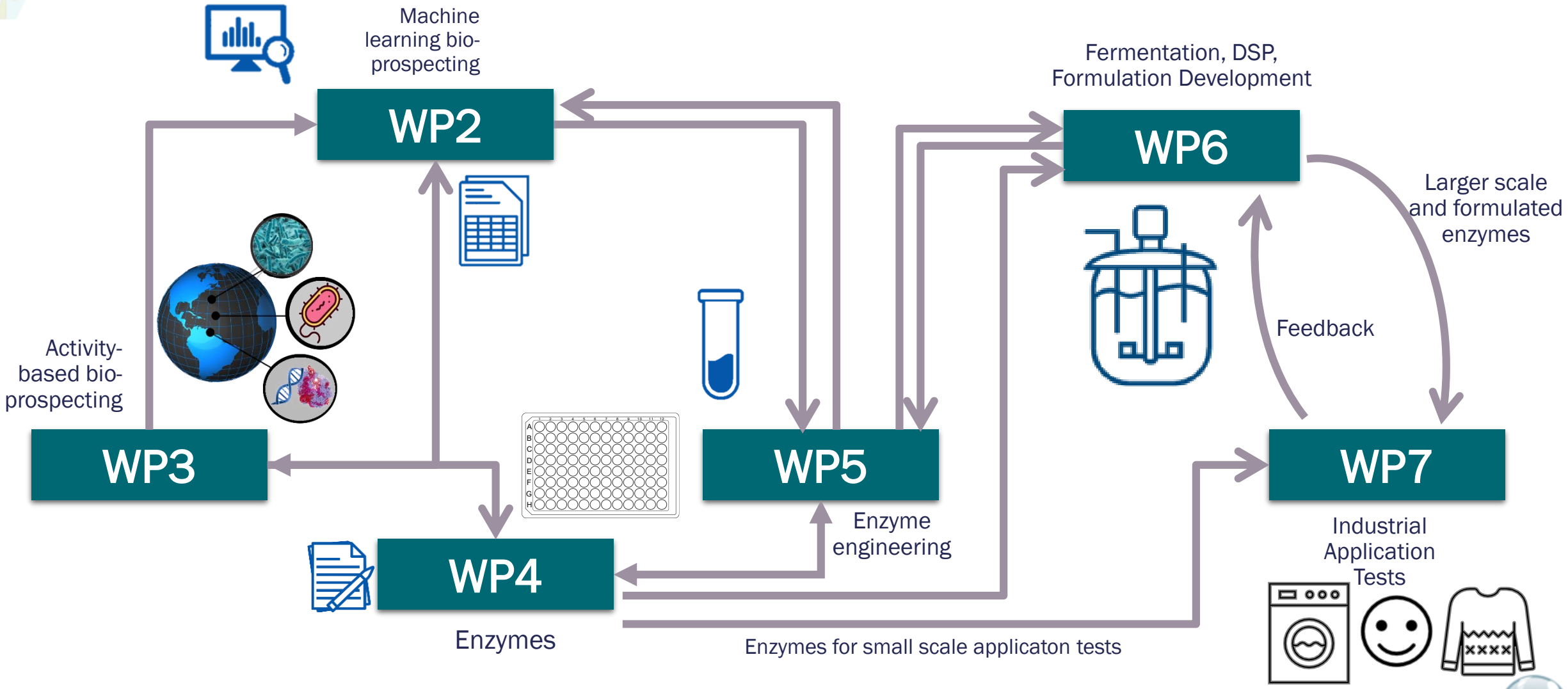
ITB, Consorzio Italbiotec

Bio_Ch, Bio-Chem Solutions SRL

EUC, Eucodis Bioscience GMBH now Biosynth GmbH

INOFEA, Inofea AG

WP6 - Interactions



Task 6.1: How to achieve objectives, and work done

Fermentation and DSP of best 18 project enzymes (M16 – M42)

- Objective:
  - To establish scalable fermentation and DSP methods for the priority enzymes selected in WP4 and WP5 and provide samples for applications tests of the WP7 partners
  - To optimize media (e. g. carbon and nitrogen sources and trace elements), growth conditions and identify the best conditions for oxygen, shear stress, and temperature to obtain the best production performance under minimal energy consumption and waste generation

- Progress undertaken and outputs achieved (07/2023)
  - CSIC: First two lead candidates (FE_Lip9 and FE_ID9) expressed in gram scale in *E. coli*
  - EUC/Biosynth: First three lead candidates expressed secreted in *Pichia pastoris* and shipped to partners
  - EUC/Biosynth: Gene optimization for secreted, recombinant expression completed
  - IST-ID: Lipase and Hyaluronidase activity tests for characterization of lead candidates established
  - Bio_Ch: working on native strains, transferred by partners, for fermentation development

WP6 - Development and supply of best enzyme prototypes. (23 PM)

BIOSYNTH®

For questions: Jan or Marc

Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

Highest priority enzymes cloned into *Pichia*

ID	Name	Partner	Priority	Signal Peptide	Nagoya protocol	Homology (%) patent database	Application	Cloning for secreted expression
1	Kest3 (lipase)	Bangor	No	No	Information provided	33.87	Detergent	
2	FE_Lip9 (lipase)	CSIC	Yes (1st)	Yes	Information provided	99.45	Detergent, textile	Pichia (Euc)
3	FE_ID9 (lipase)	CSIC	Yes (1st)	No	Information provided	100	Detergent	Pichia (Euc)
4	FE_polur1 (lipase)	CSIC	Yes (2nd)	No	Information provided	97.3	Detergent	Pichia (Euc)
5	EstLip_Dim_#008 (lipase)	UDUS	Yes (1st)	No	Information provided	100	Detergent	Pichia (Euc)
6	EstLip_Paes_TB035 (lipase)	UDUS	Yes (2nd)	Yes	Information provided	41.98	Detergent	Pichia (Euc)
7	EstLip_PtEst1 (lipase)	UDUS	Yes (2nd)	No	Information provided	58.63	Detergent	Pichia (Euc)
8	EstLip_TBEc304 (lipase)	UDUS	No	No	Information provided	62.54	Detergent	
9	PEH_Paes_PE-H_Y250S (PETase)	UDUS	Yes (1st)	Yes	Information provided	62.88	Detergent, textile	Pichia (Euc)
10	PEH_Pbau_PE-H (Lipase, PETase)	UDUS	No	Yes	Information provided	61.70	Detergent, textile	
11	PEH_Pform_PE-H (Lipase, PETase)	UDUS	No	Yes	Information provided	69.08	Textile	
12	PEH_Poce_PE-H (Lipase, PETase)	UDUS	No	Yes	Information provided	62.5	Detergent, textile	
13	GEN0105 (Lipase, PETase)	Bangor	No	No	Information provided	61.69	Detergent	
14	GEN0095 (cellulase)	Bangor	No	No	Information provided	52.5	Textile	
15	VD_PL9 (hyaluronidase)	CNR	Yes	Yes	Information provided	88.89	Cosmetic	Pichia (Euc)
16	VD_PL22 (hyaluronidase)	CNR	No	No	Information provided	69.23	Cosmetic	
17	VA_PL9 (hyaluronidase)	CNR	No	Yes	Information provided	32.38	Cosmetic	
18	Hyal_HRDSV_2334 (hyaluronidase)	CNR	No	No	Information provided	100	Cosmetic	
19	V. diabolicus V4; V. alginolyticus #23 (hyaluronidase)	CNR	Yes	-	Information provided	-	Cosmetic	
20	FE_EH37 (esterase)	CSIC	No	No	Information provided	49.09	Predictive tools	
21	FE_Lip5 (lipase)	CSIC	No	No	Information provided	43.52	Detergent	
22	TR_2E_2 (PluriZyme)	CSIC	No	No	Information provided	66.74	All	
23	EH_{1AB1C} (PluriZyme)	CSIC	No	No	Information provided	64.19	All	
24	X11_mut1 (PluriZyme)	CSIC	No	No	Information provided	64.78	All	

Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

Lead candidates: Secreted expression in *Pichia*

- ✓ **GENE DESIGN AND SYNTHESIS**
  - Sequence/codon optimization for *Pichia pastoris*
  - Addition of secretion signals (used for 1st round: α-Mating factor, OST1)
  - Synthesis of genes at external provider, cloning into Eucodis plasmids
  - Transformation into *Pichia pastoris* cells
  - Selection of Zeocin plates for multiple integration events
- ✓ **LIPASE ACTIVITY SCREENING:**
  - 24-well expression
  - Induction with methanol
  - Actvitiy based screening with pNP-butyrate
- ✓ **LIPASES & HYALURONIDASE EXPRESSION ANALYSIS:**
  - Expression analysis by SDS-PAGE

Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

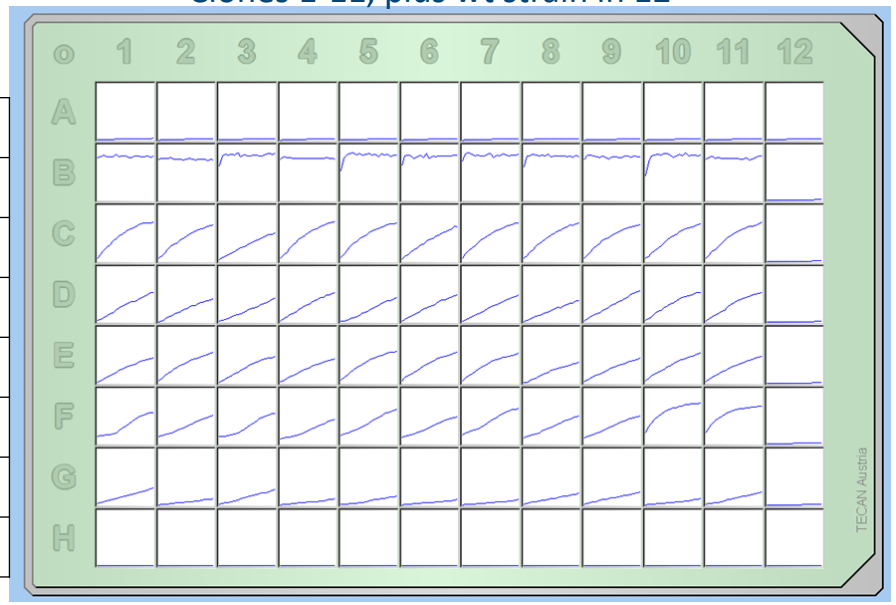
24-well Expression: Lipase activity Screening

Expressionculture 13.-16.03.2023

11 individual clones screened in pNP-butyrate assay (time course of pNP release shown)

A	1- pPichia57ost1_FE-ID9
B	2- pPichia57ost1_EstLip-Paes-TB035
C	3- pPichia57ost1_FE-Lip9
D	4- pPichia57ost1_PEH-Peas-PE-H-Y250S
E	5- pPichia57ost1_EstLip-Dim-#008
F	6- pPichia57ost1_EstLip-PtEst1
G	13- pPichia57ost1_FE-Polur1
H	(empty)

Clones 1-11, plus wt strain in 12



- All lead lipases except FE-ID9 show good to excellent activity in the supernatant.

Task 6.1: Progress 07-2023

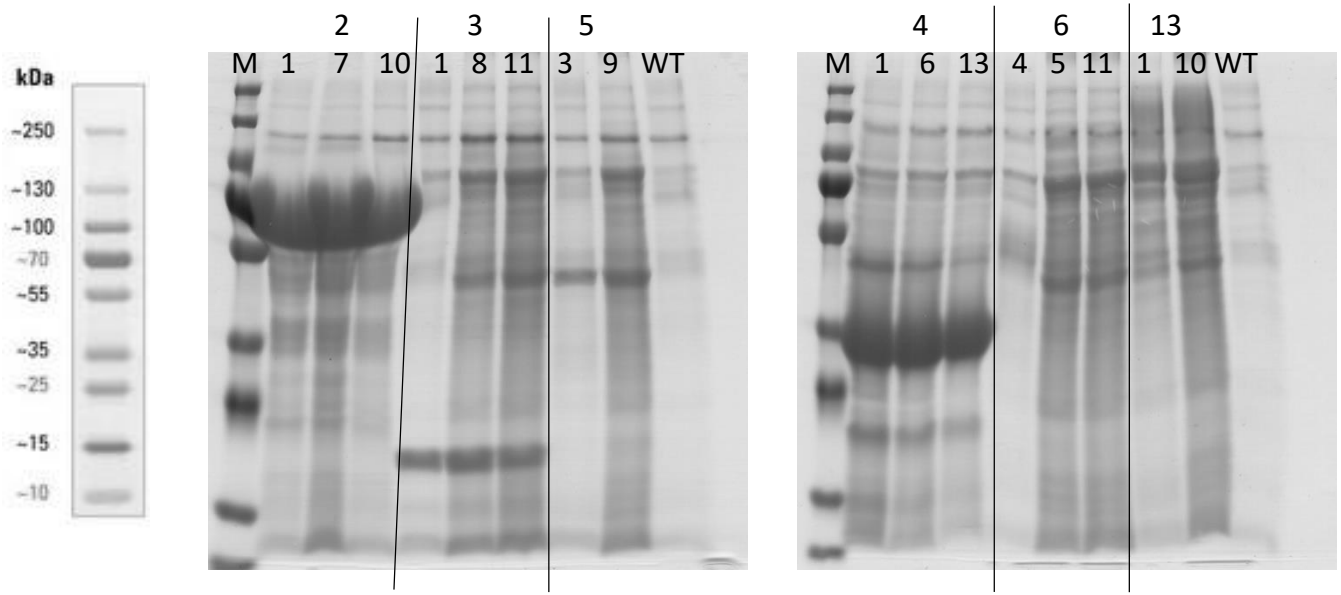
Fermentation and DSP of best 18 project enzymes (M16 – M42)

24-well Expression: SDS-PAGE analysis

Expression culture 13.-16.03.2023

2-3 clones of each construct analyzed
10x concentrated via 10kDa UF, load 10 µL
12% TGX-Gel

2. pPichia57ost1_EstLip-Paes-TB035 (~ 56 kD)
3. pPichia57ost1_FE-Lip9 (~ 22 kD)
4. pPichia57ost1_PEH-Peas-PE-H-Y250S (~30 kD)
5. pPichia57ost1_EstLip-Dim-#008 (~ 43 kD)
6. pPichia57ost1_EstLip-PtEst1 (~ 39 kD)
13. pPichia57ost1_FE-Polur1 (~ 67 kD)



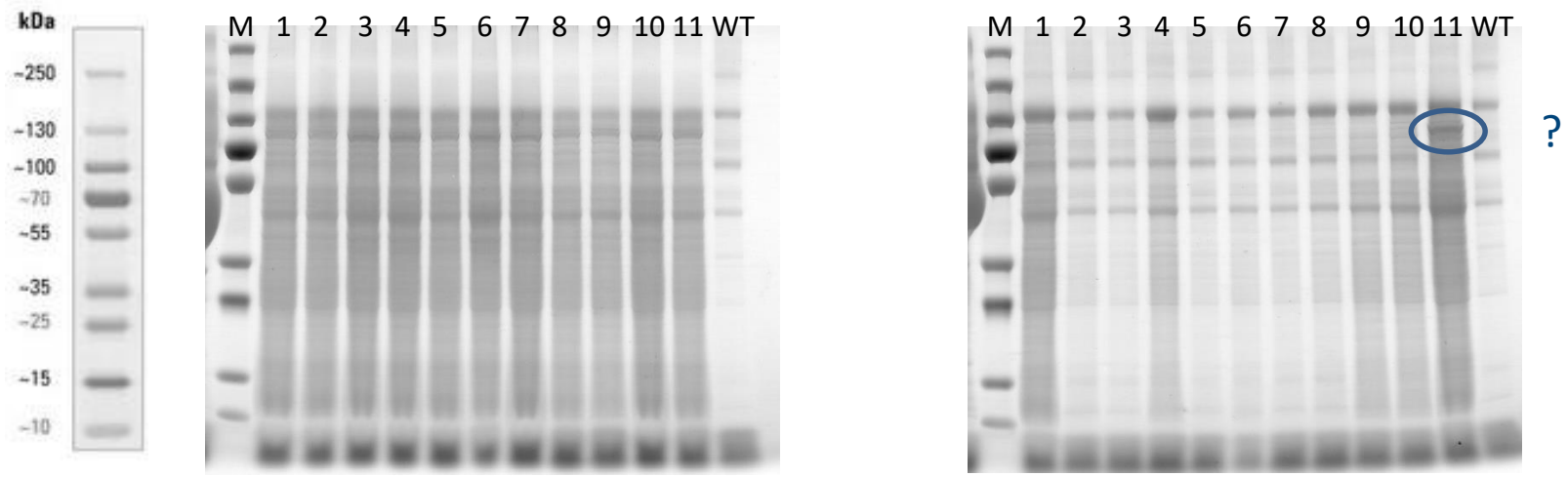
Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

24-well Expression: SDS-PAGE analysis

Expression culture 13.-16.03.2023

11 clones of each construct analyzed
200 µL concentrated via 10kDa UF, load all
4-20% SurePAGE



1. pPichia57ost1_FE-ID9 (~ 40 kD)
Lipase

14. pPichia57ost1_VD-PL9 (~ 80 kD)
Hyaluronidase

Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

Summary: Secreted expression in *Pichia*

ID	Name	Partner	Priority	Signal Peptide	Nagoya protocol	Cloning for secreted expression	Expression successful	Activity confirmed
2	FE_Lip9 (lipase)	CSIC	Yes (1st)	Yes	Information provided	Pichia (Euc)	++	++
3	FE_ID9 (lipase)	CSIC	Yes (1st)	No	Information provided	Pichia (Euc)	-	-
4	FE_polur1 (lipase)	CSIC	Yes (2nd)	No	Information provided	Pichia (Euc)	++	+
5	EstLip_Dim_#008 (lipase)	UDUS	Yes (1st)	No	Information provided	Pichia (Euc)	++	++
6	EstLip_Paes_TB035 (lipase)	UDUS	Yes (2nd)	Yes	Information provided	Pichia (Euc)	+++	+++
7	EstLip_PtEst1 (lipase)	UDUS	Yes (2nd)	No	Information provided	Pichia (Euc)	+	++
9	PEH_Paes_PE-H_Y250S (PETase)	UDUS	Yes (1st)	Yes	Information provided	Pichia (Euc)	+++	++
15	VD_PL9 (hyaluronidase)	CNR	Yes	Yes	Information provided	Pichia (Euc)	(?)	Assay establishment
19	V. diabolicus V4; V. alginolyticus #23 (hyaluronidase)	CNR	Yes	-	Information provided			

Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

Next steps: Secreted expression in *Pichia*

- ✓ **ALL LEAD LIPASES TESTED ACTIVE:**
  - Fermentation in 1 L and 10 L scale
  - Supply of samples to consortium partners
- ✓ **VD-PL9 HYALURONIDASE:**
  - Analysis of more clones to confirm expression
  - Establishment of assay in house
  - Supply of samples to consortium partners
- ✓ **FE_ID9 LIPASE (EXPRESSION FAILED):**
  - Transformation of constructs with other signal peptides
  - Expression screening

Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

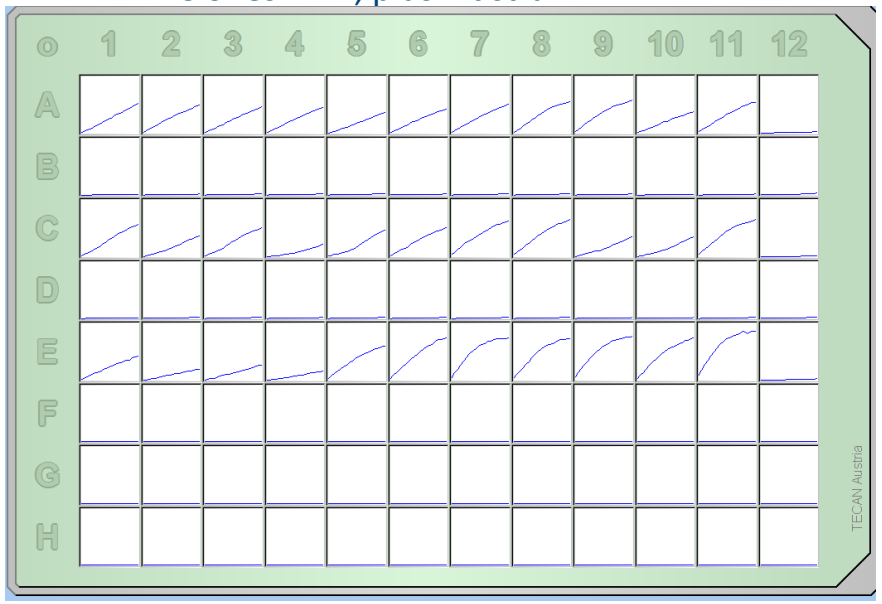
24-well Expression: Lipase activity Screening

Expression culture 24.-27.04.2023

11 individual clones screened in pNP-butyrate assay (time course of pNP release shown)

Clones 1-11, plus wt strain in 12

A	9- pPichia57aMFd_EstLip-Dim-#008
B	10- pPichia57aMFd_FE-ID9
C	12- pPichia57aMFd_FE-Lip9
D	15- pPichia57aMFd_VD-PL9 (hyaluronidase)
E	16- pPichia57aMFd_FE-Polur1
F	(empty)
G	(empty)
H	(empty)



- All lead lipases except FE-ID9 show good activity in the supernatant.
- pPichia57aMFd_Fe-Polur1 shows better activity than in previous round with OST1 signal peptide.

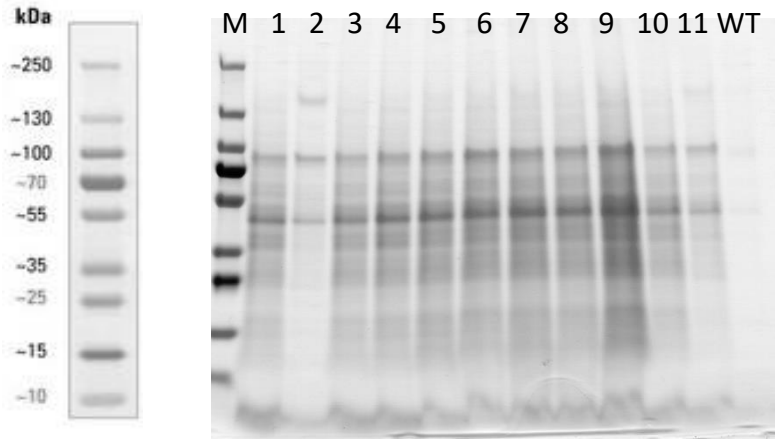
Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

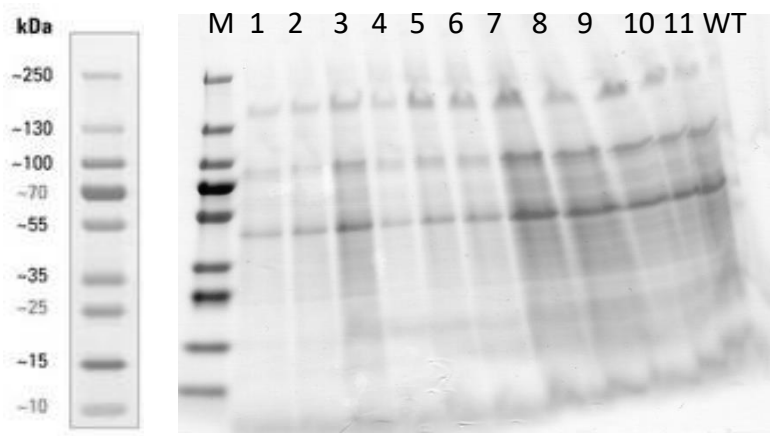
24-well Expression: SDS-PAGE analysis

Expression culture 24.-27.04.2023

11 clones of each construct analyzed
200 µL concentrated via 10kDa UF, load all
4-20% TGX



10. pPichia57aMFd_FE-ID9 (~ 40 kD)
Lipase



15. pPichia57aMFd_VD-PL9 (~ 80 kD)
Hyaluronidase

Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

10 L scale fermentation

✓ **Clones selected from 24-well screening of transformants:**

- pPichia57ost1_FE-Lip9 #8
- pPichia57ost1_EstLip-Paes-TB035 #7
- pPichia57ost1_PHE-Paes-PE-H-Y250S #11
- pPichia57ost1_EstLip-PtEst1 #11
- pPichia57ost1_EstLip-Dim-#008 #3
- pPichia57aMFd_FE-Polur1 #6

Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

Example of 10 L scale fermentation:

✓ **SELECTED CLONE:**

- pPichia57ost1_FE-Lip9 #8

Fermentation:	230504_098_FutEnzyme		
Start:	04.05.2023 09:17		
Strain:	Pichia pastoris		
Plasmid:	(3) pPichia57ost1_FE-Lip9 #8		
Medium:	4000 mL medium salt BSM		
Preculture:	~ 300 mL YPD @ 28°C		
Feeds:	internal pump 1	4 L	100% Methanol, 8 mL/L Trace
	external pump:	1.6 L	500 g/L Glycerol, 4 mL/L Biotin (0.2 g/L stock)
Feed Control:	Fed-Batch-Phase:	1350 mL feed after first pO2-peak over 10 h	
		cool-down 28-->25°C over 10 hours	
	Transition-Phase:	1 h, 20 mL/h glycerol feed, 80 mL MeOH-pulse	
	Induction-Phase:	150 mL MeOH pulses	

post sterile additions:

