Work Package 3: Activity-based bioprospecting for enzymes

Meeting #2

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 number [9]	WP3	Lead beneficiary [10]	3 - BANGOR
Work package title	Activity-based bio-prospecting for enzymes		
Start month	1	End month	36

Objectives

Entirely novel enzymes, which have no homologues in databases or with homology to known enzymes lower than ~20%, will escape the computational pre-screening in WP2. In order to circumvent this problem, WP3 combines three main pillars: bio-resources, technical capabilities for bio-resource handling and management, and activity-based multi-screens and Next Generation Sequencing (NGS), with a major objective: To screen for novel enzymes which, because their novelty, could not have been predicted using the BLAST, HMM and computational screens of WP2. To this end, the consortium has established a large collection of environmentally, geographically and taxonomically diverse bio-resources: 1. Cultured microbial isolates from (non)-extreme environments, many representing novel lineages; 2. Expression libraries from DNA from uncultivable microbial communities. 3. Genome sequences of cultivable microbial isolates; 4. Shotgun sequences of microbial communities; 5. Enzymes available in expression systems. The consortium has also at its disposal technical facilities and tools for: 1. Microbial handling and cultivation, including extremophiles, bioprospecting and sampling; 2. Handling and cloning DNA from uncultivable microorganisms; 3. High throughput screening supported by robotic and single cell manipulation workstations; 4. Multiple complementary analytics; 5. DNA sequencing (Illumina MiSeq and Oxford Nanopore instruments); 6. Bioinformatics analysis.

Description of work and role of partners

WP3 - Activity-based bio-prospecting for enzymes [Months: 1-36]
BANGOR, CSIC, UHAM, UDUS, IST ID, CNR
In WP3 we propose 3 major tasks, through which we will implement sophisticated activity-based platforms to exploit available and new ad hoc bio-resources for entirely novel enzymes demanded by the detergent, textile and cosmetic sectors.

Task 3.1 Exploitation of the FuturEnzyme bio-resource collections M1-M24
Task Lead Partner – IST-ID
Participants: CSIC, UHAM, UDUS, BANGOR, CNR

Task 3.2 Sampling extreme environments for generating new microbial bio-resources M6-M30
Task Lead Partner – CNR
Participants: IST-ID

Task 3.3 Next Generation Sequencing for generating sequences of target enzymes M1-M36
Task Lead Partner – BANGOR
Participants: CSIC, UHAM, CNR

Participation per Partner

Partner number and short name	WP3 effort
1 - CSIC	5.00
3 - BANGOR	17.00
4 - UHAM	10.00
5 - UDUS	4.00
6 - IST ID	21.00
7 - CNR	18.00
Total	75.00

List of deliverables

Deliverable Number[14]	Deliverable Title	Lead beneficiary	Type[15]	Dissemination level[16]	Due Date (in months)[17]
D3.1	Bio-resources prepared and exchanged	3 - BANGOR	Other	Confidential, only for members of the consortium (including the Commission Services)	2
D3.2	Standard assays, analytics and calculations for monitoring enzymatic performance	4 - UHAM	Report	Confidential, only for members of the consortium (including the Commission Services)	2
D3.3	Set of 100 best clones, 10 isolates, and 10 enzymes shortlisted for sequencing or transfer to WP2	6 - IST ID	Other	Confidential, only for members of the consortium (including the Commission Services)	10
D3.4	Sequence, activity, and stability datasets from best positive bio-resources	3 - BANGOR	data sets, microdata, etc	Confidential, only for members of the consortium (including the Commission Services)	18
D3.5	Set of new bio-resources to screen or sequence	7 - CNR	Other	Confidential, only for members of the consortium (including the Commission Services)	24
D3.6	Complete set of positive naïve screened enzymes and sequences and their datasets	3 - BANGOR	Report	Confidential, only for members of the consortium (including the Commission Services)	32

Task 3.1 Exploitation of the FuturEnzyme bio-resources collections (M1-M24)

- A list of bio-resources available within the consortium have been prepared and exchange (D3.1 – month M2)
  - All partners implicated

D3.1. BIO-RESOURCES PREPARED AND EXCHANGED

ENZYMES AVAILABLE
1353 entries, representing highly diverse enzymes relevant to FuturEnzyme, available in expression systems, from single (meta)genomes; the enzymes have been isolated and characterized for purposes others than those in FuturEnzyme, and will be now screened with project-relevant substrates and conditions.

ISOLATES AVAILABLE
1387 entries, representing psychrophilic, mesophilic, thermophilic, hyper-thermophilic, thermo-acidophilic, alcaliphilic, extreme halophilic, obligate anaerobic and facultative (micro)aerobic sulphur-respiring microorganisms. The collection includes strains growing at temperatures from 0° to 92°C, pH from 1.5 to 10.0, salinity up to 490 g/L, and pressure up to 50 Mpa.

ISOLATES WITH GENOMES AVAILABLE
197 entries, representing genomes from isolates representing lineages of (non)-extremophiles growing from 0 to 92°C, pH from 1.5 to 9.0, salinity up to 492 g/L, pressure up to 50 MPa.

METAGENOMIC LIBRARIES
28 entries, representing DNA material from communities inhabiting extreme environments (low pH from 1.1 to 4.4; high pH of 9.3-9.6; high salinity from 200 to 490 g/L; pressure up to 300 MPa; temperature up to 98°C) and non-extreme environments, including contaminated sites (close to neutral pH, low to moderate salinity (up to 50 g/l), temperatures from 4 to 30°C, up to 10.1 MPa).

ENRICHMENT CULTURES
41 entries, derived from samples originated from multiple locations and representing enriched microorganisms of at least 16 different genera.

ISOLATES WITH PROVEN ACTIVITY
55 entries.

SHOTGUN METAGENOME SEQUENCES
61 entries, corresponding to at least 16 different types of extreme and non-extreme environments.

These QR codes are confidential and available within the FuturEnzyme consortium. In order to increase the security, they have been blocked with a password (FuturEnzyme€01/06/2021). They will also be included in the private area of the FuturEnzyme website (www.futurenzyme.eu), in the section Shared material. This private area that serves as a repository for the project is accessible to the members of the consortium through user and password.

Submitted to the EU portal on 30th july 2021

Task 3.1 Exploitation of the FuturEnzyme bio-resources collections (M1-M24)

- A number of assays for functional screens have been defined and shared (D3.2 – Month 2)
  - All partners implicated

D3.2. STANDARD ASSAYS, ANALYTICS AND CALCULATIONS FOR MONITORING ENZYMATIC PERFORMANCE

18x Protocols for DETERGENT APPLICATIONS
4x pH shift liquid protocols for quantifying esterase-lipase activity
3x Liquid protocols for quantifying esterase-lipase activity with chromogenic esters
2x Liquid protocols for quantifying esterase-lipase activity with non-chromogenic esters
9x Agar plate protocols

23x Protocols for TEXTILE APPLICATIONS
7x Agar plate polyesterase screening assays
2x Agar plate protease protocols
1x Agar plate cellulase protocol
1x Agar plate oxidoreductase protocol
12x Liquid protocols

48 common and standardised protocols

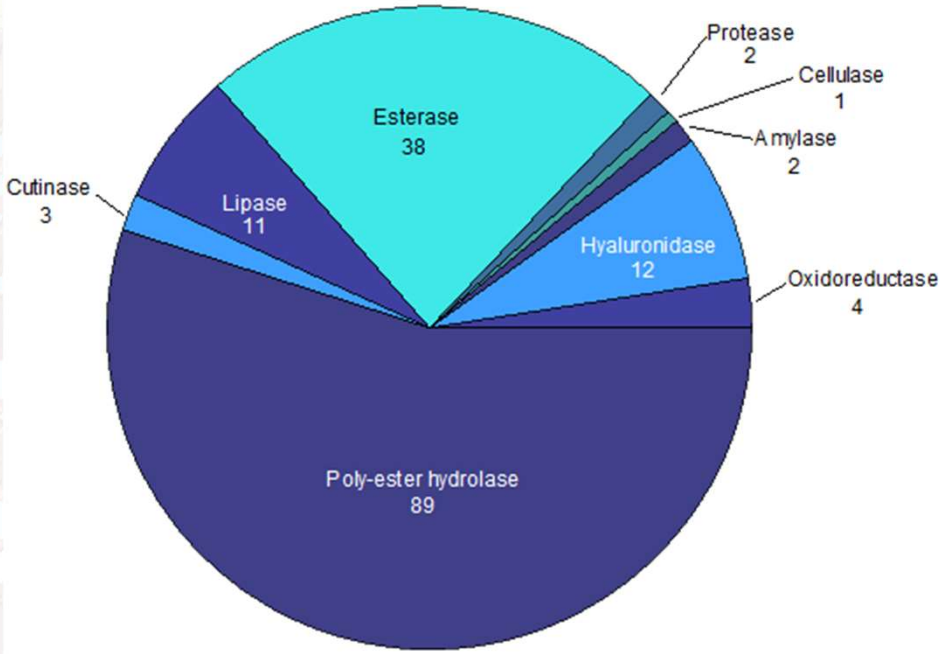
7x Protocols for COSMETIC APPLICATIONS
4x Liquid colorimetric assays for hyaluronidase activity
1x Liquid analysis of hyaluronic acid oligosaccharides by HPAEC-PAD
1x Liquid analysis of hyaluronic acid oligosaccharides by SEC-ELSD
1x Agar assay for the determination of hyaluronidase activity

Submitted to the EU portal on 30th july 2021

Task 3.1 Exploitation of the FuturEnzyme bio-resources collections (M1-M24)

- A list of best clones, isolates and enzymes have been prepared and shortlisted for sequences (D3.3 – month M10)
  - All partners implicated

D3.3. 100 Best clones, 10 isolates, and 10 enzymes shortlisted for sequencing or transfer to WP2



Distribution by activity of the enzymes and microorganisms selected in this deliverable

The 154 prospects (up to 162, noticing that some isolates present different activities, so most probably several enzymes) have been settled apart out of 120 genomes from isolates, metagenomes from 47 microbial communities, 1200 microbial strains, 30 metagenome libraries and 500 enzymes (as mentioned in Task 3.1, Grant Agreement, Annex 1, part A).

Isolates/Microorganisms

			Number of isolates with activity/ies	First priority for (industrial partner/s)	Second priority for (industrial partner/s)
Oxidoreductase			3	Schoeller	Henkel
Hydrolase	Glucosidase	Hyaluronidase	10	Evonik	
		Amylase	1	Scholler	Henkel
	Peptidase	Protease	1	Scholler	Henkel
	Ester-hydrolase	Esterase	4	Henkel/ Schoeller	
		Lipase	8	Henkel/ Schoeller	
		Cutinase	3	Henkel/ Schoeller	

Enzymes

				Number of enzymes with activity	First priority for (industrial partner/s)	Second priority for (industrial partner/s)
EC 1	Oxidoreductase	Laccase, Cu-oxidase		1	Schoeller	Henkel
EC 3	Hydrolase	Glucosidase	Hyaluronidase	2	Evonik	
			Amylase	1	Scholler	Henkel
			Cellulase	1	Schoeller	
		Peptidase	Protease	1	Scholler	Henkel
		Ester-hydrolase	Esterase	34	Henkel/ Schoeller	
			Lipase	3	Henkel/ Schoeller	
			Poly-ester hydrolase	89	Schoeller	Henkel

D3.3. 100 Best clones, 10 isolates, and 10 enzymes shortlisted for sequencing or transfer to WP2

The following QR code directs to the full list of **candidate isolates**

The following QR code directs to the full list of **candidate enzymes**

Task 3.1 Exploitation of the FuturEnzyme bio-resources collections (M1-M24)

- Data set of sequence, activity and stability from best positive bio-resources (D3.4 – month M18) *in progress*

UDUS

- WP3
  - ➢ Task 3.1 Exploitation of the FuturEnzyme bio-resource collections

✓ A set of 88 esterases and lipases including 16 cutinase-like enzymes from previous projects collected

	1	2	3	4	5	6	7	8	9	10	11	12
A	LCC (WCCG)	Psab PE-H	Abo_LipA (CE02)	Paes TB045 (CE15)	CalB	Est24c11	TBEc350	Hyd8c31	Sma LipA	Aku GDSL1 (CE17)	Hic	Hyd20c2
B	LCC	Paby PE-H	Hyd18c13	Abo_LipC (CE09)		TBEclH8	TBEc321	Hyd18c8	Hyd4c6	Est8c8	ED30_ K329E	Lip10c11
C	PETase	Ppac PE-H	TBEc157	Hyd13c2	Aku Est3 (CE20)	FsCut	Est9c19	Lip3c12	Hyd3c14	Abo_LipE (CE10)	PT35_ E121Q	Est20c28
D	Plit PE-H	Poce PE-H	TBEc310	Abo_LipD (CE07)	Est9c28	Hyd10c19	TBEc314	Aku Est2 (CE19)	Abo_LipG (CE11)	Abo_LipI (CE12)	Cyc	Est13c9
E	Pbau PE-H	Paes PE-H Y250S	TBEc304	Hyd33c4	Lip1c6	Est29c9	Hyd20c11	Paes TB035 (CE13)	1,4-D#003	Hyd22c10	Dim008	Est30c5
F	Ppel PE-H	Paes PE-H (CE16)	CycTB025	Abo_Est3 (CE03)	MHETase	Est51c6	Aku Est1 (CE18)	TBEc305	Hyd23c15	TBEc308		EstP
G	POIL-1 PE-H	Pxin PE-H	Hyd7c19	EV	Dim-008 (CE01)	Paes TB037 (CE14)	Est16c36	ED30	Est24c4	Hyd19c35	Est65c2	
H	Abo_Est7 (CE05)	Paes TB072(CE24)	Dim004	Abo_Est1	Paes TB040 (CE22)	Sep029-D9	PaesTB074 (CE25)	Dim001	Paes TB081 (CE23)	Abo Est2 (CE04)	Paes TB001 (CE21)	Abo Est12 (CE08)

Table of ready to use UDUS/UHAM esterases & lipases at UDUS for FuturEnzyme activities

