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Deliverable 7.5

Novel or improved expression and screening systems (both vectors and hosts)

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Deliverable Report 7.5

Summary

The Micro B3 deliverable D7.5 'Novel or improved expression and screening systems (both vectors and hosts), for high-throughput discovery of new bioactive compounds from marine metagenomic libraries' is linked to the tasks 7.4 and 7.5 and aims at developing novel or improved tools to enable more effective screening of marine metagenomic libraries for more diverse and novel bioactive compounds. The current delivery report has contributions from partners BIOMERIT, Bio-Iliberis and Univ. Bangor.
In order to facilitate metagenomic library construction and screening in a broader range of hosts a novel broad-host range shuttle fosmid, pCCERI, has been developed in BIOMERIT and is available to all partners. A compatible helper plasmid has also been constructed.
At Bangor a functional screening system has been developed to detect 4´-phosphopantetheinyl transferase (PTTase) activity associated with non-ribosomal peptide synthetase (NRPS) and/or type I polyketide synthase (PKS) in fosmid-based metagenomic libraries. At Bio-Iliberis R&D metagenomic libraries from extreme marine sites have been constructed in the fosmid vector pCC1FOS and screened for biotransformation activities, while work is ongoing to develop improved expression vectors and hosts to allow the expression of otherwise silent genes in metagenomic libraries from extreme conditions.

Detailed Description

7.5 A. Novel or improved expression and screening systems for high-throughput discovery of new bioactive compounds from marine metagenomic libraries

A primary objective of work package 7 (WP7) is to develop, validate, and apply laboratory-screening protocols for the cultivation-independent discovery, description, and exploitation of new gene functions, enzyme reactions, biosynthetic pathways, and bioactive compounds with practical applications. To achieve this, novel / improved expression and screening systems have been developed for mining metagenomic libraries. Metagenomic expression systems aimed at larger inserts and at a broader host range than *E. coli*, only, have been developed at BIOMERIT, Bio-Iliberis and Bangor.

***Development of a novel broad-host range fosmid (BIOMERIT)*:** A novel broad-host range shuttle fosmid, pCCERI, has been developed in BIOMERIT for metagenomic library construction (Fig 1). pCCERI facilitates large DNA inserts (~40kb) and can be efficiently conjugated to *Streptomyces* sp. and *Pseudomonas putida* strains where it integrates onto the chromosome via site-specific recombination. A compatible helper plasmid has also been constructed.

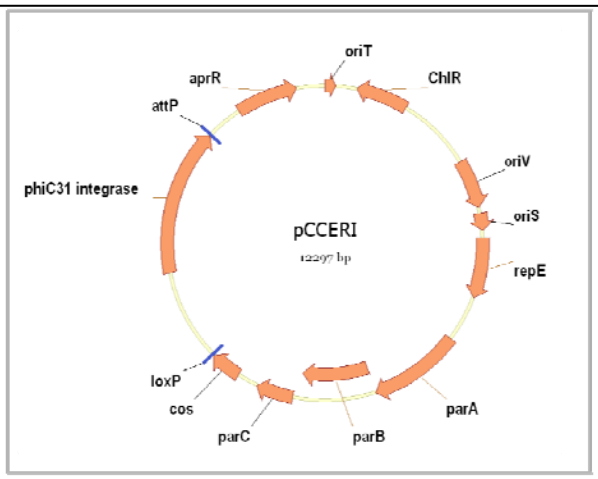


Fig. 1: Broad host range shuttle fosmid pCCERI

Features of pCCERI BAC/fosmid shuttle expression system:

- oriT allows interspecies conjugation
- ϕC31 integrase and attP site allow for integration onto host chromosome
- Apramycin and chloramphenicol antibiotic markers
- Amplifiable from 1 to ~50 copies
- Insert size: Fosmid ~40 kb insert / BAC - 200 kb+ insert
- Host Strains: *E. coli*, *S. lividans*, *P. putida*

Originally metagenomic libraries from deep sea and shallow water sponges were generated in the fosmid vector pCC1fos [1,2,3,4]. More recently metagenomic libraries have been generated from high molecular weight DNA isolated from the three marine sponges *Haliclona simulans* (circa 40,000 clones), *Stelleta normani* (circa 11,500 clones), and *Axinella dissimilis* (circa 19,000 clones), using the broad host range shuttle-fosmid pCCERI (developed at UCC). The libraries have been cloned in a range of hosts including *E. coli*, *Streptomyces* and *P. putida* strains.