32 mL Biotin

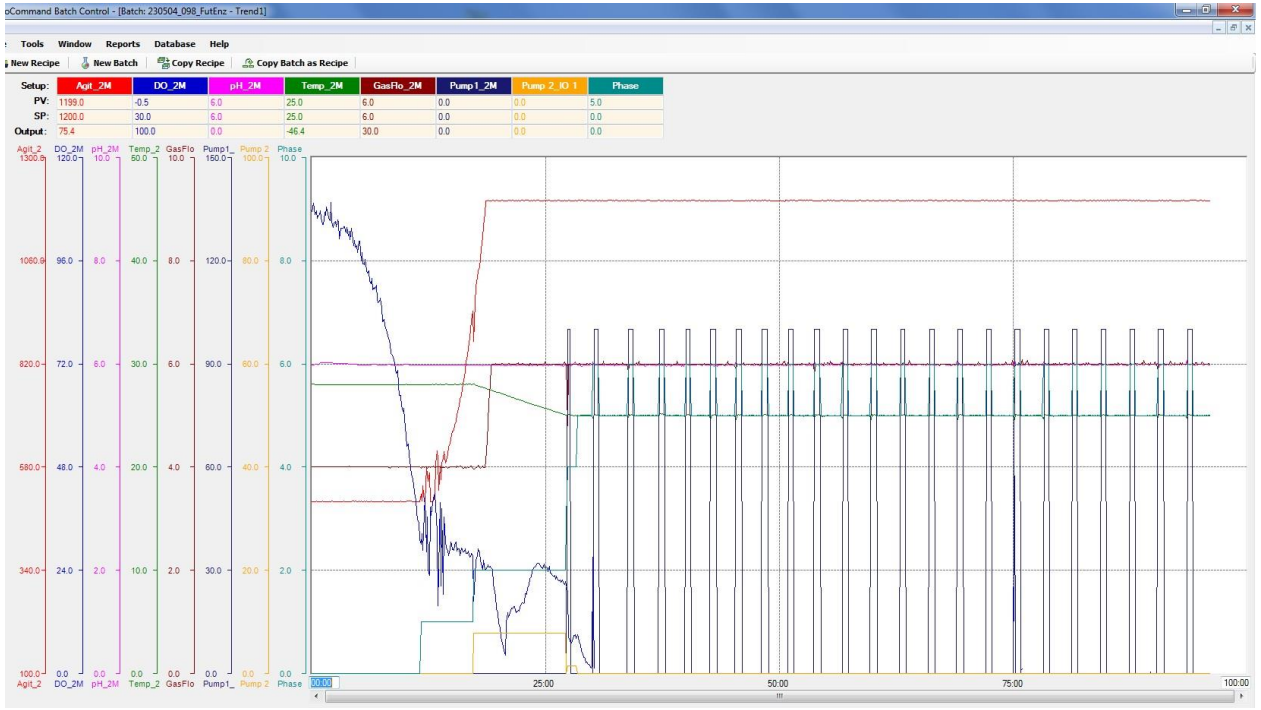
32 mL BSM Trace

Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

Example of 10 L scale fermentation: FE-Lip9

- ✓ **SELECTED CLONE:**
  - pPichia57ost1_FE-Lip9 #8



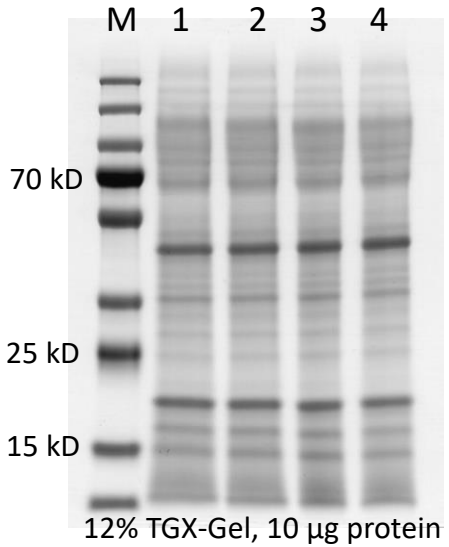
Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

10 L scale fermentation of FE-Lip9:

✓ **DSP of pPichia57ost1_FE-Lip9 #8**

- 0.2 µm filtered Supernatant (7500 mL)
- Concentration via 10 kDa (5000 mL)
- Buffer change to 25 mM Kpi, 50 mM NaCl, pH 6.3 (1900 mL)
- Lyophilization



12% TGX-Gel, 10 µg protein

	SN (1)	Concentrate (2)	Final (3)	Lyo (4)
Protein concentration	0.72 mg/mL	1.17 mg/mL	2.79 mg/mL	0.084 mg/mg
Activity	1.33 U/mL	2.55 U/mL	6.00 U/mL	0.19 U/mg

Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

Summary of top priority lead candidates

Done and shipped:

- ✓ pPichia57ost1_FE-Lip9 #8
- ✓ pPichia57ost1_EstLip-Paes-TB035 #7
- ✓ pPichia57ost1_PHE-Paes-PE-H-Y250S #11

Ongoing (finished next week):

- pPichia57ost1_EstLip-PtEst1 #11 (10 L failed, feed protocol needs optimization)
- pPichia57ost1_EstLip-Dim-#008 #3 (analysis open)

Open:

- pPichia57aMFd_FE-Polur1 #6 (this weekend)

Activity to be tested:

- pPichia57aMFd_VD-PL9 (hyaluronidase)

Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

Shipment of current lead candidates

19.06.2023	Eucodis	BANGOR	Enzyme lyophilisate	3x 100 mg	FE-Lip9, EstLip-Paes-TB035, PHE-Paes-PE-H-Y250S
19.06.2023	Eucodis	CNR	Enzyme lyophilisate	3x 100 mg	FE-Lip9, EstLip-Paes-TB035, PHE-Paes-PE-H-Y250S
19.06.2023	Eucodis	CSIC	Enzyme lyophilisate	3x 100 mg	FE-Lip9, EstLip-Paes-TB035, PHE-Paes-PE-H-Y250S
19.06.2023	Eucodis	Henkel	Enzyme lyophilisate	3x 100 mg	FE-Lip9, EstLip-Paes-TB035, PHE-Paes-PE-H-Y250S
19.06.2023	Eucodis	INOFEA AG	Enzyme lyophilisate	3x 2 g	FE-Lip9, EstLip-Paes-TB035, PHE-Paes-PE-H-Y250S
19.06.2023	Eucodis	IST-ID	Enzyme lyophilisate	3x 5 g	FE-Lip9, EstLip-Paes-TB035, PHE-Paes-PE-H-Y250S
19.06.2023	Eucodis	Schoeller	Enzyme lyophilisate	3x 10 g	FE-Lip9, EstLip-Paes-TB035, PHE-Paes-PE-H-Y250S
19.06.2023	Eucodis	UDUS	Enzyme lyophilisate	3x 500 mg	FE-Lip9, EstLip-Paes-TB035, PHE-Paes-PE-H-Y250S
19.06.2023	Eucodis	UHAM	Enzyme lyophilisate	3x 100 mg	FE-Lip9, EstLip-Paes-TB035, PHE-Paes-PE-H-Y250S

Task 6.1: Progress 07-2023

Fermentation and DSP of best 18 project enzymes (M16 – M42)

Next round of candidates

ID	Name	Partner	Priority	Signal Peptide	Homology (%) patent database	Application	Cloning for secreted expression
1	Kest3 (lipase)	Bangor	No	No	33.87	Detergent	2nd round
3	FE_ID9 (lipase)	CSIC	Yes (1st)	No	100	Detergent	2nd round
8	EstLip_TBEc304 (lipase)	UDUS	No	No	62.54	Detergent	2nd round
10	PEH_Pbau_PE-H (Lipase, PETase)	UDUS	No	Yes	61.70	Detergent, textile	2nd round
11	PEH_Pform_PE-H (Lipase, PETase)	UDUS	No	Yes	69.08	Textile	2nd round
12	PEH_Poce_PE-H (Lipase, PETase)	UDUS	No	Yes	62.5	Detergent, textile	2nd round
13	GEN0105 (Lipase, PETase)	Bangor	No	No	61.69	Detergent	2nd round
14	GEN0095 (cellulase)	Bangor	No	No	52.5	Textile	2nd round
15	VD_PL9 (hyaluronidase)	CNR	Yes	Yes	88.89	Cosmetic	2nd round
16	VD_PL22 (hyaluronidase)	CNR	No	No	69.23	Cosmetic	2nd round
17	VA_PL9 (hyaluronidase)	CNR	No	Yes	32.38	Cosmetic	2nd round
18	Hyal_HRDSV_2334 (hyaluronidase)	CNR	No	No	100	Cosmetic	2nd round
20	FE_EH37 (esterase)	CSIC	No	No	49.09	Predictive tools	2nd round
21	FE_Lip5 (lipase)	CSIC	No	No	43.52	Detergent	2nd round
22	TR_2E_2 (PluriZyme)	CSIC	No	No	66.74	All	
23	EH_{1AB1C} (PluriZyme)	CSIC	No	No	64.19	All	
24	X11_mut1 (PluriZyme)	CSIC	No	No	64.78	All	
25	LC1Hm_4133	CSIC				Cosmetic	2nd round
26	LC1Hm_4133 cut	CSIC				Cosmetic	2nd round
27	pVec11 (peroxidase)	Bangor		No	69.8	Textile	2nd round

WP6 - Development and supply of best enzyme prototypes. (23 PM)

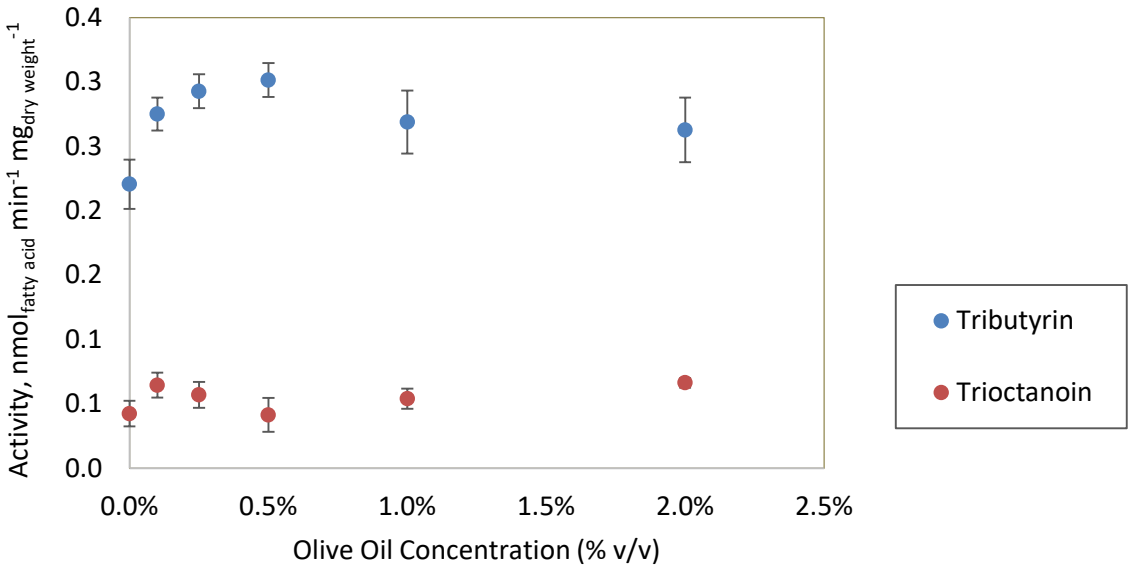
IST-ID
Associação do Instituto Superior Técnico para a Investigação e Desenvolvimento