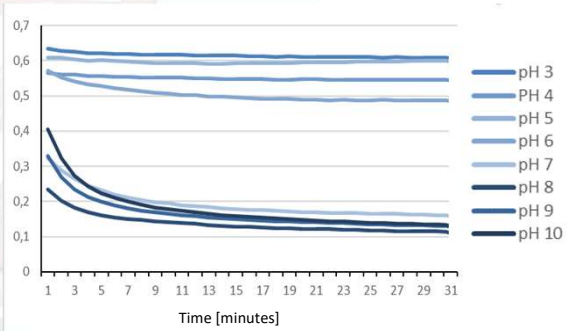
UDUS

- WP3
  - ➢ Task 3.1 Exploitation of the FuturEnzyme bio-resource collections

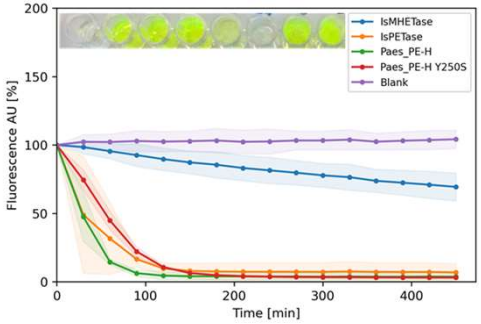
✓ a set of 88 esterases and lipases including 16 cutinase-like enzymes from previous projects collected

✓ MTP assay development

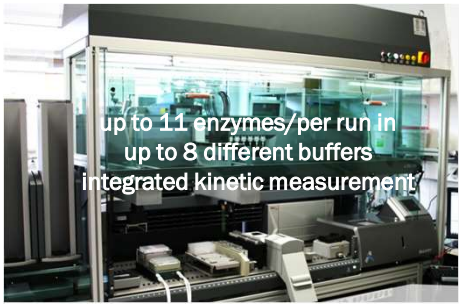
Turbidity measurements for cutinase characterization



pH indicator assay for hydrolysis of solid substrates



Implemented automated characterization



UDUS

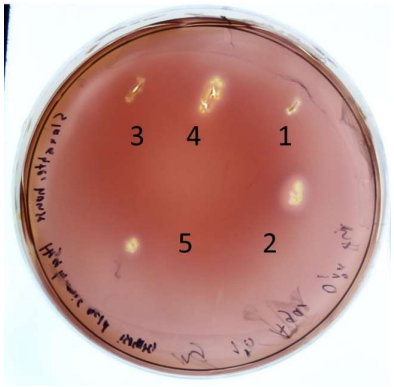
- WP3
  - ➢ Task 3.1 Exploitation of the FuturEnzyme bio-resource collections

✓Enrichment cultures of slaughterhouse metagenome bank with hyaluronic acid

➢Several strains with *in silico* put. hyaluronic acid lyases

➢Mainly from clade of gamma proteobacteria,
e.g., *Proteus* sp. or *Raoultella* sp.

M9 medium
+0.1% hyaluronic acid
stained with iodide



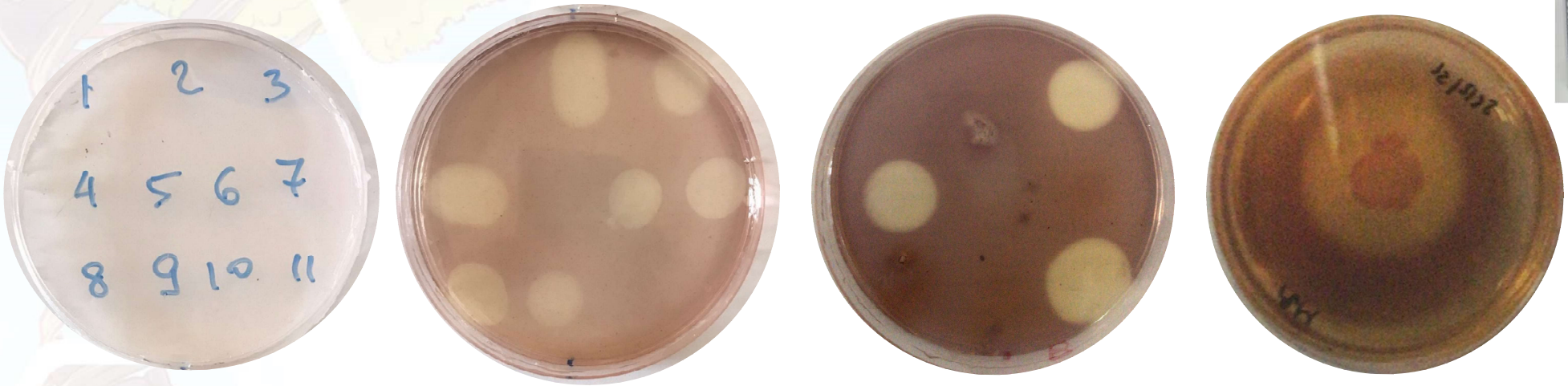
UDUS

- WP3 Deliverable 3.3 Resources

	Source	ID-status	Genome status	Enzyme candidates
isolates showing hyaluronic acid hydrolysis				
Proteus sp.	Slaughterhouse drain	Partial 16S rDNA sequence	Not sequenced, Sequence of related strains available	*In silico:* Chondroitin lyase Put. Hyaluronic acid AC Lyase
Raoultella sp.	Slaughterhouse drain	Partial 16S rDNA sequence	Not sequenced, Sequence of related strains available	*In silico*: Put. heparinase II/III family protein
Enterobacteria	Slaughterhouse drain	Partial 16S rDNA sequence	Not sequenced, Sequence of related strains available	
Spirosoma sp.	Slaughterhouse drain	Partial 16S rDNA sequence	Not sequenced, Sequence of related strains available	*In silico:* Put. polysaccharide lyase
strains exceptionally enriched in esterases				
Halopseudomonas aestusnigri	Oil polluted coast (spain)	Type strain	draft	2 Polyesterases (1 confirmed), 12 confirmed additonaly esterases
Halopseudomonas litoralis	Coastal waters (spain)	Type strain	Closed genome available.	2 Polyesterases (1 confirmed)
Halopseudomonas oceani	Deep Sea	Type strain	draft	2 Polyesterases (1 confirmed)
Halopseudomonas bauzanensis	Polluted industrial site soil (italy)	Type strain	draft	2 Polyesterases (1 confirmed)

Task 3.1 Exploitation of the FuturEnzyme bio-resources collections (M1-M24)

- Two hyaluronic-acid degrading isolates have been identified by CNR, when using EVO hyaluronic acid as substrate for screening



- *Halorhabdus* sp. SivX81 (genome sequenced)
- *Vibrio alginolyticus* from anoxic sediments of meromictic brackish Lake Faro, Messina

List of hydrolytic halo- and halonatronoarchaea

Strain	Lakes	Affiliation	Substrate	Activity	Growth
H-hyl	Cl	Halobacteria (new genus?)	Hyaluronic acid (HA)	+	+
RMX81	Cl	Halorhabdus sp.	Coco oil, xylan. HA	+	+
RMX62	Cl	Halorhabdus uthaensis	Coco oil, skim milk, xylan, HA	+	+
Siv8X	Cl	Halorhabdus uthaensis	Coco oil, skim milk, xylan HA	+	+
HArcel-Eu2	Cl	Halomicrobium sp.	Hyaluronic acid, cellulose	+	+
HArcel2**	Cl	Halosimplex sp.	Cellulose	+	+
HArcel3**	Cl	Halomicrobium sp.	Cellulose	+	+
Harc-L1	Cl	Halobacteria (unidentified)	Olive oil	+	+
Harc-L2	Cl	Halobacteria (unidentified)	Olive oil	+	+
BNX81	Cl	Halococcoides cellulosivorans	Cellulose, xylan HA	+	+
LCL711		Halorhabdus sp.	Xylan, hyaluronic acidHA	+	+
AB-hyl1	SL	Paracoccus sp.	Hyaluronic acid (HA)	+	+
AArcel7	SL	Natrarchaeobius sp.	Hyaluronic acid (HA)	+	-
AArc-St1-1*	SL	Natranaeroarchaeum aerophilum	Hyaluronic acid (HA)	+	-
AArc-L1	SL	Natrarchaeobaculum aegyptiacus	Olive oil	+	+
AArc-L2	SL	Natronolimnohabitans innermongolicus	Olive oil	+	+
AArc-LBj	SL	Halobacteria (unidentified)	Olive oil	+	+

S - soda lakes; Cl - chloride lakes;

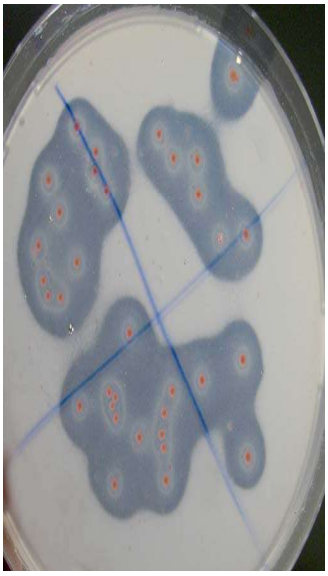
* - extremely high carotenoid content - suitable for production?

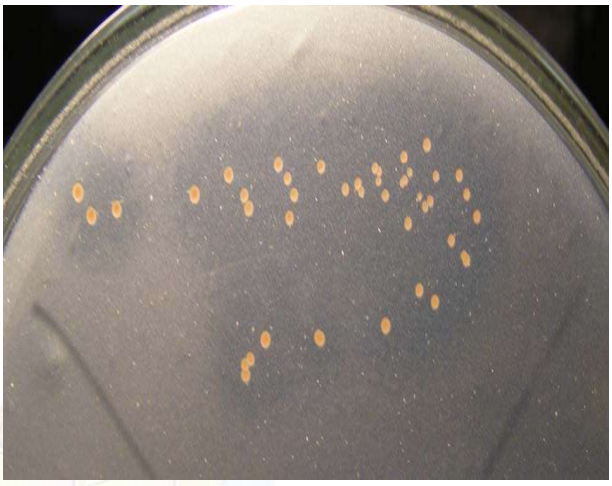
** - for genomes

Bold – genome sequenced

AArc-L1

AArc-LBj

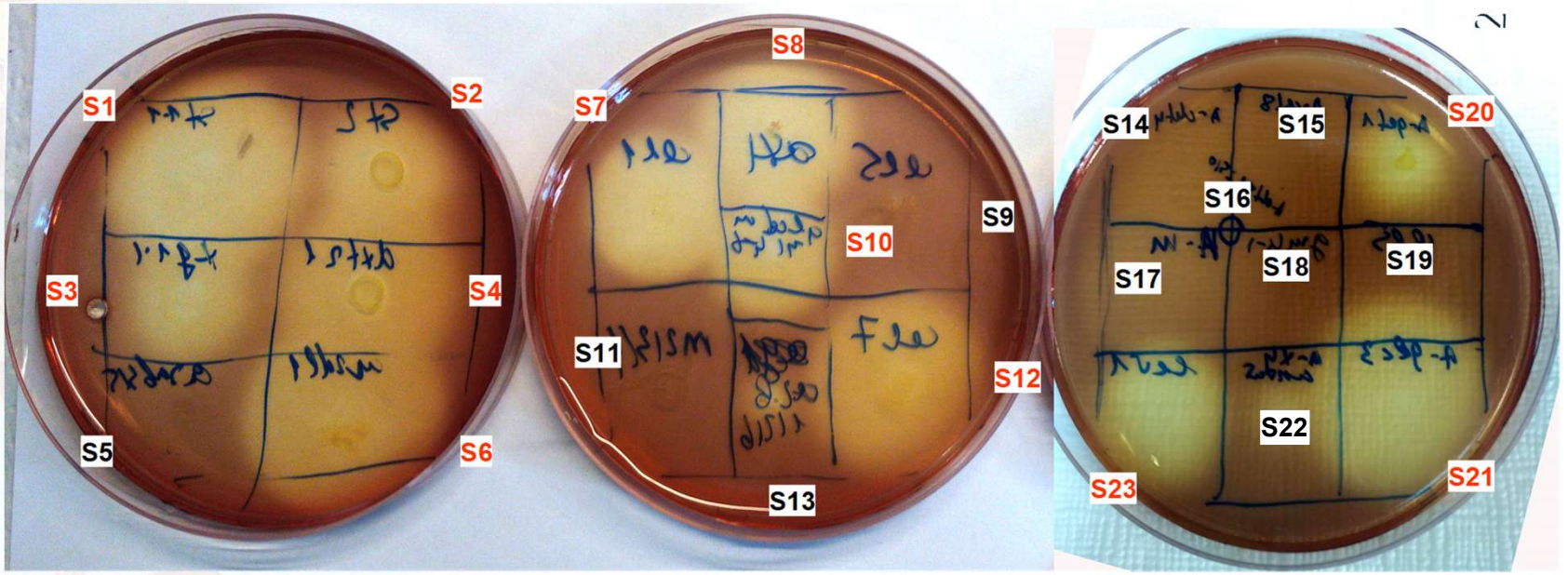




AArc-L2

Lipase activity in colonies of natronoarchaea

Natronoarchaeal HA+ isolates grown at 4 M Na^+/pH 9.5 (Hyaluronic acid+ye) / 37°C/ 9d)