Functional screening of metagenomic libraries targeting enzymatic activities (BIOMERIT):

Lipase activity: Functional analysis of the *Stelleta normani* library through laccase and lipase screen gave 10 clear lipase positive clones in *E. coli* (Figure 2). Initial end-sequencing of the 10 lipase-positive clones indicated that likely origins of clones were diverse; *Chloroflexi*, alpha-, beta-, and delta-*Proteobacteria* and *Firmicutes*. One particular hyper lipase-producing clone was identified. All clones are currently being sequenced and further characterised.

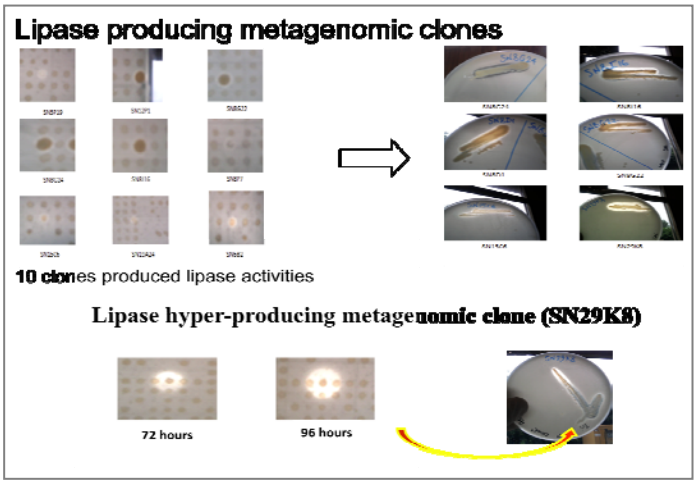


Figure 2: Ten clones from the *Stelleta normani* metagenomic library showed lipase activity

Protease / Transaminase activity: The metagenomic library from *Axinella dissimilis* was screened for protease and transaminase activities (Figure 3). Out of 9600 clones screened one candidate protease clone was identified. The activity was confirmed using plate and colorimetric assays and the metagenomic clone is currently being sequenced and further characterized. 8640 clones were screened for transaminase activity and 6 positive clones were identified. Using a substrate specific for ω-transaminase activity (α-methyl–benzyl-amine) one of the 6 clones was positive. It is currently being sequenced and will undergo further characterisation.