For questions: Carla

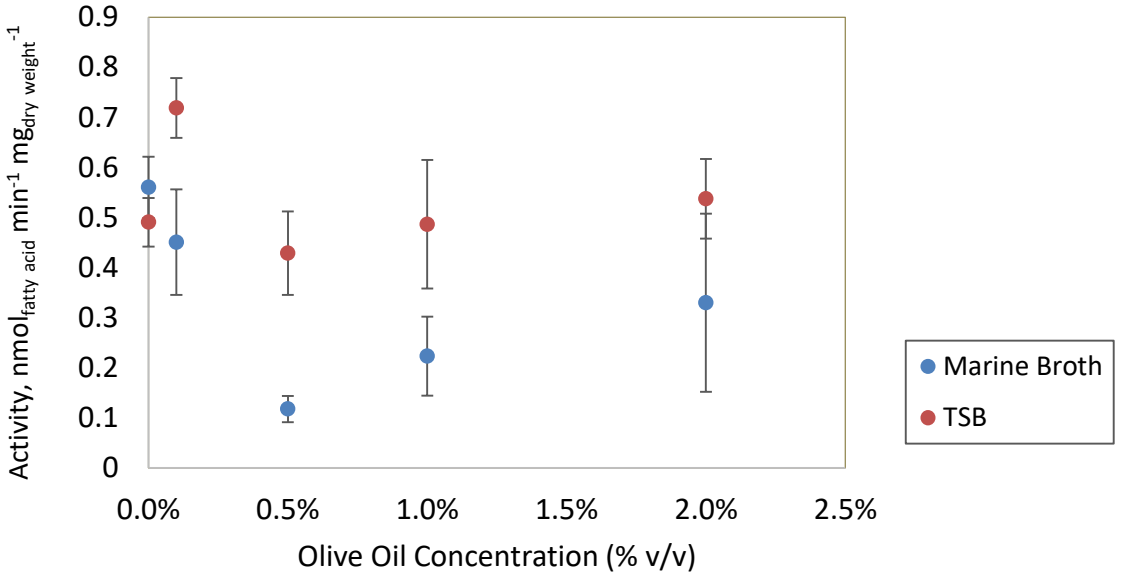
WP6 - Development and supply of best enzyme prototypes. (23 PM)

Lipase activity assays

Serratia quinivorans



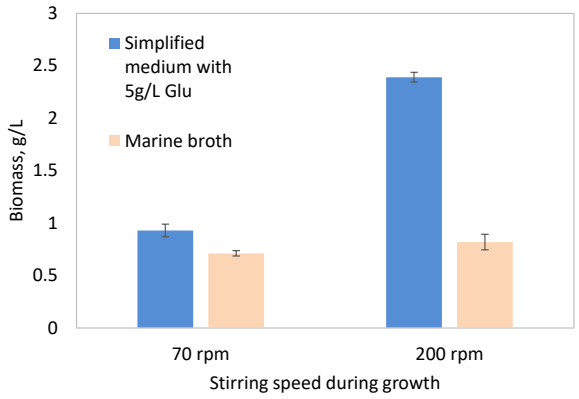
Pseudomonas protegens

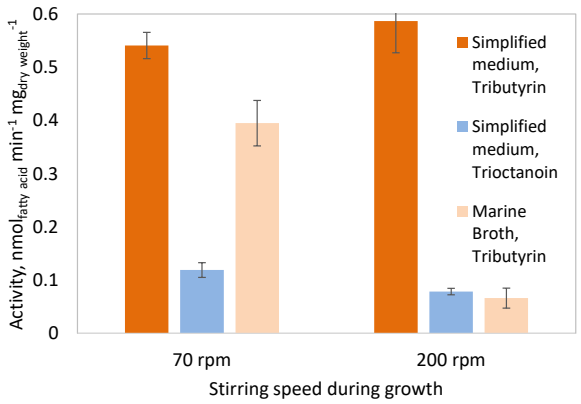


WP6 - Development and supply of best enzyme prototypes. (23 PM)

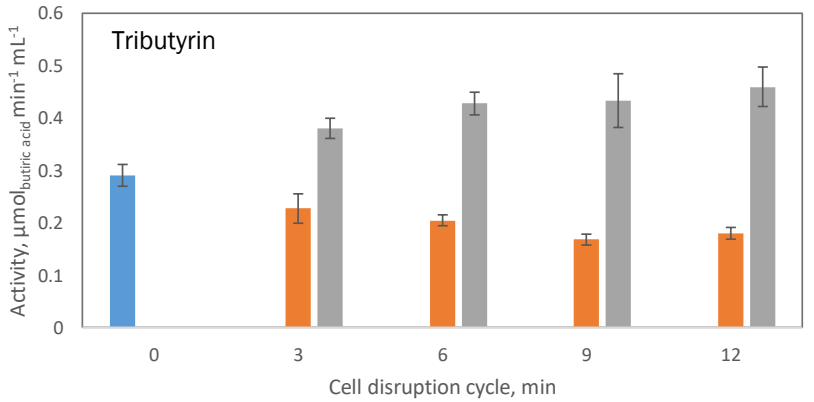
Lipase activity assays

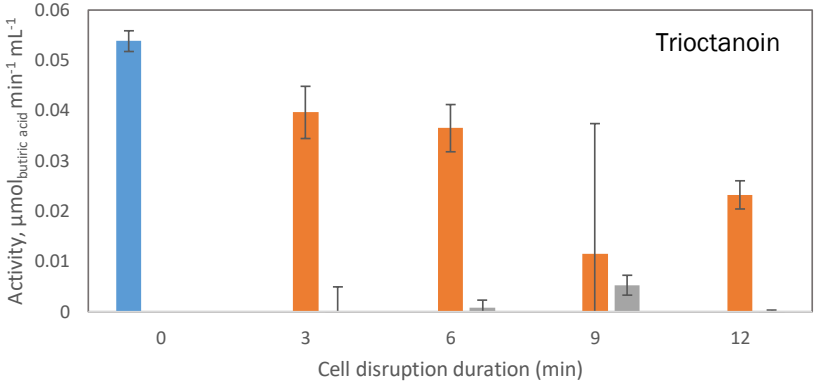
Effect of reaction and growth conditions on cell activity. Isolate 790 (*Serratia quinivorans*)