Soda lake natronoarchaea

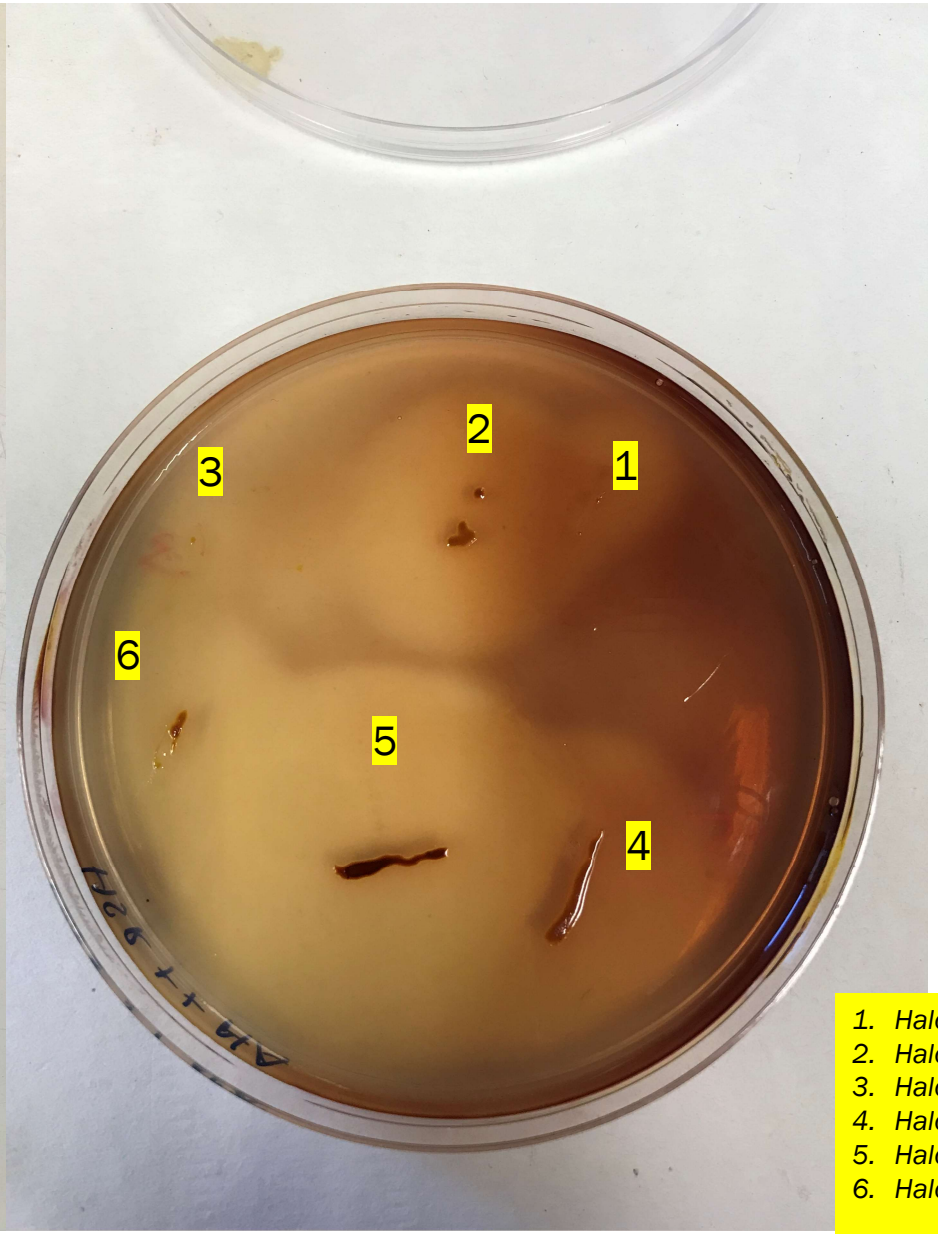
S1: AArc-St1-1 (amylolytic)
S2: AArc-St2 (amylolytic)
S3: AArc-xg1-1 (xyloglycan)
S4: AArc-dxtr1 (dextran)
S5: AArc-arb3/5 (arabinan)
S6: AArc-curdl1 (curdlan)
S7: AArcel1 (*Natronolimnobius*; cellulo-xylan)
S8: AArc-ax1(*Natronolimnobius*; arabinoxylan)
S9:AArcel5 (*Natronobiforma*; cellulo-xylan)
S10: AArc-glctm3/4/8 (*Natronococcus*; glct-mannan)
S11:AArc-m2/3/4 (mannan-cellulo)
S12: AArcel7 (chitin)
S13: AArc-arb1/2/6 (*Natronolimnobius*; arabinan)

S13

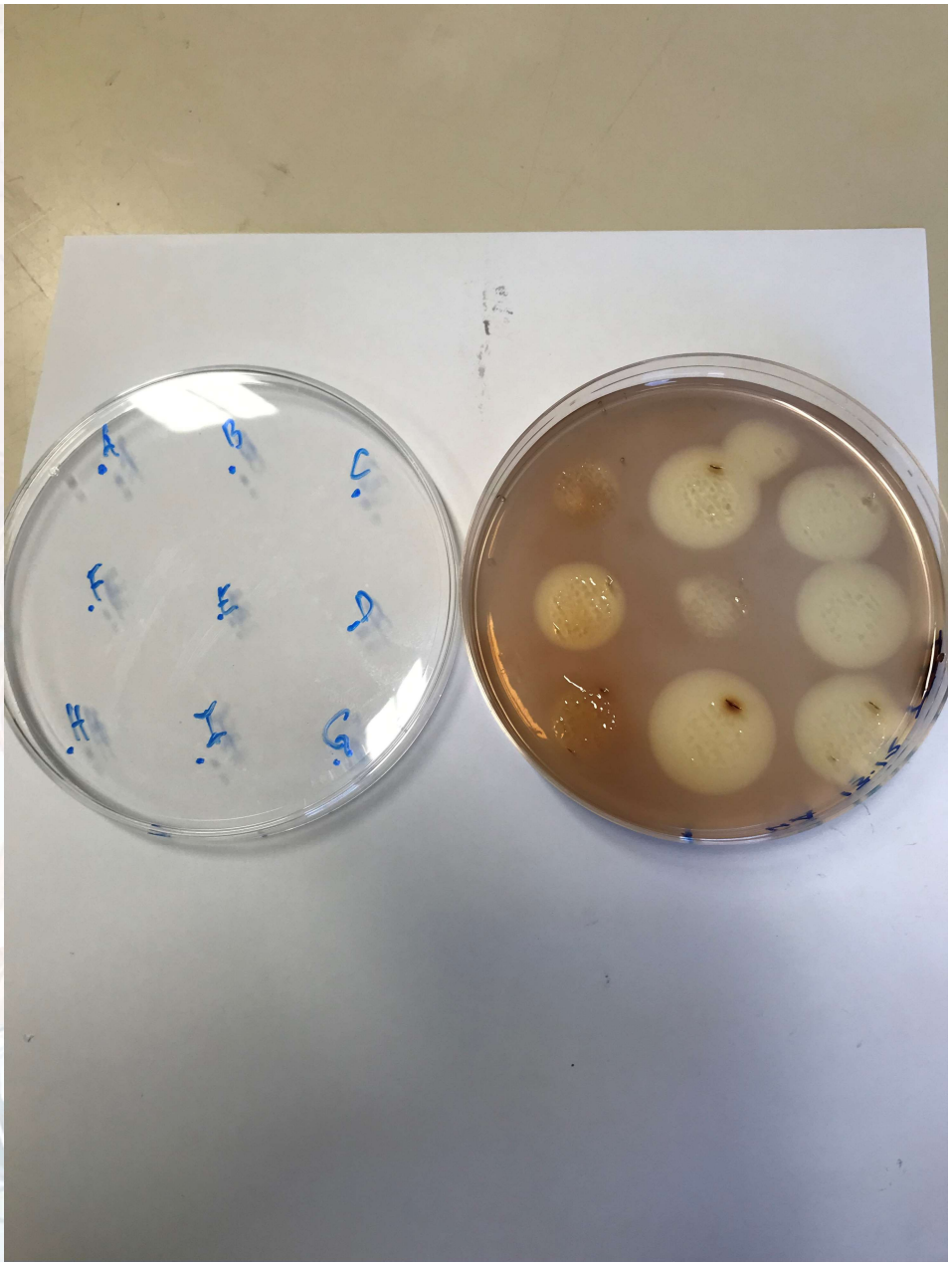
S14: AArcht4 (*Natrarchaeobius chitinivorans*)
S15: AArcht8 (*Natrarchaeobius chitinivorans*)
S16: AArcht-Sl (*Natrarchaeobius chitinivorans*)
S17-19: Mannan-utilizing natronoarchaea
S20:AArc-glct1 (galactan)
S21: AArc-glc3 (*Natronorubrum tibitense*; glycogen)
S22: AArc-X4 (*Halomicrobium* sp; cellulo)
S23: AArc-lev1 (levan)[~Aarc-St1-1]

Only **AArcel7** showed moderate growth with Hyl in liquid culture without ye. But it faded after second transfer
Colony growth: S1, S2, S3, S4, S6(weak), S7, S8, S9(w), S10, S11, **S12,** S13; Cl6

Halorhabdus sp. LCL711



1. *Halomicrobium* sp. LC1Hm
2. *Halorhabdus uthaensis* Siv8X
3. *Halomicrobium* sp. Harcel-Eu2
4. *Halococcoides cellulosivorans* BNX81
5. *Halorhabdus* sp. RMX81
6. *Halorhabdus uthaensis* RMX62



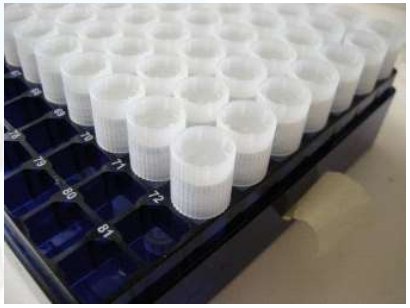
A. Haloferax lucertense SVX82
B. Halosimplex sp. HArcel2
C. Halomicrobium sp. Harcel3
D. Halobacteria (new genus) H-hyl
E. Haloferax alexandrinus BNX82
F. Halorhabdus sp. KCL-HA6
G. Halococciodes sp. BariCL
H. *Haloferax* sp. SVXCL
I. *Halorhabdus* sp. KCL5

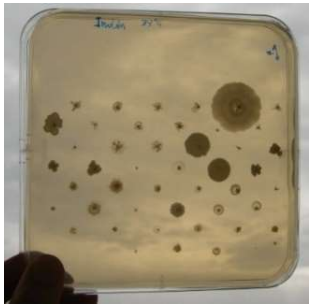
Hyaluronidase activity by agar-diffusion test of haloarchaeal cultures grown with cellobiose + hyaluronate for one week. Fraction of 50 µl of supernatant was placed on filter discs and incubated on the plates with hyaluronic acid (400 mg l^{-1}) at pH 7.5, 4 M total Na^+, 37 °C, 24h;

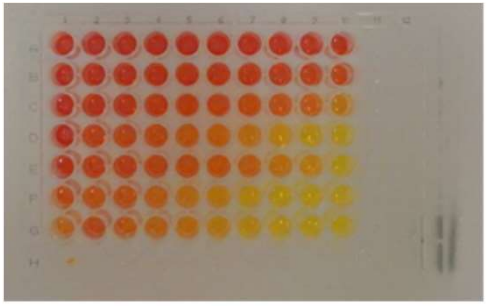
IST-ID The Association of Instituto Superior Técnico for Research and Development.

- WP3 - Activity-based bio-prospecting for enzymes. (21 PM)

- sampling for new bio-resources; identifying novel microbes and enzyme activities; screening our microbial collection for efficient enzymes







	Isolate #	Lipase, 30°C
Bacillus subtilis spizizenii	83	++
Not identified	422	++
Bacillus circulans	435	++
Bacillus licheniformis	603	++
Bacillus pumilus	637	++
Bacillus mycoides	82	+
Bacillus longisporus	110	+
Bacillus longisporus	145	+
Bacillus longisporus	174	+
Bacillus longisporus	177	+
Bacillus longisporus	217	+
Bacillus longisporus	225	+
Bacillus longisporus	231	+
Bacillus pumilus	323	+

Task 3.1 Exploitation of the FuturEnzyme bio-resource collections

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

Table MS9.1 – Isolates with lipase activity at 30ºC.

	Isolate #	Lipase, 30ºC
Bacillus subtilis spizizenii	83	++
Not identified	422	++
Bacillus circulans	435	++
Bacillus licheniformis	603	++
Bacillus pumilus	637	++
Bacillus mycoides	82	+
Bacillus longisporus	110	+
Bacillus longisporus	145	+
Bacillus longisporus	174	+
Bacillus longisporus	177	+
Bacillus longisporus	217	+
Bacillus longisporus	225	+
Bacillus longisporus	231	+
Bacillus pumilus	323	+
Bacillus megaterium	348	+
Bacillus megaterium	349	+
Bacillus megaterium	374	+
Bacillus subtilis	375	+
Bacillus subtilis spizizenii	381	+
Bacillus megaterium	383	+
Bacillus megaterium	384	+
Not identified	389	+
Bacillus pumilus	390	+
Bacillus megaterium	394	+
Bacillus megaterium	399	+
Arthrobacter globiformis	431	+
Bacillus licheniformis	481	+
Bacillus megaterium	484	+
Brevibacillus centrospor	562	+
Alcaligenes faecalis	563	+
Bacillus licheniformis	564	+
Bacillus longisporus	568	+
Bacillus pumilus	572	+
Bacillus pumilus	575	+
Bacillus licheniformis	594	+
Bacillus longisporus	641	+
Bacillus gibsonii	651	+
Bacillus longisporus	655	+
Bacillus longisporus	671	+
Bacillus longisporus	689	+

Table MS9.2 – Isolates with protease activity at 30 and 37ºC.

	Isolate #	Protease, 30°C	Protease, 37°C
Bacillus longisporus	217	+	++
Bacillus longisporus	225	+	++
Bacillus cereus	347	+	++
Bacillus subtilis spizizenii	381	+	++
Bacillus thuringiensis	382	+	++
Bacillus megaterium	394	+	++
Bacillus megaterium	399	+	++
Bacillus megaterium	484	+	++
Bacillus pumilus	572	+	++
Bacillus licheniformis	594	+	++
Bacillus longisporus	641	+	++
Bacillus longisporus	655	+	++
Alcaligenes faecalis	634		++
Virgibacillus pantothenticus	596	++	+
Bacillus mycoides	82	+	+
Bacillus subtilis spizizenii	83	+	+
Bacillus longisporus	110	+	+
Bacillus longisporus	145	+	+
Bacillus longisporus	174	+	+
Bacillus longisporus	177	+	+
Bacillus longisporus	231	+	+
Bacillus pumilus	323	+	+
Bacillus megaterium	348	+	+
Bacillus megaterium	349	+	+
Bacillus megaterium	374	+	+
Bacillus subtilis	375	+	+
Bacillus megaterium	383	+	+
Bacillus megaterium	384	+	+
Not identified	389	+	+
Not identified	422	+	+
Bacillus licheniformis	481	+	+
Brevibacillus centrospor	562	+	+
Alcaligenes faecalis	563	+	+
Bacillus licheniformis	564	+	+
Bacillus longisporus	568	+	+
Bacillus pumilus	575	+	+
Bacillus licheniformis	603	+	+
Bacillus pumilus	637	+	+
Bacillus gibsonii	651	+	+
Bacillus marisflavi	668	+	+
Bacillus longisporus	671	+	+
Bacillus longisporus	689	+	+
Alcaligenes faecalis	266	++	
Bacillus thuringiensis	346B	++	
Bacillus circulans	370	+	
Bacillus pumilus	390	+	
Arthrobacter globiformis	431	+	
Bacillus circulans	435	+	

Table MS9.3 – Isolates with inulinase activity at 30 and 37ºC.