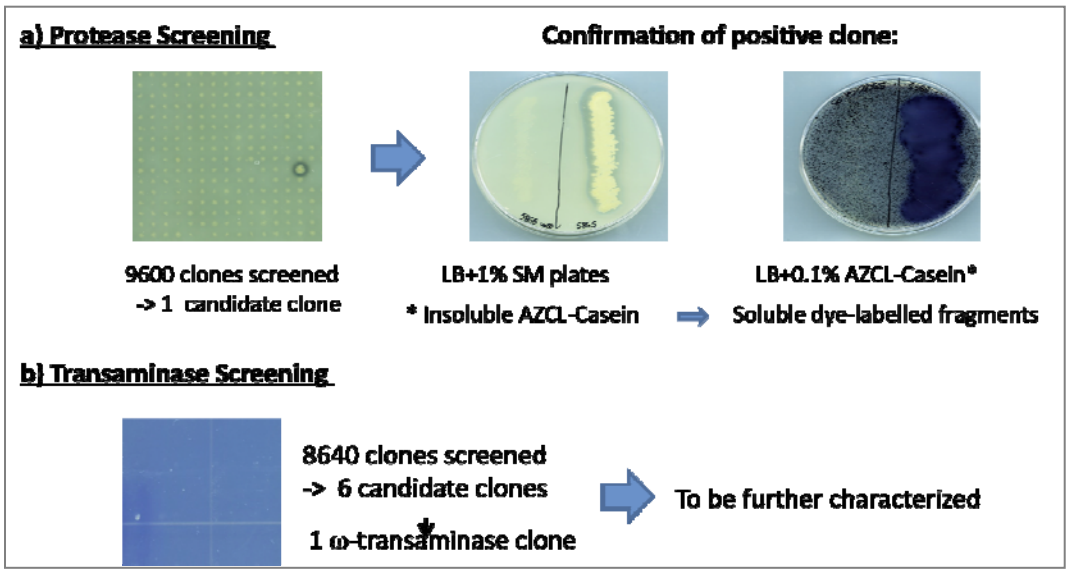


Figure 3: Protease and transaminase activities identified in the metagenomic library from *Axinella dissimilis*

Functional screen targeting PPTase activity (Bangor): The development of a functional screening system to target PPTase genes in fosmid-based metagenomic libraries carried out at Bangor included several steps and involved the generation of a variety of constructs.
Initially, we constructed the strain *E. coli* EPI300-T1^R lacking an endogenous *entD* gene. The *entD* gene was deleted from the chromosome of *E. coli* EPI300-T1^R by using the one-step chromosomal gene inactivation technique[5]. For this purpose, plasmid pKD4 was used as template to amplify the kanamycin resistance marker (*kan*^{R}) flanked by FLP-recombinase recognition sites. The sequences at the 5´end of each primer were homologous to genomic regions targeted for deletion. The amplified PCR product was purified and electroporated into *E. coli* EPI300-T1^R strain in which the λ-Red recombinase expression plasmid pKD46 was introduced. Homologous recombination and integration of the PCR product into the genomic DNA resulted in deletion of the gene of interest and replacement by the Km^R cassette. After electroporation, 1 mL SOC + 10 mM arabinose (to induce recombinase expression) was added and electroporated cells were incubated for 1h at 37 ºC before spreading onto agar plate to select Km^R transformants by incubation at 37 ºC overnight.
The obtained colonies were PCR screened to verify recombination of the *kan*^{R} gene into the *entD* locus. The diagnostic primers used for PCR verification were locus-specific and primed from regions upstream of the PCR integration sites. Positive colonies were then cured of pKD46 by incubation at 43ºC. In the second step, the constructed *E.coli* EPI300-T1^R with *entD* deletion strain was transformed with the plasmid expressing wild-type *bpsA*. Positive clones, which were selected by appropriate antibiotic, were chosen for detection of PPTase genes in metagenomic libraries.

The final step was to verify the utility of the constructed strain. We selected one of the fosmid-based metagenomic libraries available in our laboratory. Using standard methods, the purified tested fosmid-based metagenomic library was transformed into prepared electrocompetent *E. coli* EPI300-T1^{R} cells harbouring *entD* deletion and the pCR2.1TopoTA plasmid harbouring *bpsA* from *Streptomyces lavendulae*. After electroporation, cells were incubated for 1h at 37 °C with shaking. After primary determination of the optimal plating for screening, the volume of cells for plating was adjusted to obtain between 5000 and 10,000 clones per plate. Cells were plated on auto-induction agar containing L-glutamine for observation of indigoidine synthesis. Incubation of plates was first done at 37 °C for 12 h and then continued at room temperature until a blue colour developed. Colonies that appeared blue were taken for further analysis (Fig.4).

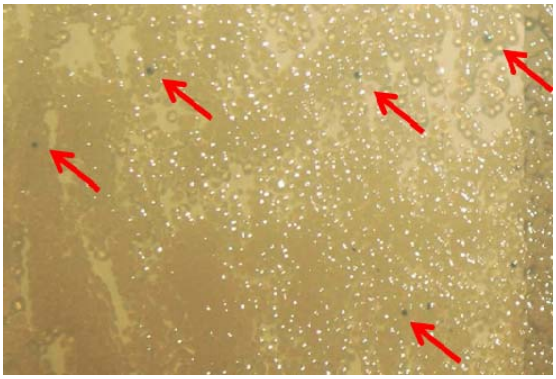


Figure 4: Indigoidine-positive clones from a fosmid library emerged after 7 days of incubation on the test agar. Positive clones are indicated by arrows

Selected clones (12) were subjected to fosmid isolation and sequencing of inserts. The sequencing revealed that the origins of genomic fragments were from *Vibrio* spp. and *Pseudomonas* spp., with the closest relatives being the *Vibrio* sp. Ex25 from deep-sea hydrothermal vent and well-known *Pseudomonas putida* KT2440 respectively. All twelve clones were affiliated to above organisms in a ratio 4/8, respectively. Fragments have been subjected to the annotation using the online server at antiSMASH.secondarymetabolites.org .