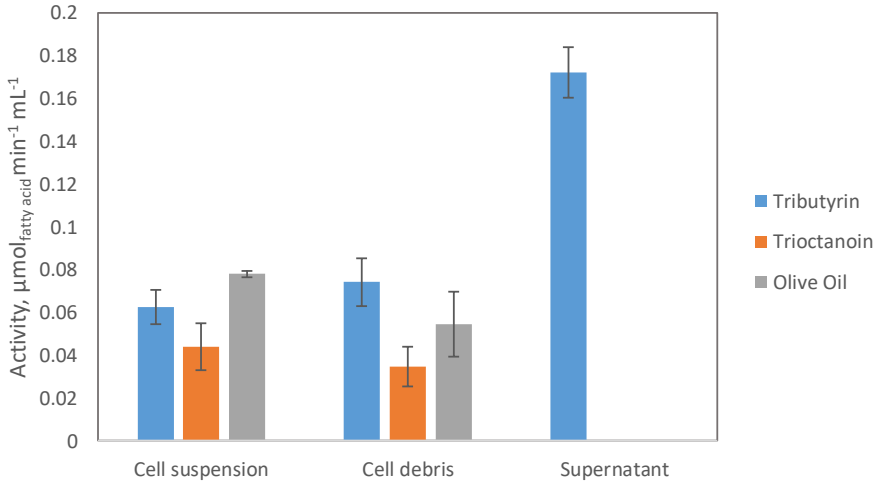


Simplified medium





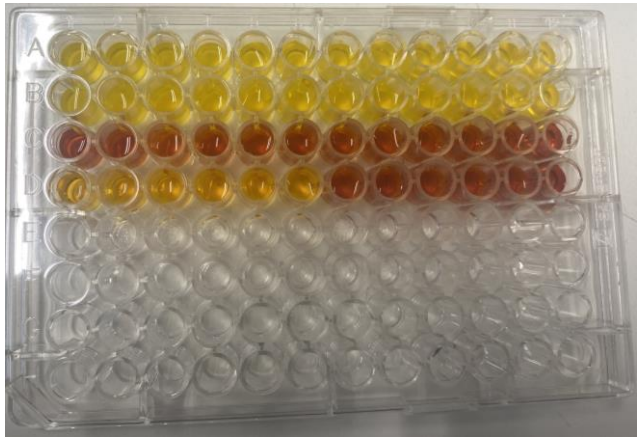
MB+0.5% olive oil



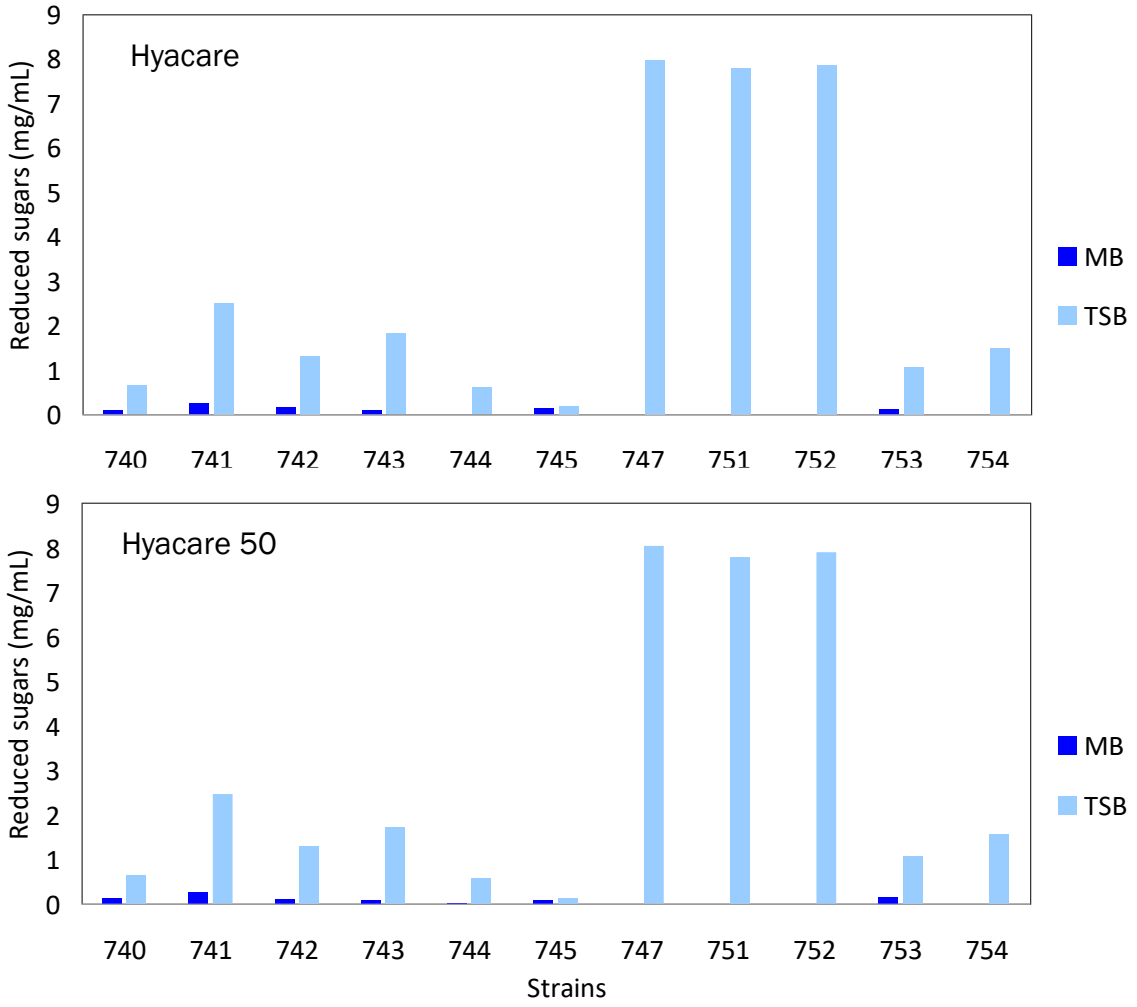
Cell Suspension Debris Supernatant

WP6 - Development and supply of best enzyme prototypes. (23 PM)

Hyaluronidase activity – DNS method



Reduced sugars measured after 48h of enzymatic reaction using the supernatants of cells grown in MB and TSB.



WP6 - Development and supply of best enzyme prototypes. (23 PM)

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Hyaluronidase activity –paper-based method (Zhao et al. 2022)

analytical chemistry

pubs.acs.org/ac Article

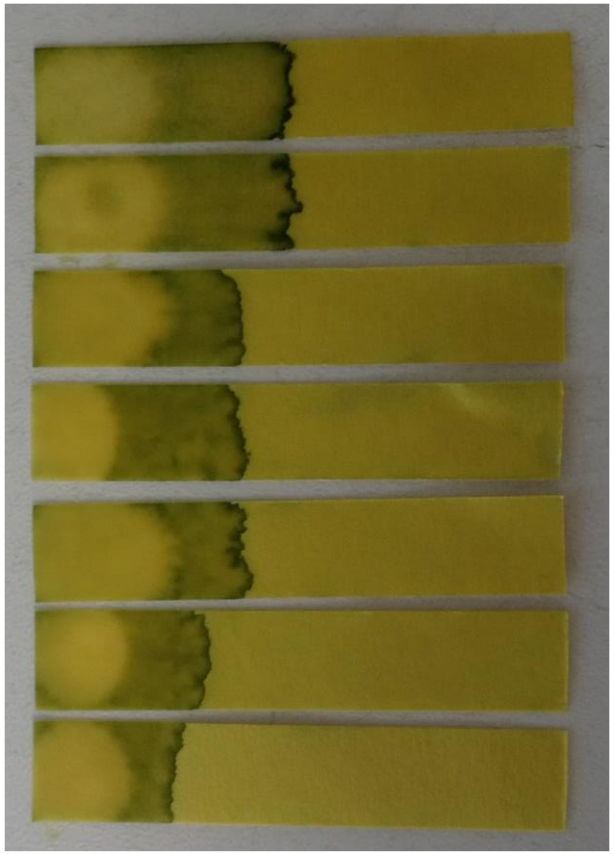
Paper-Based Flow Sensor for the Detection of Hyaluronidase via an Enzyme Hydrolysis-Induced Viscosity Change in a Polymer Solution

Binglu Zhao, Lubin Qi,* Wenjun Tai, Mei Zhao, Xiangfeng Chen, Li Yu, Jianguo Shi, Xiao Wang, Jin-Ming Lin, and Qiongzheng Hu*

Cite This: *Anal. Chem.* 2022, 94, 4643–4649 Read Online

HA (mg/mL)

0
1.56
1.78
2.89
5
5.78
10



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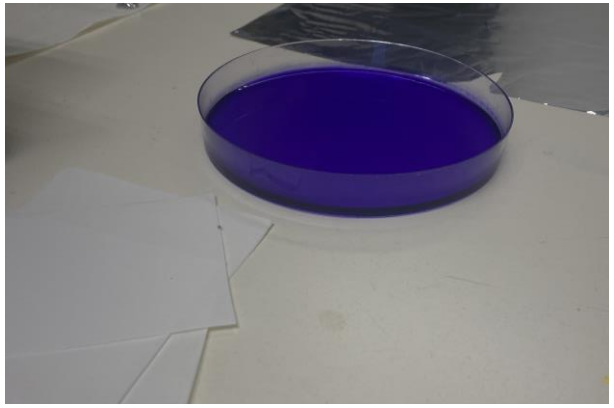
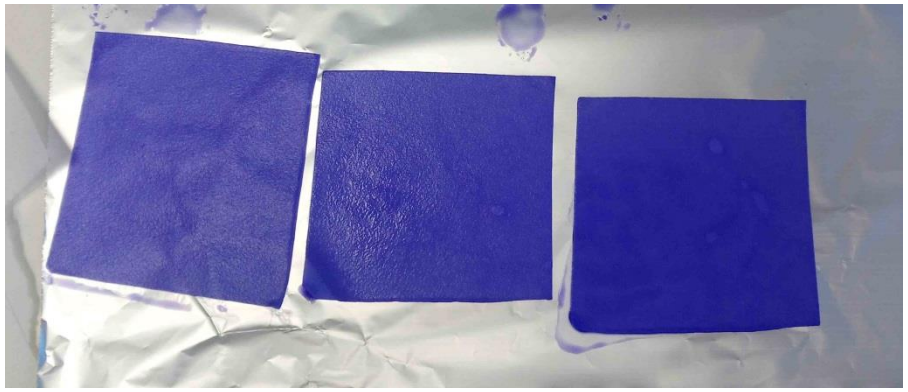
Hyaluronidase activity –new paper-based method





HA (mg/mL)