	Isolate #	Inulinase, 30 °C	Inulinase, 37 °C
Bacillus subtilis spizizenii	83	+	++
Bacillus longisporus	217	+	++
Bacillus longisporus	225	+	++
Bacillus circulans	370	++	++
Bacillus thuringiensis	382	++	++
Bacillus mycoides	82	+	+
Bacillus longisporus	110	+	+
Bacillus longisporus	145	+	+
Bacillus longisporus	174	+	+
Bacillus longisporus	177	+	+
Bacillus longisporus	231	+	+
Bacillus pumilus	323	+	+
Bacillus cereus	347	++	+
Bacillus megaterium	348	+	+
Bacillus megaterium	349	+	+
Bacillus megaterium	374	+	+
Bacillus subtilis	375	+	+
Bacillus subtilis spizizenii	381	+	+
Bacillus megaterium	383	+	+
Bacillus megaterium	384	+	+
Not identified	389	+	+
Bacillus megaterium	394	+	+
Bacillus megaterium	399	+	+
Not identified	422	+	+
Bacillus circulans	435		+
Bacillus licheniformis	481	++	+
Bacillus megaterium	484	+	+
Brevibacillus centrospor	562	+	+
Alcaligenes faecalis	563	+	+
Bacillus licheniformis	564	+	+
Bacillus longisporus	568	+	+
Bacillus pumilus	572	+	+
Bacillus pumilus	575	+	+
Bacillus licheniformis	594	+	+
Virgibacillus pantothenticus	596		+
Bacillus licheniformis	603	+	+
Bacillus pumilus	637	+	+
Bacillus longisporus	641	+	+
Bacillus gibsonii	651	+	+
Bacillus longisporus	655	+	+
Bacillus marisflavi	668	++	+
Bacillus longisporus	671	+	+
Bacillus longisporus	689	++	+
Bacillus pumilus	390	++	
Arthrobacter globiformis	431	+	
Bacillus thuringiensis	346B	++	

Table MS9.4 – Isolates with amylase activity at 30 and 37ºC.

	Isolate #	Amylase, 30 °C	Amylase 37 °C
Bacillus mycoides	82	++	++
Virgibacillus pantothenticus	596	+	++
Bacillus subtilis spizizenii	83	+	+
Bacillus longisporus	110	+	+
Bacillus longisporus	145	+	+
Bacillus longisporus	174	+	+
Bacillus longisporus	177	+	+
Bacillus longisporus	217	+	+
Bacillus longisporus	225	+	+
Bacillus longisporus	231	+	+
Bacillus pumilus	323	+	+
Bacillus cereus	347	+	+
Bacillus megaterium	348	+	+
Bacillus megaterium	349	+	+
Bacillus megaterium	374	+	+
Bacillus subtilis	375	+	+
Bacillus subtilis spizizenii	381	+	+
Bacillus megaterium	383	+	+
Bacillus megaterium	384	+	+
Not identified	389	+	+
Bacillus megaterium	394	+	+
Bacillus megaterium	399	+	+
Not identified	422	+	+
Bacillus licheniformis	481	+	+
Bacillus megaterium	484	+	+
Brevibacillus centrospor	562	+	+
Alcaligenes faecalis	563	+	+
Bacillus licheniformis	564	+	+
Bacillus longisporus	568	+	+
Bacillus pumilus	572	+	+
Bacillus pumilus	575	+	+
Bacillus licheniformis	594	+	+
Bacillus licheniformis	603	+	+
Bacillus pumilus	637	+	+
Bacillus longisporus	641	+	+
Bacillus gibsonii	651	+	+
Bacillus longisporus	655	+	+
Bacillus marisflavi	668	+	+
Bacillus longisporus	671	+	+
Bacillus longisporus	689	+	+
Alcaligenes faecalis	266	++	
Bacillus circulans	370	++	
Bacillus thuringiensis	382	++	
Bacillus pumilus	390	+	
Arthrobacter globiformis	431	+	
Bacillus circulans	435	++	
Alcaligenes faecalis	634	++	
Bacillus thuringiensis	346B	+	

Table MS9.5 – Isolates with transaminase activity at 30 and 37ºC.

	Isolate #	Transaminase, 30ºC
Bacillus longisporus	225	++
Not identified	232	++
Marinobacter psychrophilus	240	++
Lacticigenium naphtae	256	++
Aeromonas caviae	257	++
Not identified	521	++
Not identified	523	++

Task 3.1 Exploitation of the FuturEnzyme bio-resources collections

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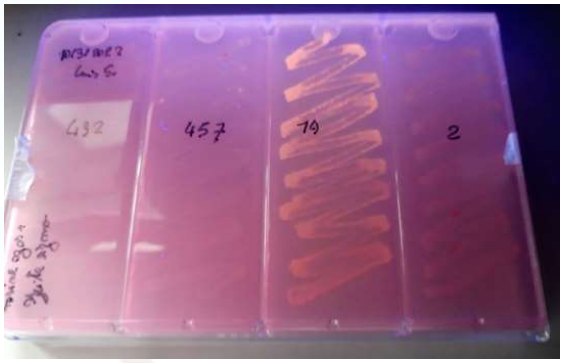
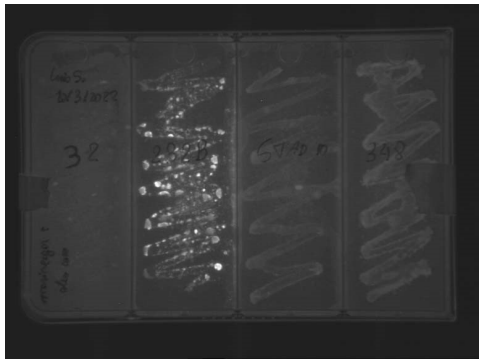
- sampling for new bio-resources; identifying novel microbes and enzyme activities; screening our microbial collection for efficient enzymes

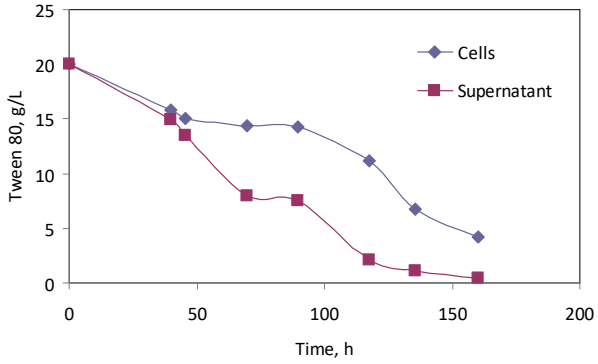
Lipase/Esterase activity

Bacterial collection	Tributyrin or Tween 80	Cononut oil	Palm oil	Olive oil
Existing	40*	5	0	6
FuturEnzyme (new)	to be det.	11	0	10

* Mostly *Bacillus* sp.; presented in MS9







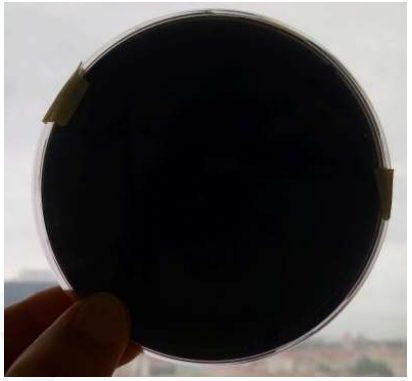
Task 3.1 Exploitation of the FuturEnzyme bio-resources collections

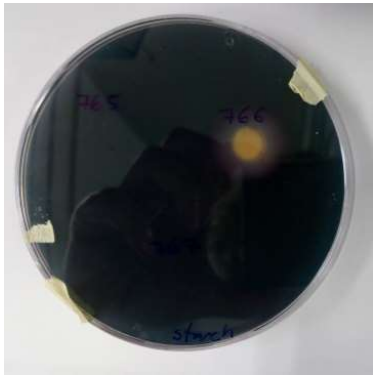
- sampling for new bio-resources; identifying novel microbes and enzyme activities; screening our microbial collection for efficient enzymes

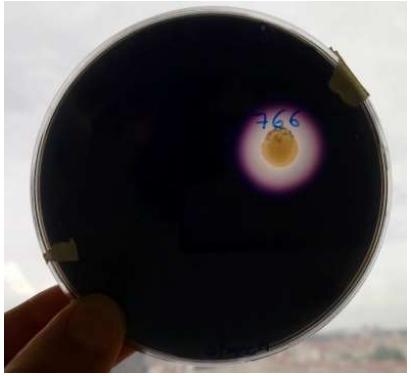
Amylase activity

Bacterial collection	Starch
Existing	48*
FuturEnzyme (new)	7

* Mostly *Bacillus* sp.; presented in MS9







Task 3.1 Exploitation of the FuturEnzyme bio-resources collections

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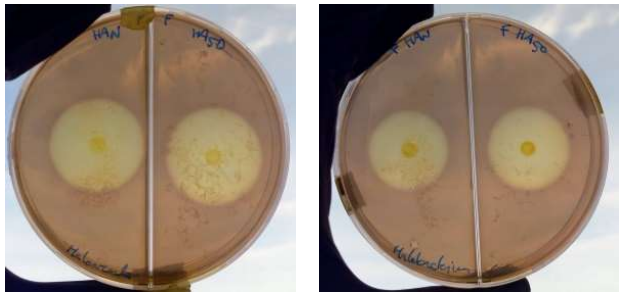
- sampling for new bio-resources; identifying novel microbes and enzyme activities; screening our microbial collection for efficient enzymes

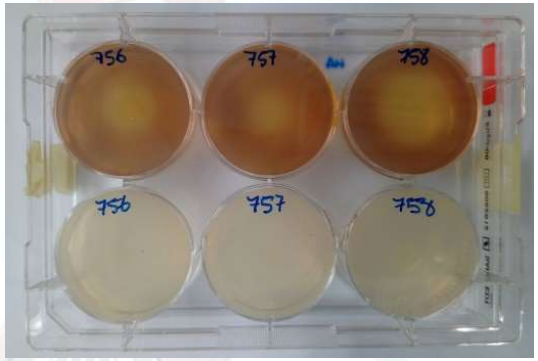
Hyaluronidase activity

Bacterial collection	HA (Hyacare)	HA5 (Hyacare)
Existing	2*	2*
FuturEnzyme (new)	14	14

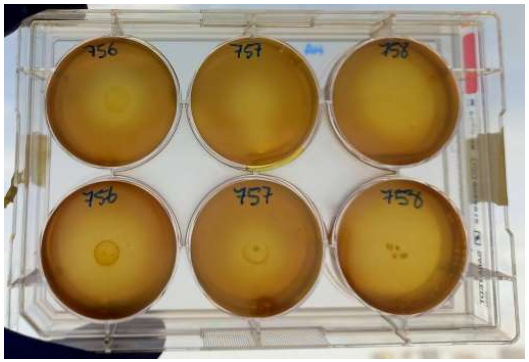


* Archaea species

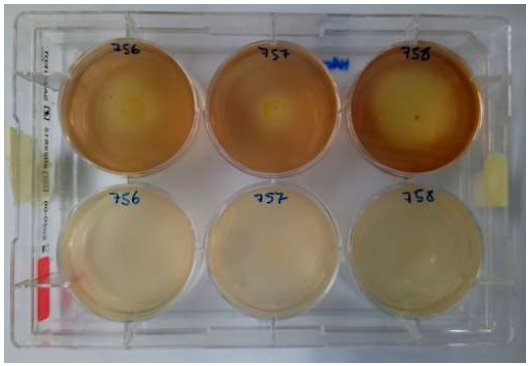
HA – 1 week



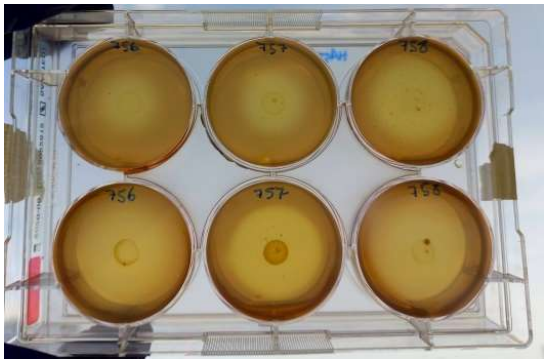
HA – 2 week



HA50 – 1 week



HA50 – 2 week



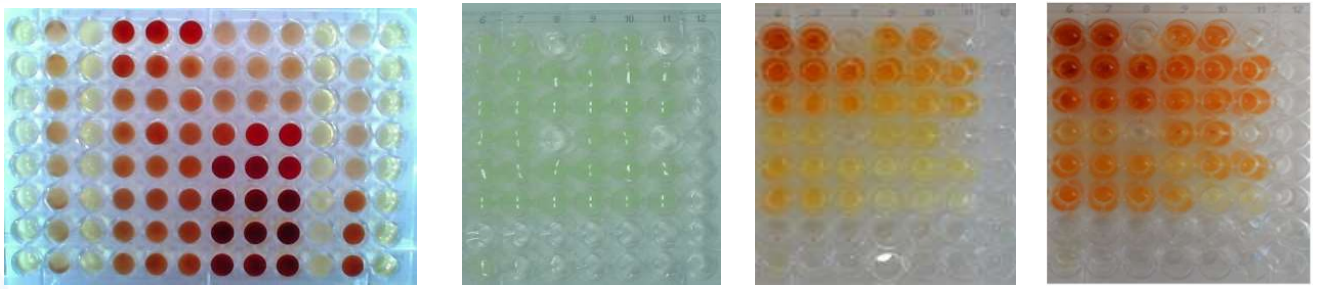
Task 3.1 Exploitation of the FuturEnzyme bio-resources collections

- sampling for new bio-resources; identifying novel microbes and enzyme activities; screening our microbial collection for efficient enzymes

Other activitites

Bacterial collection	Protease	Inulinase	Transaminase
Existing	48*	46*	7*

* Mostly *Bacillus* sp.; presented in MS9



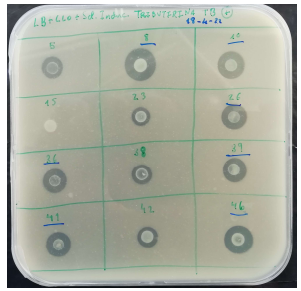
Task 3.1 Exploitation of the FuturEnzyme bio-resources collections (M1-M24)

- Three lipases have been identified by **CSIC**, when screening two fosmid libraries made at BANGOR using tributyrin, olive oil, egg yolk, cocoa and coconut
  - *2 clones from D2 library (bone [turkey femur]-degrading microbiome; 11.12.2017;* Byfjorden (60,238185N, 5,181210E) were found positive for *tributyrin, egg yolk and cocoa*
    - *D2 pCCFOS fosmid library has a titre of 9000 clones max.*
  - *1 clone from I3 library (bone [cow tibia]-degrading microbiome; 11.12.2017;* Byfjorden (60,238185N, 5,181210E) were found positive for *tributyrin, egg yolk, olive oil and cocoa*
    - *I3 pCCFOS fosmid library has a titre of 2000 clones max.*