Results showed no putative PKS-related gene clusters, but some small-metabolites-related gene clusters in *Vibrio* sp. genomic fragments (Fig.5).

Figure 5: antiSMASH report on prediction of gene clusters in the "positive" fosmid clone (indicated as query sequence).

The results of sequencing the twelve fosmid inserts from the blue colonies however did not show any hit (identity) with PPTase. The reason for the occurrence of false positives can be related to the occurrence of small chain dehydrogenase/reductase on both pseudomonad- and vibrio-derived fragments, which could lead to the formation of blue product (due to the conversion of e.g. tryptophane in the medium). The occurrence of false positives has also been reported earlier [6]. Therefore, this assay seems non-specific under tested conditions and requires further adjustment.

7.5 B. Development of improved expression vectors and hosts for metagenomic libraries exploring extremophilic and rare biotransformation activities

Application of enhanced host-vector systems for screening of metagenomic libraries from extreme environments

Bio-Iliberis has performed the sampling, DNA extraction and preparation of 3 metagenomic libraries. The samples were taken from: **Tinto River estuarine (Huelva)**, **salty underground waters polluted by hydrocarbons (Murcia)** and a **water sample from Motril harbour (Granada)**. Following the extraction of high molecular weight DNA, DNA was cloned into the vector pCC1FOS and transferred through EPI300™ cells. After checking the library efficiency, clones were stored with 33% glycerol for later use in functional screening (Figure 6).