0

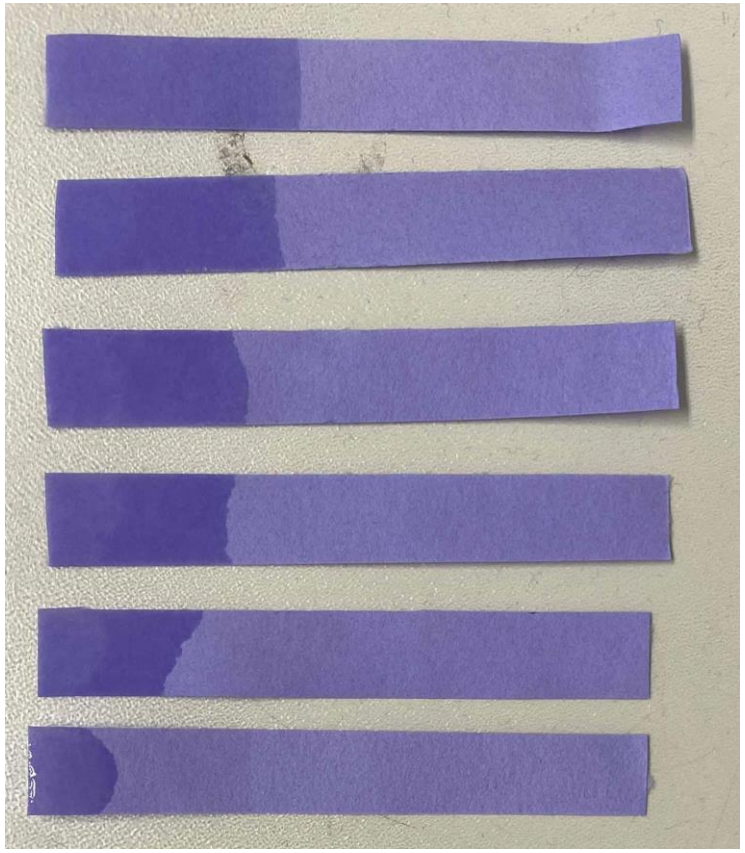
0.625

1.25

2.50

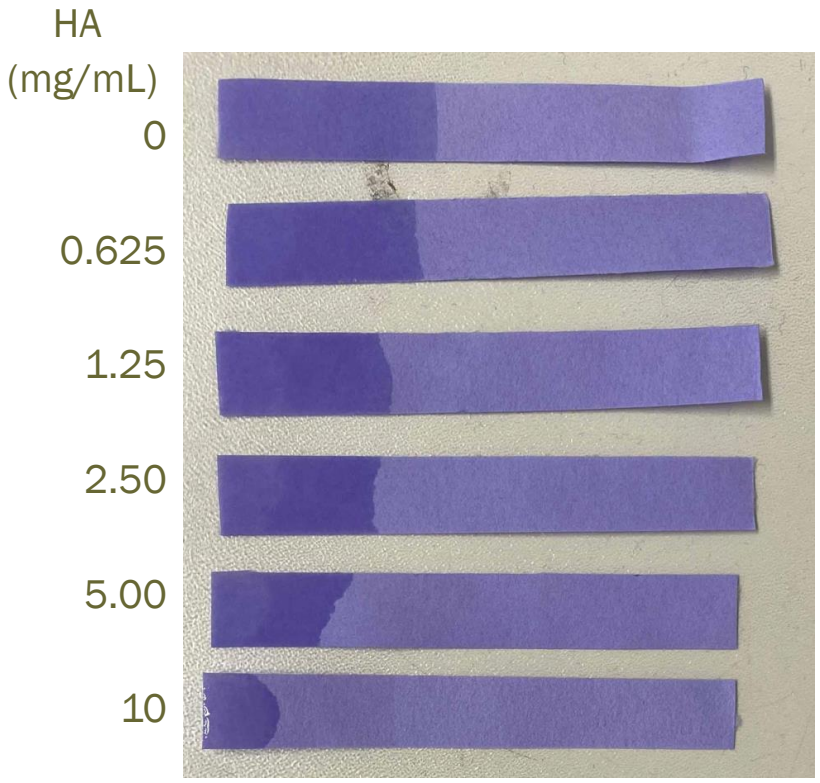
5.00

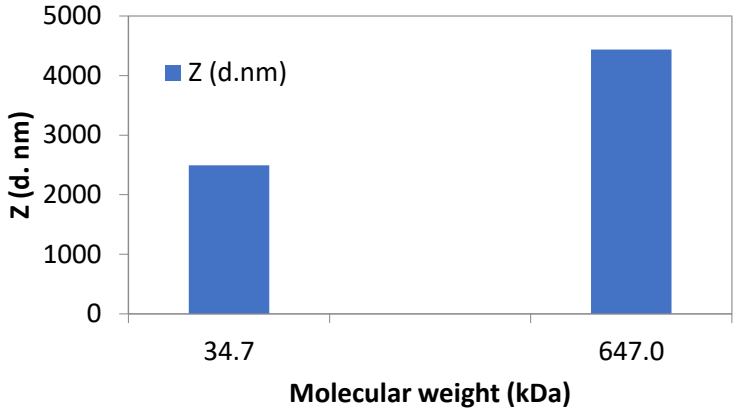
10

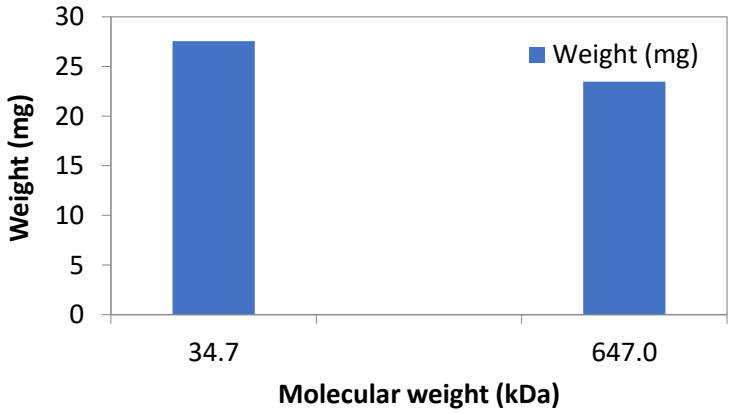


WP6 - Development and supply of best enzyme prototypes. (23 PM)

Hyaluronidase activity –new paper-based method - validation







WP6 - Development and supply of best enzyme prototypes. (23 PM)

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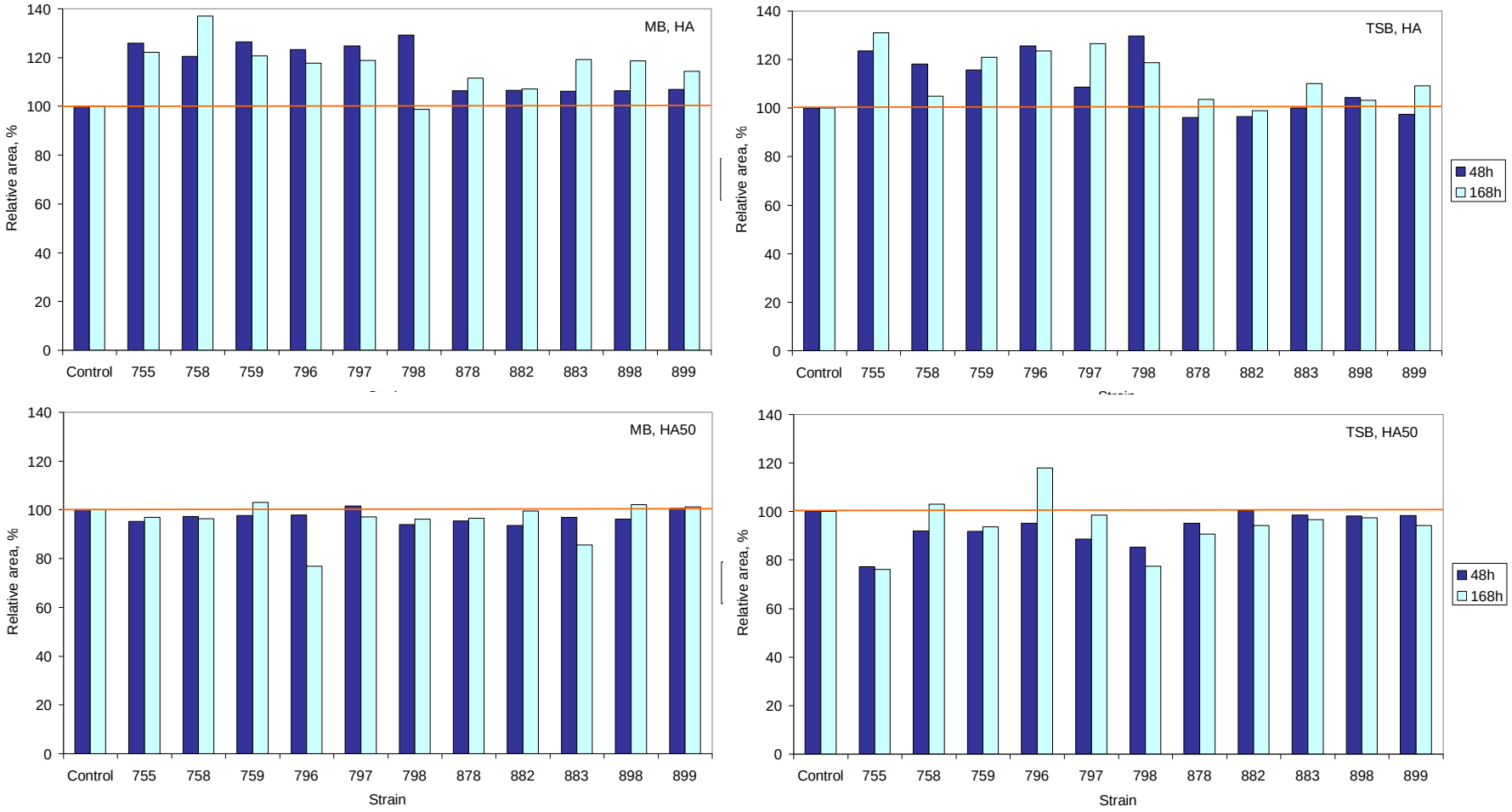
Hyaluronidase activity –new paper-based method



WP6 (& WP7)

Future experiments

Detergents: to test the 3 enzymes we've received in June with the stained textiles under conditions simulating a washing cycle (new reactors under development to simulate textile "rubbing"

Textiles: to test the 3 enzymes we've received in June to clean the dyeing liquid after dyeing process and to remove the solvents in the textiles; to use our strains for the same purpose

Cosmetics: to use our strains in small reactors until hyaluronidases are received.