Task 3.1 Exploitation of the FuturEnzyme bio-resources collections (M1-M24)

- 34 lipases have been identified by CSIC, when screening 5 fosmid libraries made at BANGOR using tributyrin, olive oil, egg yolk, cocoa and coconut

# Site-library	Nr. Clones	Nr of positives
D2 library (bone [turkey femur]-degrading microbiome; 11.12.2017; Byfjorden (60,238185N, 5,181210E)	9000	2
I3 library (bone [cow tibia]-degrading microbiome; 11.12.2017; Byfjorden (60,238185N, 5,181210E)	2000	1
MedSea clone library (Ancona port, Italy, 43°37′N; 13°30′15″E)	10300	7
Acid mine dranage system (Spain; 43°15′47″N, 5°46′9″W)	11600	13
TB (Thermophillic Bacteria) (mix genomes)	11800	11
TOTAL	44700	34



Task 3.1 Exploitation of the FuturEnzyme bio-resources collections (M1-M24): BU

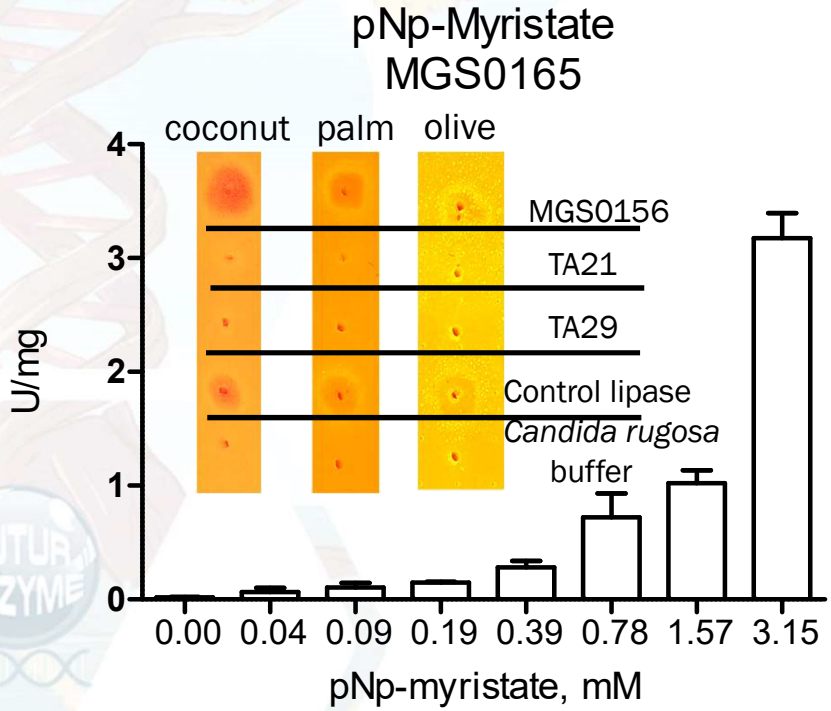
PRIFYSGOL BANGOR UNIVERSITY

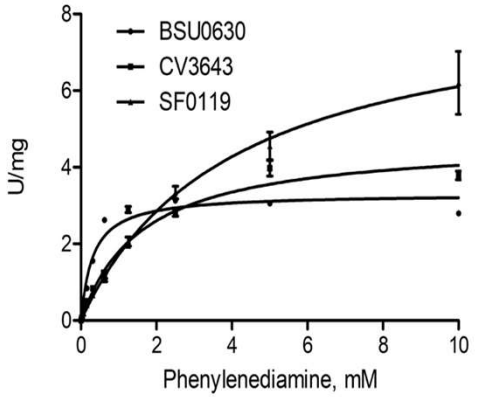
Laccases and Peroxidases-catalases (**21** soluble enzymes purified, **6** found active with ABTS/Phenylenediamine/sinapic acid

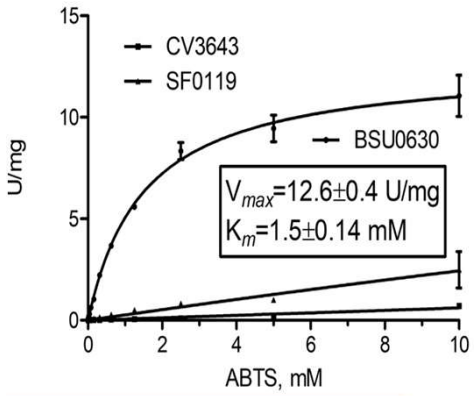
Cellulases: **3** soluble, none active with model pNp-substrates, **24** transformed, not expressed

Lipase: **1**soluble and active with coconut, olive and palm oils, pNp-models substrates C2-C18

Amylases: 3 soluble amylases, none active with model pNP substrates







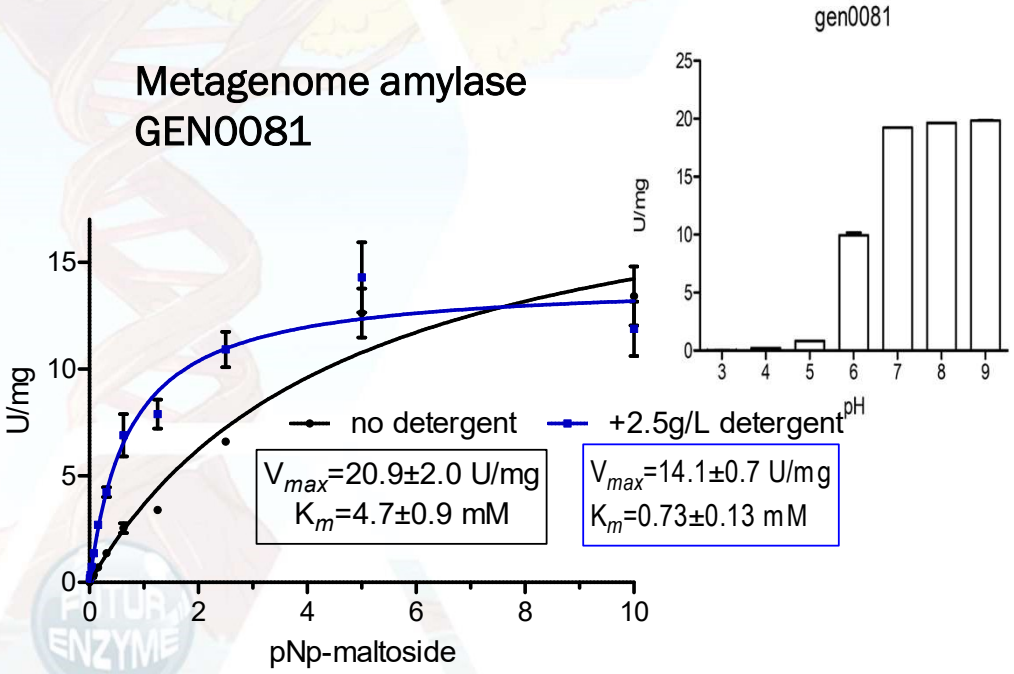
Laccase	V_{max} U/mg	K_m mM
BSU0630	3.3±0.1	0.3±0.04
CV3643	4.7±0.18	1.6±0.18
SF0119	8.5±0.55	4.0±0.58

Task 3.1 Exploitation of the FuturEnzyme bio-resources collections (M1-M24)

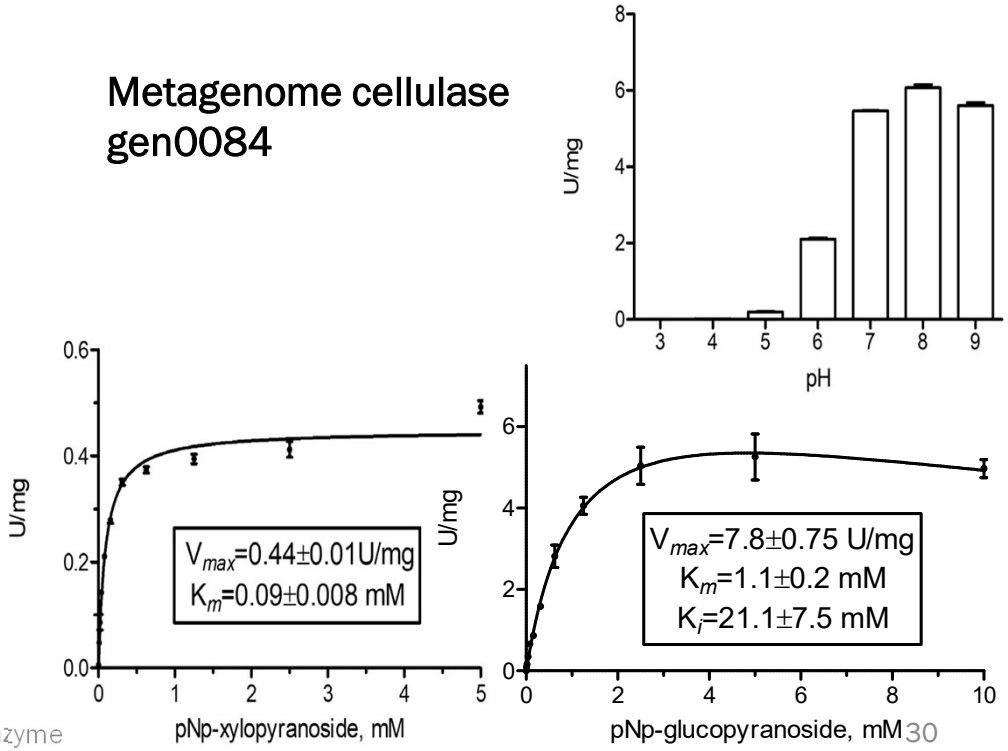
PRIFYSGOL BANGOR UNIVERSITY

BU: collection of 37 uncharacterized metagenomic glycoside hydrolases 14 purified soluble, screened for cellulase and amylase activity with 22 pNp-substrates: 1 active cellulase, 1active amylase found

Metagenome amylase GEN0081



Metagenome cellulase gen0084



Task 3.1 Exploitation of the FuturEnzyme bio-resources collections (M1-M24)

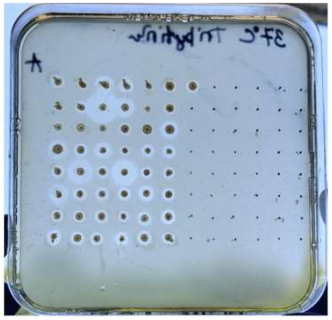
PRIFYSGOL BANGOR UNIVERSITY

Screening of Ischia hot vents BBP-enrichment fosmid library (Ischia,Italy) (10000 clones max) identified:

- 12 fosmid clones positive for tributyrin, 2 fosmids clones positive for tributyrin, coconut oil, palm oil
- 16 fosmid clones with amylase activity for starch
- 3 fosmid clones with laccase activity for syringol and 3 fosmid clones with protease activity for skim milk

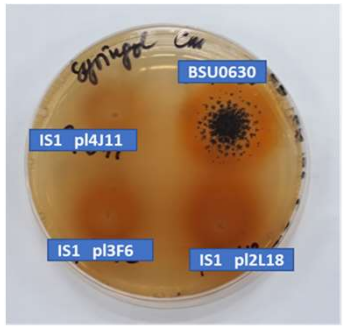
Screening of soil fosmid library (9000 clones max) identified:

- 16 fosmid clones were positive for tributyrin

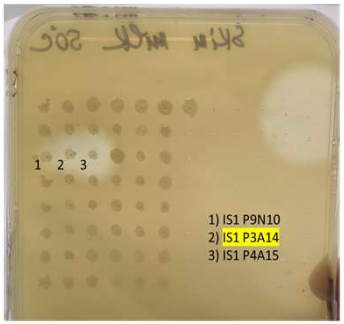


tributyrin

starch



syringol



skim milk

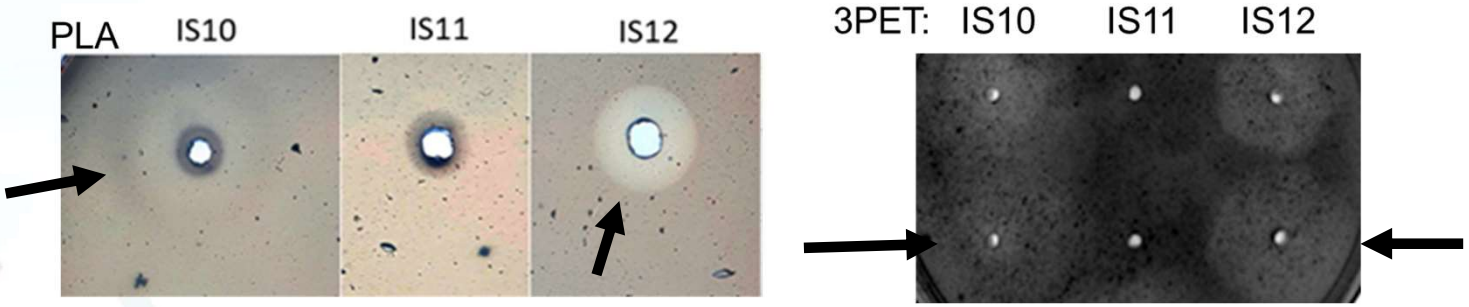
Task 3.1 Exploitation of the FuturEnzyme bio-resources collections (M1-M24): Bangor University (BU)

PRIFYSGOL BANGOR UNIVERSITY

Three thermophilic metagenomic carboxylesterases from Ischia hot vents BBP-enrichment fosmid library (Ischia,Italy) (10000 clones max) were identified:

- IS10: putative α/β hydrolase (314 aa, Chloroflexi), maximal activity at 70°C
- IS12: putative α/β hydrolase (318 aa, Chloroflexi), maximal activity at 70°C-80°C
- IS11: β-lactamase-like and lipocalin domains (455 aa, Dehalococcoidea), at 80°-90°C

Polyesterase activity: detected for IS10 and IS12



- 10 IS12 esterase homologues were cloned
- 23 IS12 esterase homologs were mined from BU resources, expressed in 1L and purified

IS11: thermophilic metagenomic carboxyl esterase with β-lactamase and lipocaline domains