For questions: Fabrizio

Hyaluronidase production by strain *Vibrio sp.* IAMC-CNR#23

Screening of cultivation media from the BCSMedDat database

- Media BCS365 and BCS366 identified as suitable for growth and hyaluronidase (extracellular production)
- Hyacare used as inducer

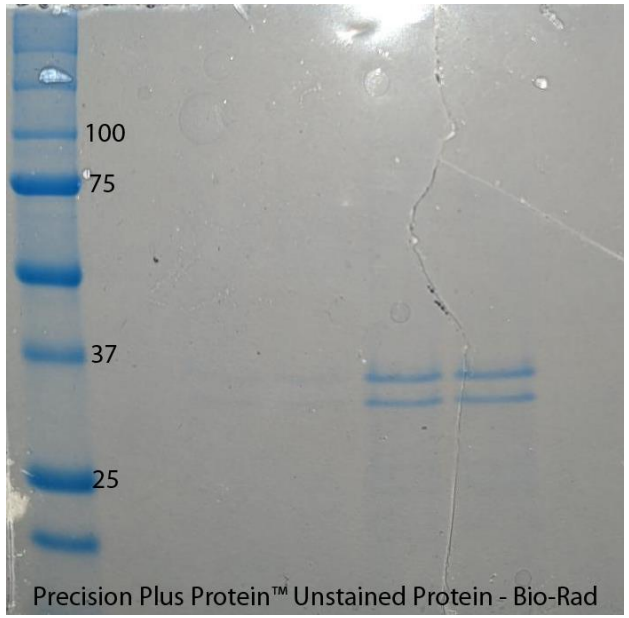
Medium	OD600 max	Hyaluronidase activity (IU/ml)	Notes
DSM2216	5,8	0,023	control
BCS365	4,6	0,028	
BCS366	8,7	0,028	

Hyaluronidase assay – notes on methodology

- Activity assay adapted from Dorfman, A. (1955) Methods in Enzymology, Volume I, 166-173 – current
- Activity assay adapted from Zhao et al. (2022) Anal. Chem. 2022, 94, 4643−4649 – in progress
- Activity considerably lower than reported in studies with other strains/sources. Qualitatively much evident
- pH has been modified from the method
- Study performed on the Hyacare substrates supplied by Evonics

Fermentation Hyaluronidase 15 L

- Activity peaks in 48 hours
- Concentration performed on 30 KDa ultrafiltration membranes
- No signal on SDS-PAGE coherent with the estimated Hyaluronidase size
- Other fermentations in progress
- Samples to be sent to CSIC



Fermentation of *Coriolopsis gallica*

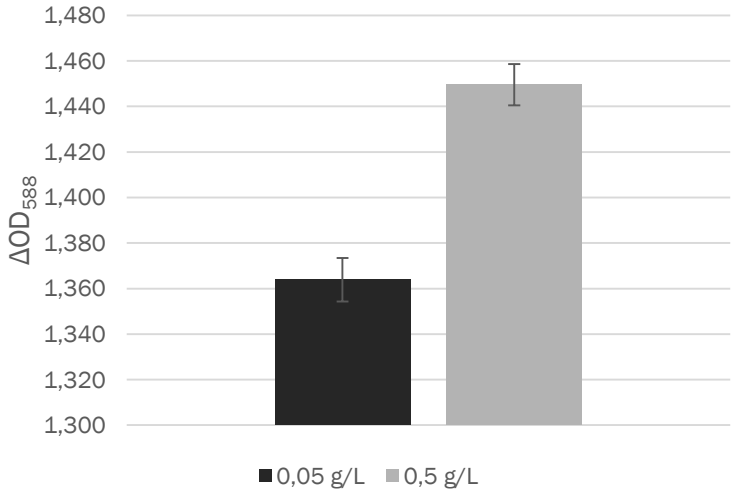
- High level of laccase activity
- Affordable fermentation and DSP process
- Concentration performed by ultrafiltration and (NH4)2SO4 precipitation
- Oxydation of wastewater dyes from Sholler

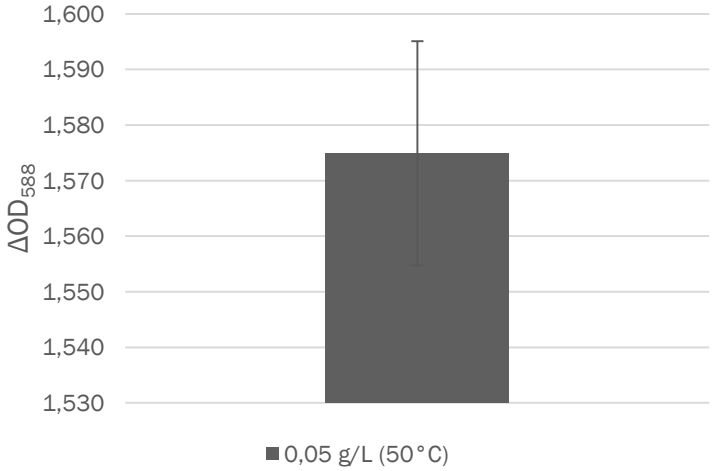
Fermentation *Coriolopsis gallica*

- Dye degradation test (Bangor)
- 10% liquid dye (Sholler)
- The assay was performed in 1mL solutions obtained by addition of 100 µL of dye, 100 µL of enzyme stock solution and 800 µL of ultrapure water. Absorbance changes were measured 588 nm after incubation at 30°C or 50°C for 50 minutes.

Fermentation *Coriolopsis gallica*

- samples treated with 0,05 µg/µL of laccases showed absorbance decrease of 75±1% while samples treated with 0,5 µg/µL of enzyme showed decrease of 79±1%.
- using 0,05 µg/µL of enzyme solution and an incubation temperature of 50°C absorbace decrease of 87±1% was achieved
- A greysh color is retained by the solution after treatment
- The tested laccase has proved to be efficient also in the presence of tensioactives (Tween 20 and Tween 80).





Conclusions and outlook

Hyaluronidase

- Production feasible and relative easy in an industrial medium
- Other inducers?
- Enzyme concentrate to be delivered to CSIC

Laccase

- Efficient in wastewater decoloration tests
- Next steps?

WP6 – Feedback of the Advisory Board

- **Environmental impact, safety issues, sustainability** needs are to be properly considered for enzymes' production technology and also for their further applications.

- The involved industrial partners might have **preferences regarding expression hosts and plasmids**. This should be clarified with them ideally prior to the enzyme production process. And if needed, reconsideration of enzyme production strategy should be considered.

- The consortium needs to be aware that the aimed production of enzymes will be industrial. Therefore, also the final production **strains/plasmids need to have industrial licenses** at that point.

- **Sustainability** needs to be precisely considered in the supply of the best enzymes (objective 5)

WP6 – Feedback of the Advisory Board

- It is essential to **precisely define the leading enzyme candidates**. If needed we can reach out **ICRT** (International Consumer and Research Testing, a consortium of which Altroconsumo is partner, and that usually carry out independent testing) **to assess the sustainability and eco-friendliness of the new products, but also the accessibility to the products in terms of price**. This would be a different comparison than that made inside our consortium with benchmark products. Niesel offered to cooperate also for the high scale enzyme production.

- De Lorenzo wanted to know about the **expression and production platform**, since we might be putting too much effort in this aspect. The part about discovery, screening and improvement of enzymes is of high quality, innovative and cutting-edge, but the production steps are “old-style”, lacking innovation. Ferrer explained that we usually begin with E. coli, and if it does not work we can turn into other organisms such as Aspergillus, or even the cell-free expression system that our partners from UHAM have developed in the frame of the project. Other partners such as Eucodis or BioC_Chem Solutions have different expression organisms that are at the disposal of the partners. BioC_Chem is developing a method that will predict the culture media depending on the sequence and organism.

- Regarding **commercialization** of enzymes and companies of interest: the path should be clearly defined. If you get interest from 2 or 3 companies, what will the group do? Who will **own the IP**?

Thank you!