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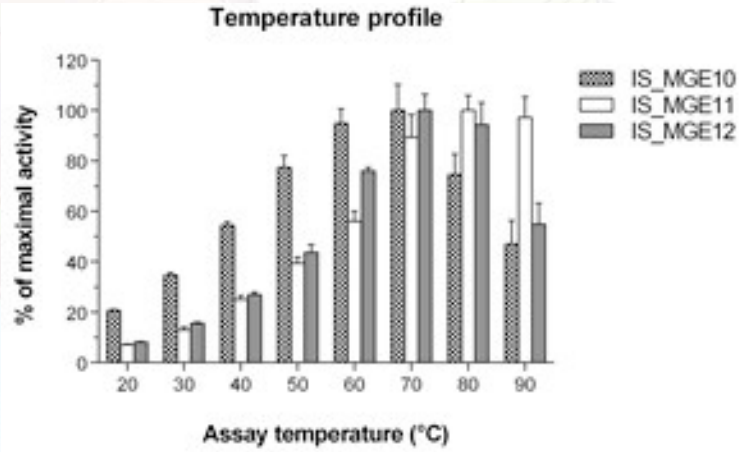
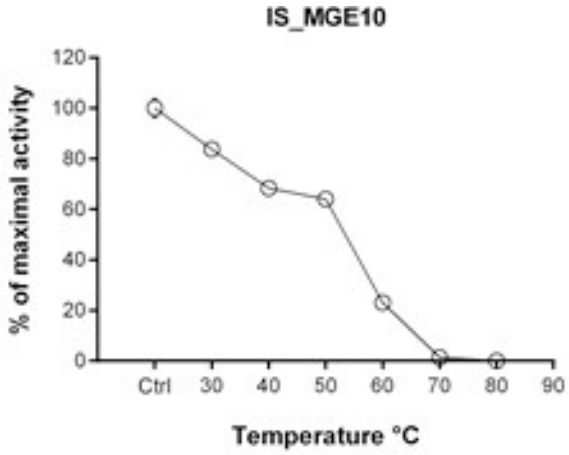
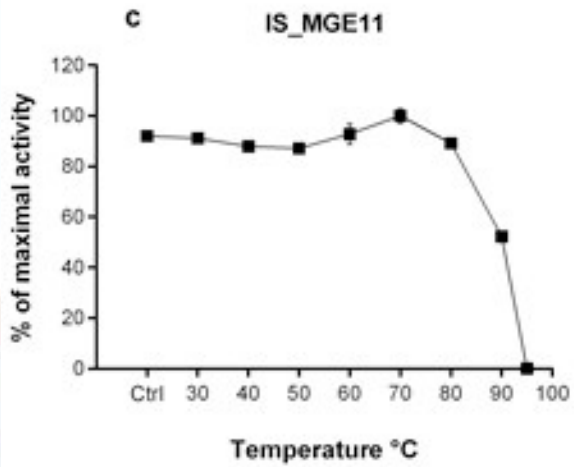
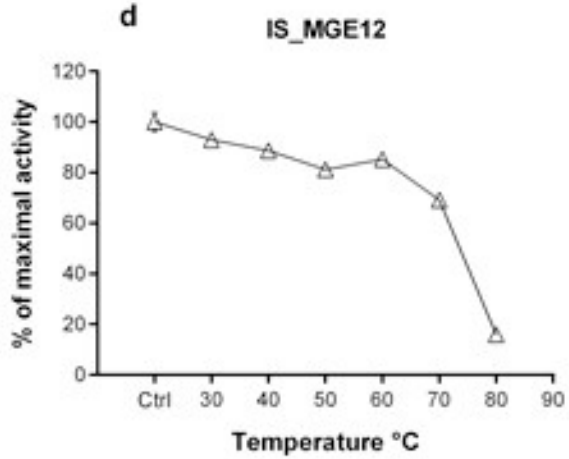
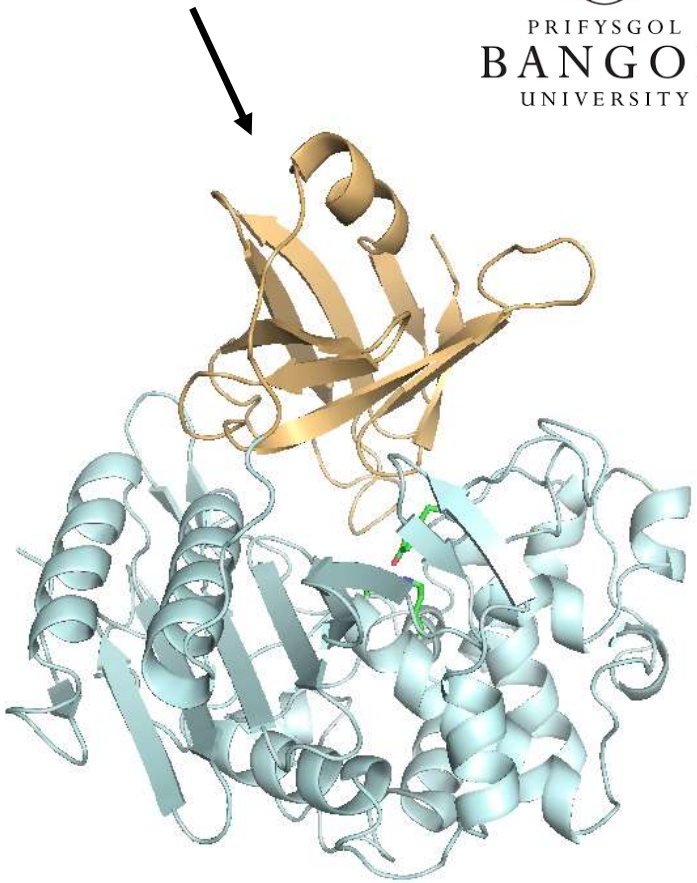










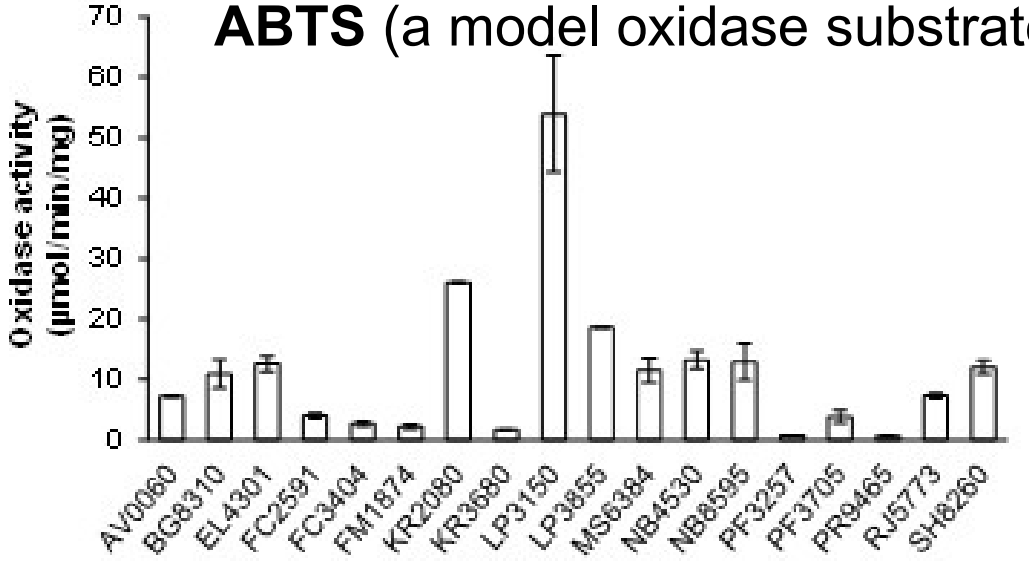
β-Lactamase domain

Bacterial peroxidases-catalases (BU)

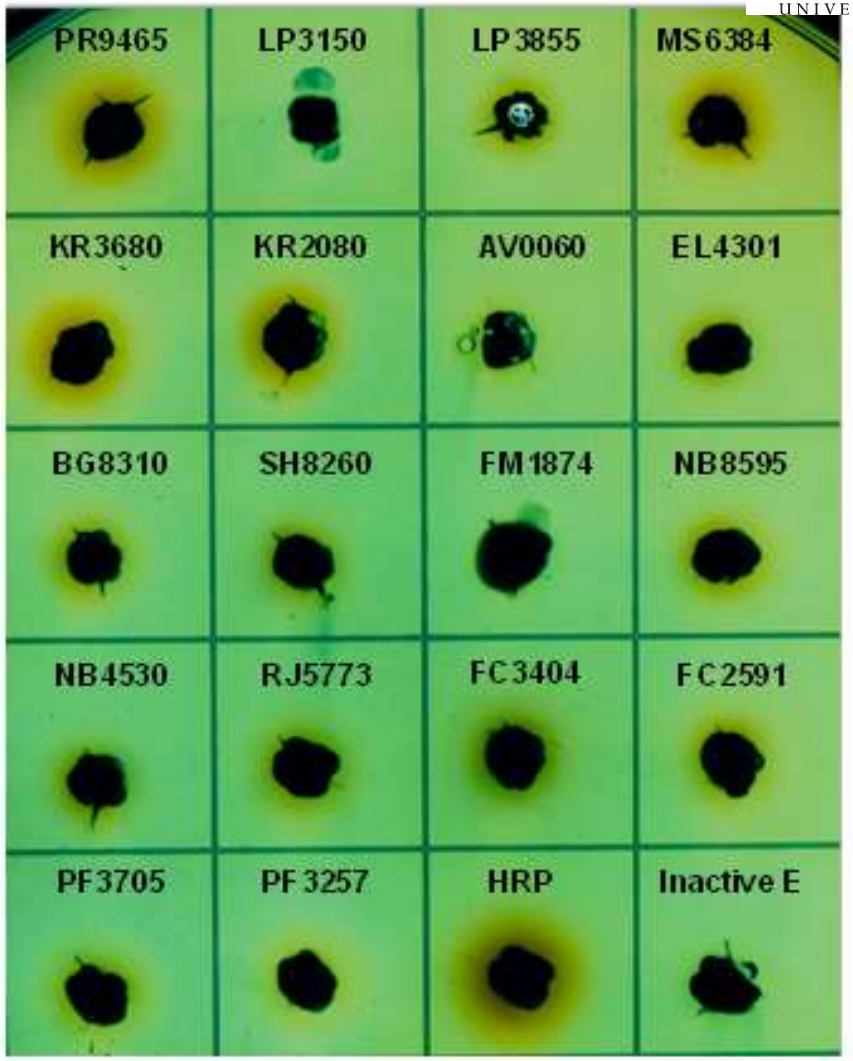
screening purified proteins against ABTS and lignin

(18 proteins)

ABTS (a model oxidase substrate)



Lignin-plate screen for oxidase activity ⟶
(yellow zone formation)



Structural characterization of bacterial heme peroxidases

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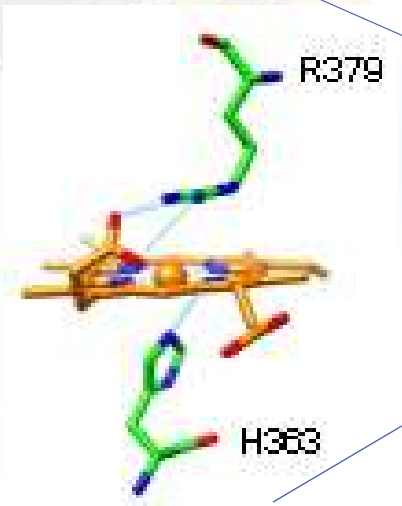
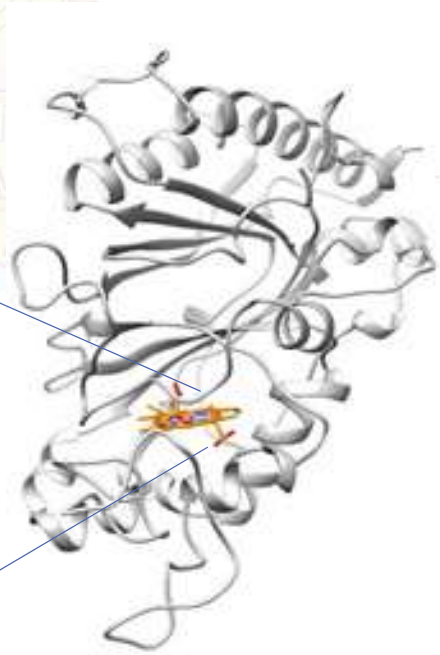


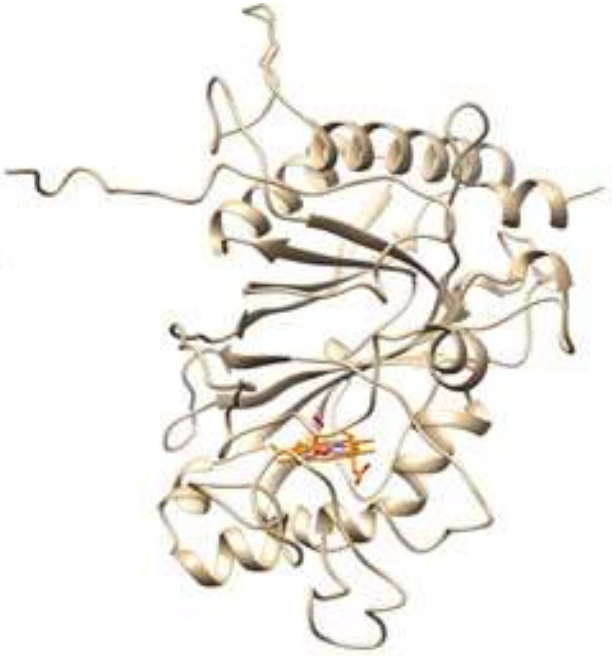
Heme coordination

FC2591



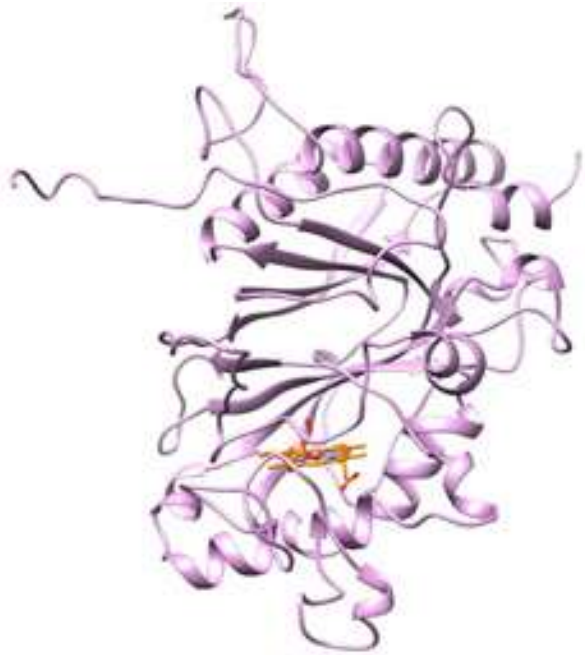
Frankia casuarinae

PF3257



Pseudomonas fluorescens

PR9465



Pseudomonas rhizosphaerae

Reaction product analysis: laccase BSU0630 and DyP heme peroxidase FC2591

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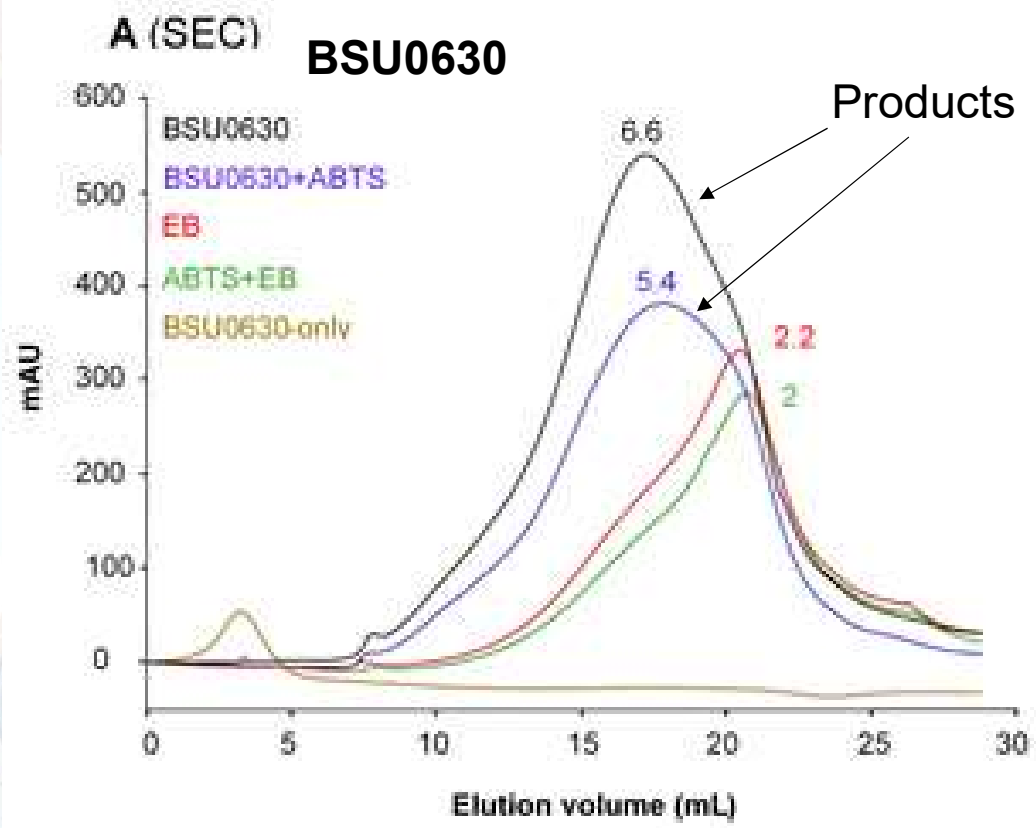
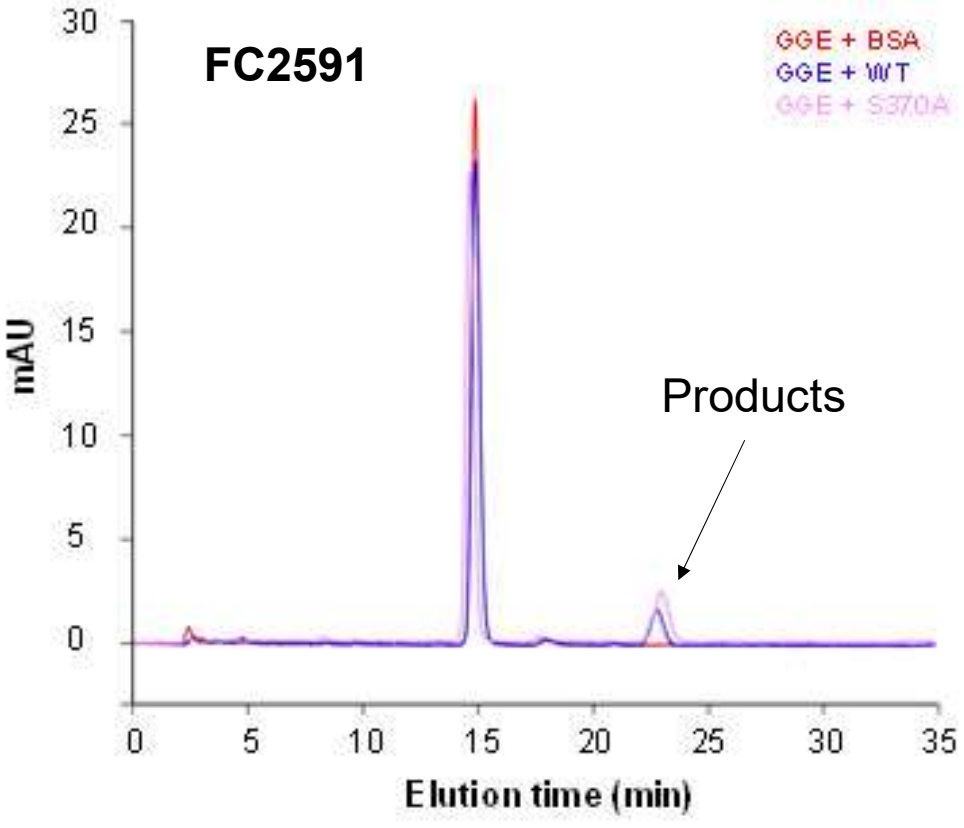
Liquid (hydrophobic) chromatography (C18 column)

Substrates: soluble (low sulfonate) kraft lignin GGE (guaiacylglycerol-β-guaiacyl ether)





Crystal structures of extremophilic glycoside hydrolases

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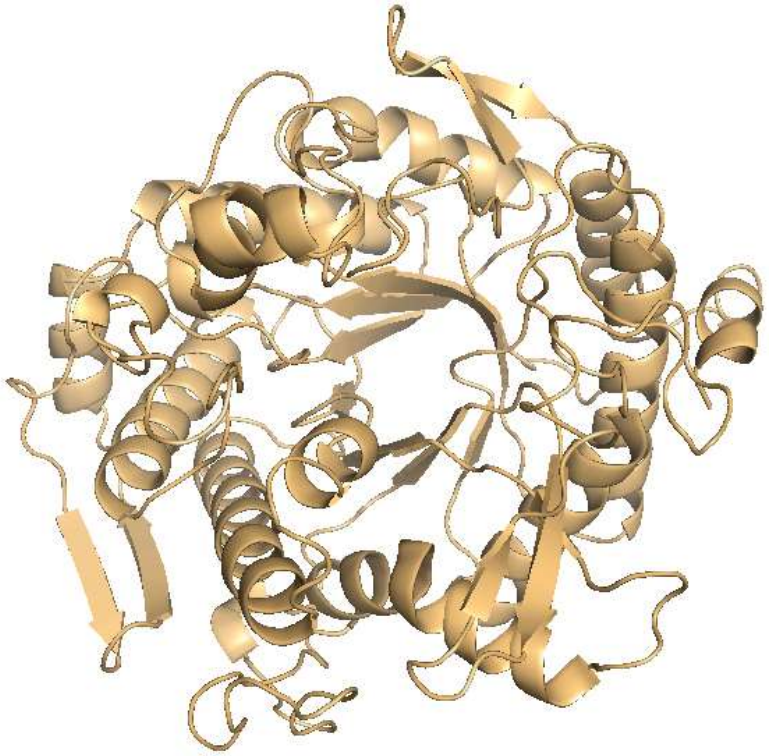
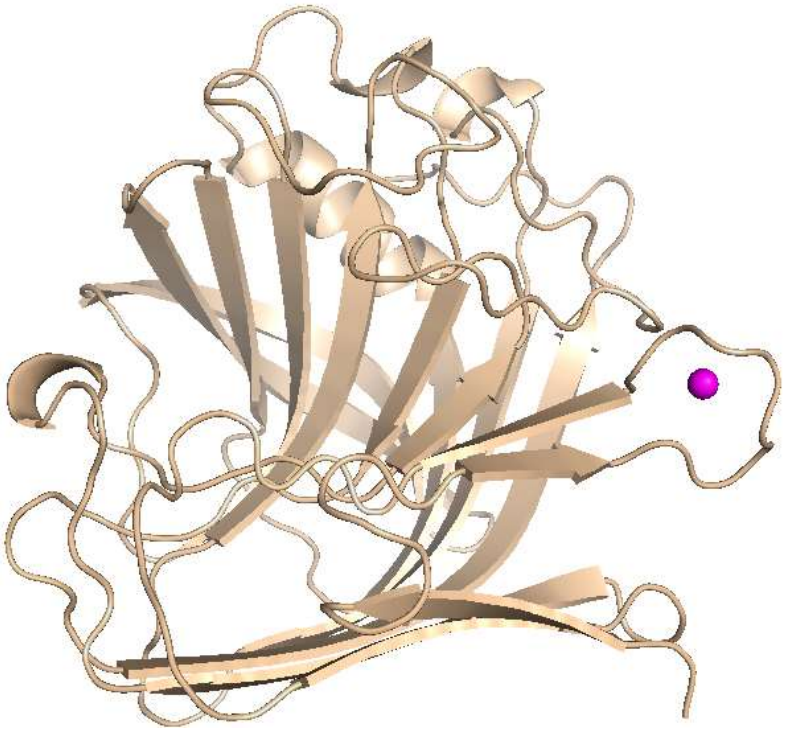
GH1
(β-glucosidase, β-galactosidase)



from *Cuniculiplasma divulgatum*

thermophilic GH12
(endo-β-1,4-glucanase)



from *Thermococcus* sp.

Task 3.2 Sampling extreme environments for generating new microbial bio-resources M6-M30

- Sampling activities are planned to generate new isolates and sequences to feed WP2/WP4 *in progress*

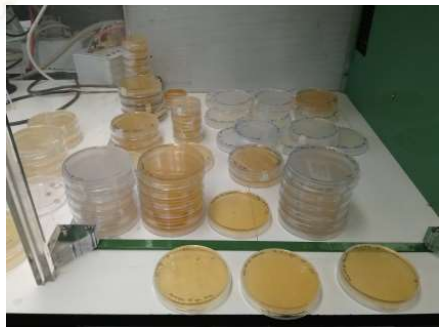
Milestone 11: *"The first sampling campaign completed"*

Task 3.2 Sampling extreme environments for generating new microbial bio-resources

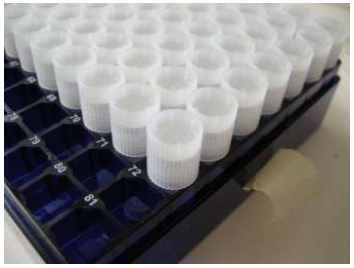
IST-ID
Associação do Instituto Superior Técnico para a Investigação e Desenvolvimento

- sampling for new bio-resources; identifying novel microbes and enzyme activities; screening our microbial collection for efficient enzymes

Rock pool at Guincho, Portugal



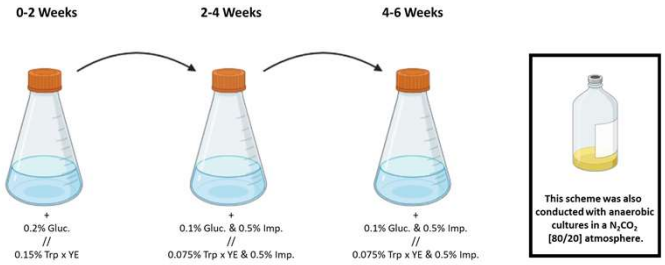
Sherlock® Microbial ID System



Screening

Enrichments for polymer-degrading microorganisms

- M9 medium / M9 + Gluc / M9 + Trp + YE
- 28 °C
- Addition of 0.5 % Impranil
- Last round without C-source, only Impranil, biotin, trace elements and casaminoacids



Enrichments for polymer-degrading microorganisms

Bacterial isolates degrading Impranil DLN so far:

Anaerobic

- *Aeromonas salmonicida*

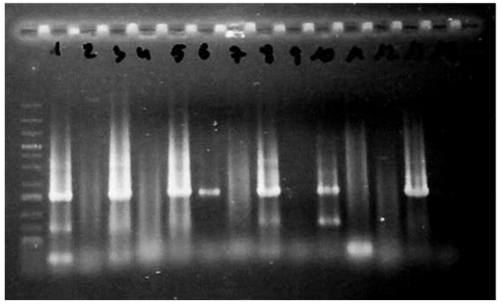
Aerobic

- *Delftia acidovorans/lacustris*
- *Sphingobacterium paramultivorum*
- *Pigmentiphaga daeguensis*
- ***Acinetobacter sp.***
- *Bacillus sp.*
- *Achromobacter spanius*
- *Rhodococcus fascians*
- *Pseudomonas putida*
- *Enterobacter sp.*
- *Pseudomonas songnenensis*
- *Brucella ciceri*
- *Pseudomonas stutzeri*

LB + DLN

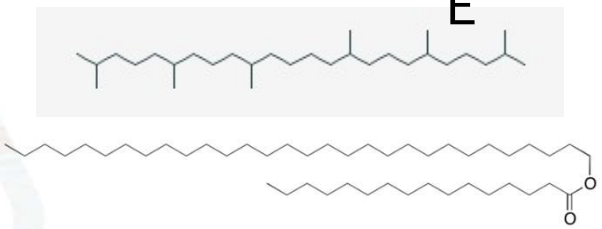
M9 + DLN

Microbial enrichments on PE and PP

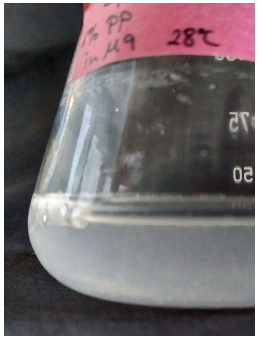


H_3C [ ] CH_3 16

$CH_3(CH_2)_{38}CH_3$ LDPE



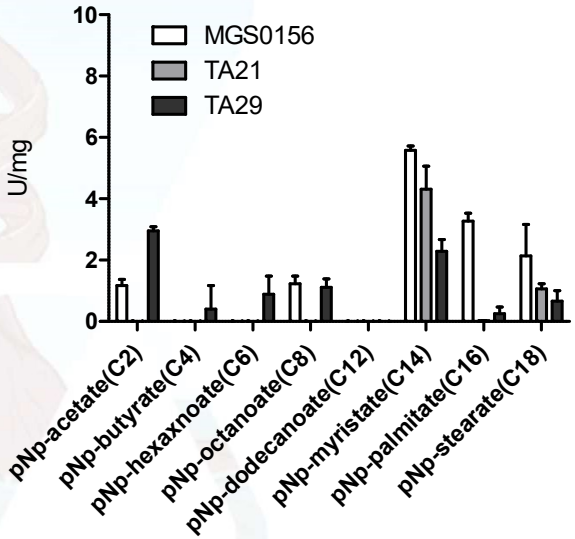
- Microscopy
- Cloning and sequencing of the 16S DNA
- Isolation of microorganisms
- Sequencing of metagenome/-transcriptome

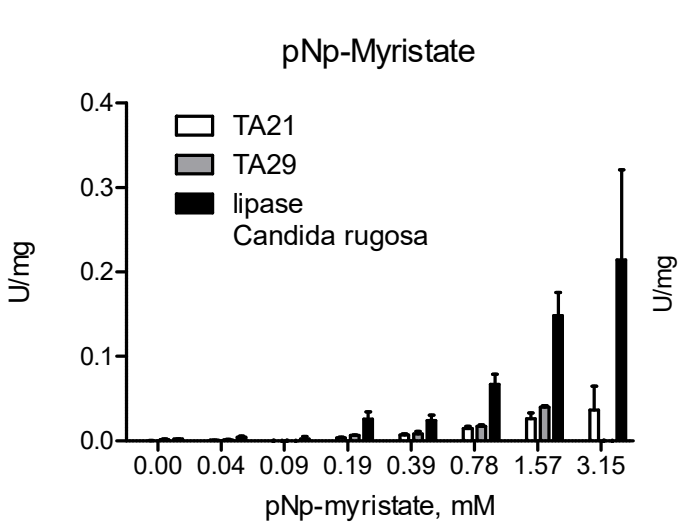


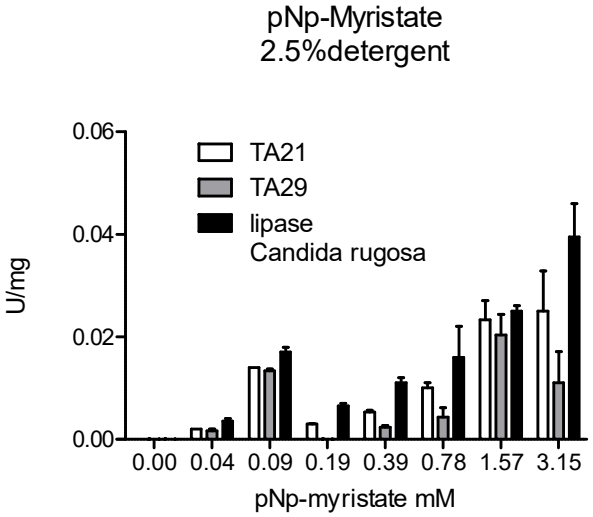
PP

Task 3.3 Next Generation Sequencing for target enzymes M1-M36

- *Thermoleophilum album* – thermophilic, obligate hydrocarbonoclastic, high-GC Gram+.
  Genome sequenced at Bangor
  26 genes were cloned, 5 soluble esterases purified, 2 lipases were found active with C14-C18 pNp-esters







Task 3.3 Next Generation Sequencing for generating sequences of target enzymes M1-M36

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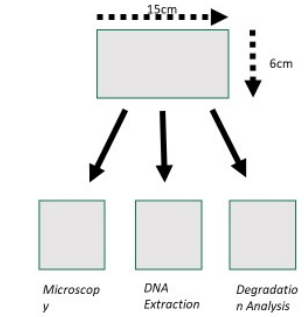
Sequences available for enzyme mining:

- 39 treatments/timepoints of microbiomes of colonisers of plastics (HDPE, LDPE, PP, PE and PET) in a transition:
  -wastewater treatment plant effluent
  -river water
  -brackish water
  -seawater (mesocosm) at the School of Ocean Sciences, Bangor University

Sequenced by Bangor (NERC 'Plastic Vector' project)
>250 Gb data available for enzyme mining **in FuturEnzyme**

Water	Time
Effluent	24 Hours
Fresh	16 Hours
Brackish	18 Hours



Task 3.3 Next Generation Sequencing for generating sequences of target enzymes M1-M36

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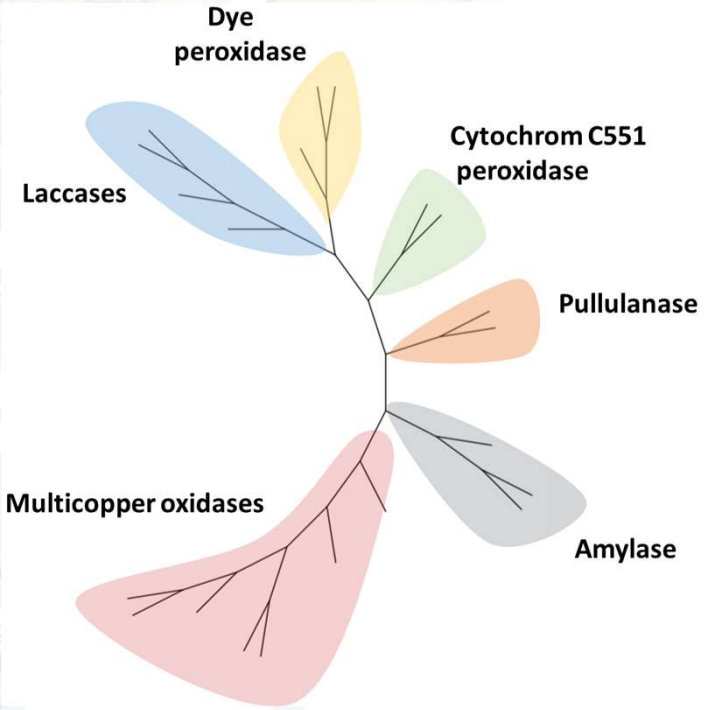
Menai Straits surface seawater an the St George Pier, School of Ocean Sciences, Bangor University
Lignin enrichment (shotgun metagenome sequenced at Bangor)
18 genes from 6 enzyme families with highest coverage were cloned



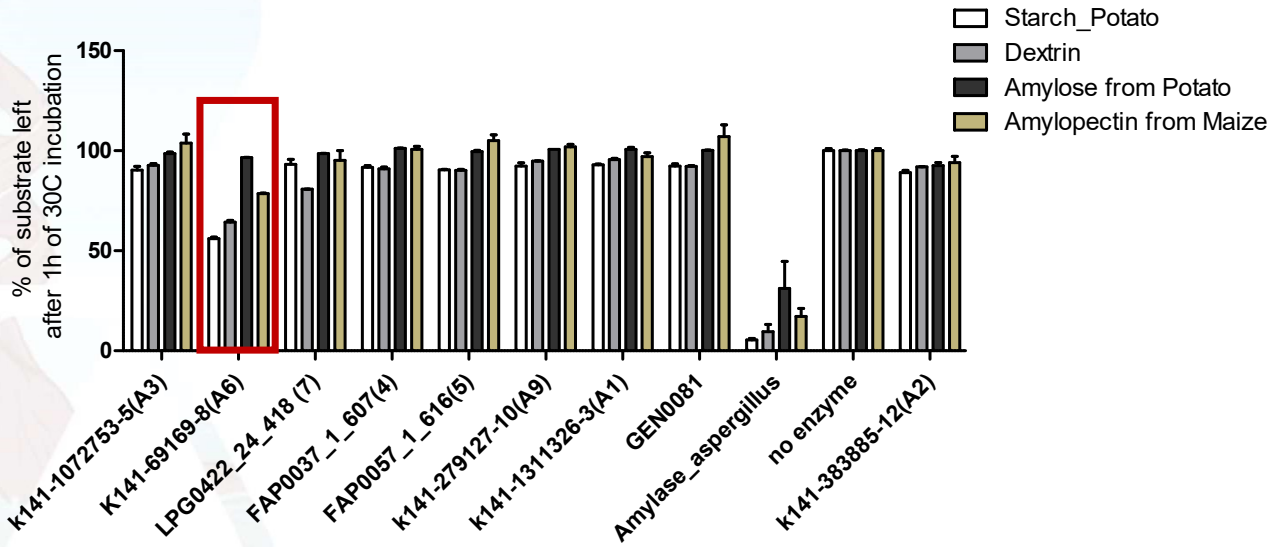
No	Protein ID	Cov-ge	Function
1	NEAIFBCB_08428	25.4	Multicopper oxidase MmcO
2	NEAIFBCB_134373	21.5	Multicopper oxidase MmcO
3	NEAIFBCB_16232	23.2	Multicopper oxidase MmcO
4	NEAIFBCB_16336	21.4	Multicopper oxidase MmcO
5	NEAIFBCB_43079	20.8	Multicopper oxidase MmcO
6	NEAIFBCB_48041	24.1	Multicopper oxidase MmcO
7	NEAIFBCB_64887	21.5	Multicopper oxidase MmcO
8	NEAIFBCB_23465	24.1	dye peroxidase DyP2
9	NABHOKAF_09411	1072.0	hypothetical protein
10	NEAIFBCB_25687	23.2	Laccase domain protein YfiH
11	NABHOKAF_13763	1029.7	Laccase domain protein YfiH
12	NEAIFBCB_104707	29.9	hypothetical protein
13	NEAIFBCB_35416	22.0	Glucoamylase
14	NABHOKAF_07137	21.2	Neopullulanase
15	NABHOKAF_09877	23.4	Pullulanase
16	NEAIFBCB_23469	22.1	hypothetical protein
17	NEAIFBCB_09304	24.7	Cytochrome c 551 peroxidase

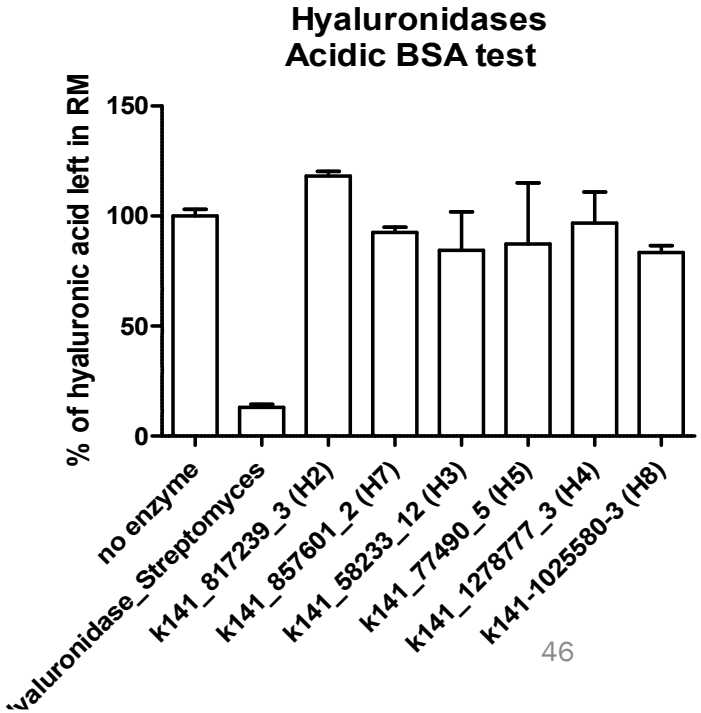
Task 3.3 Next Generation Sequencing for generating sequences of target enzymes M1-M36

- 30 treatments/timepoints of microbiomes from anaerobic bioreactors set up from Landfill in Penhesgyn Recycling Centre, Anglesey Natural Resources Wales (co-participant) 200 Gbp sequencing data

  11 amylases (most-abundant in metagenomic reads) (s. overleaf) cloned, **5** expressed soluble, **1** found active with starch substrates

  8 Hyaluronidase cloned, **6** expressed soluble.





Task 3.3 Bio-resources (Bangor) (in progress)

37 genes synthesized:

1. 20 novel glycosyl hydrolases from the **ERA Synbiogas project (**Landfill in Penhesgyn Recycling Centre, Anglesey, Natural Resources Wales) were synthesized for novel hydrolase activity screening.
2. 11 glycosyl hydrolases (amylases, from *Nanohaloarchaea* (**Partner CNR))**
3. 6 esterases from genomes of thermophilic bacteria (external partner, **INMI**)
4. 14 esterases from *Alcanivorax pacificus, A. gelatinipgagous* and *A*. sp. 24.

Deliverables

List of deliverables

Deliverable Number[14]	Deliverable Title	Lead beneficiary	Type[15]	Dissemination level[16]	Due Date (in months)[17]
D3.1	Bio-resources prepared and exchanged	3 - BANGOR	Other	Confidential, only for members of the consortium (including the Commission Services)	2
D3.2	Standard assays, analytics and calculations for monitoring enzymatic performance	4 - UHAM	Report	Confidential, only for members of the consortium (including the Commission Services)	2
D3.3	Set of 100 best clones, 10 isolates, and 10 enzymes shortlisted for sequencing or transfer to WP2	6 - IST ID	Other	Confidential, only for members of the consortium (including the Commission Services)	10
D3.4	Sequence, activity, and stability datasets from best positive bio-resources	3 - BANGOR	data sets, microdata, etc	Confidential, only for members of the consortium (including the Commission Services)	18
D3.5	Set of new bio-resources to screen or sequence	7 - CNR	Other	Confidential, only for members of the consortium (including the Commission Services)	24
D3.6	Complete set of positive naïve screened enzymes and sequences and their datasets	3 - BANGOR	Report	Confidential, only for members of the consortium (including the Commission Services)	32






Milestones

Milestone number[18]	Milestone title	Lead beneficiary	Due Date (in months)	Means of verification
MS9	First round of functional screens completed	6 - IST ID	6	Materials available – this milestone will attest the realisation of the first screens of available bio-resources.
MS10	First round of sequencing completed	3 - BANGOR	6	Data available – this milestone will attest to the realisation of the sequencing of the first selected bio-resources found to be positive in the screen tests.
MS11	The first sampling campaign completed	7 - CNR	12	Sites data, samples available - this milestone will attest completion of campaigns for sampling new bio-resources with information about sample sites available.