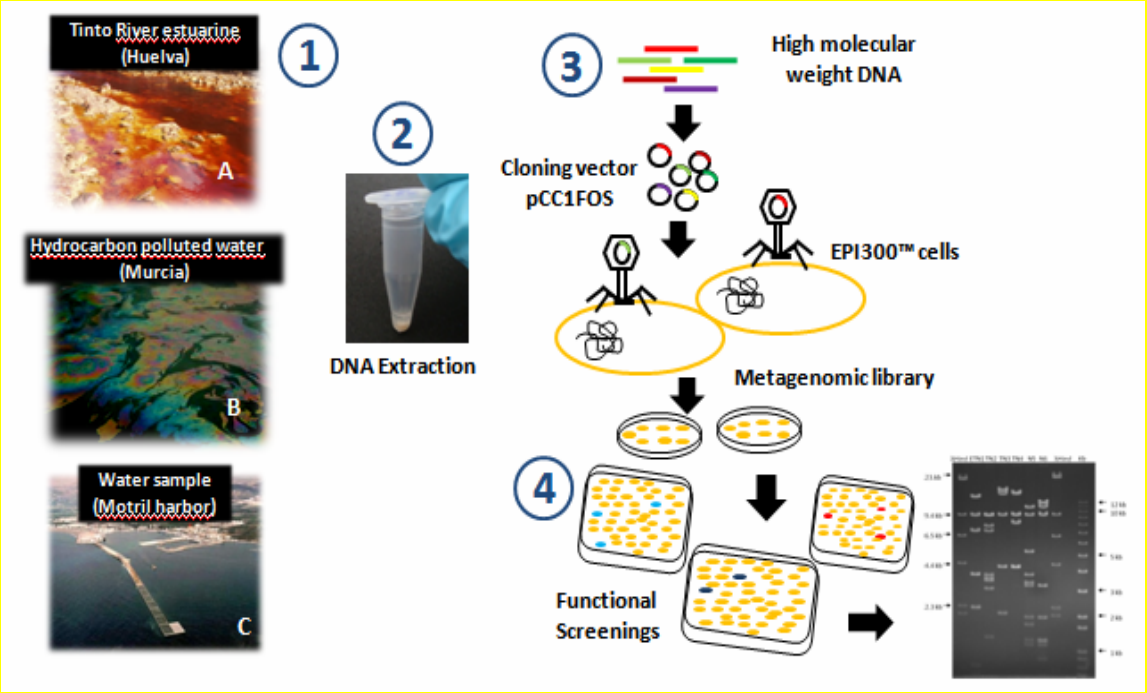


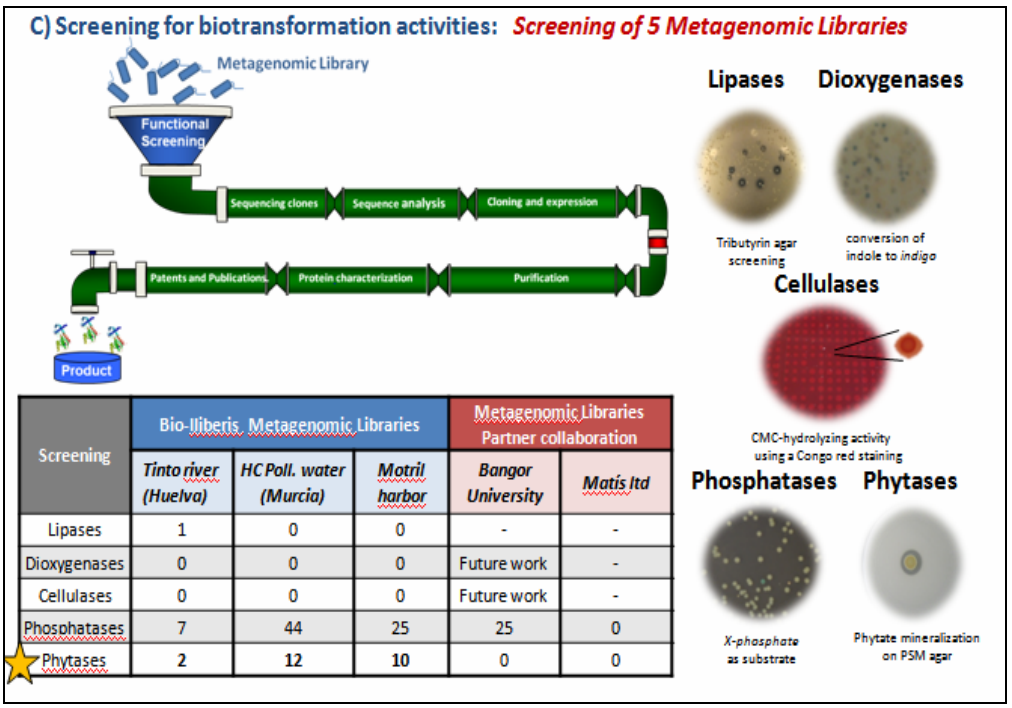
Figure 6: Application of enhanced host-vector systems for screening of metagenomic libraries from extreme environments

Screening metagenomic libraries for biotransformation activities

Bio-Iliberis has performed the functional screenings of 5 metagenomic libraries including the 3 libraries constructed by the company from extreme environment samples and 2 metagenomic libraries constructed by the partners Bangor University and Matís Ltd. (Figure

7). The search has focused on dioxygenase and oxygenase, lipase, cellulase and phytase activities based on the following tests:

Oxygenases and Dioxygenases: screening using LB agar with Indol in the reaction as substrate. To find oxygenases the protocol performed is based on the conversion of colorless indole into blue indigo, in the dioxygenases screening the reaction is based on catechol conversion into yellow semialdehyde.
Lipases: Tributyrin agar screening and olive oil and Rhodamine B screening.
Cellulases screening: Carboxyl Methyl Cellulose agar with Congo red staining.
Phosphatases: *X-phosphate* as substrate in LB-agar medium.
Phytases: Phytate mineralization on Sodium Phytate medium agar.



Screening	Bio-Iliberis Metagenomic Libraries			Metagenomic Libraries Partner collaboration	
	Tinto river (Huelva)	*HC Poll. water (Murcia)*	*Motril harbor*	*Bangor University*	*Matís ltd*
Lipases	1	0	0	-	-
Dioxygenases	0	0	0	Future work	-
Cellulases	0	0	0	Future work	-
Phosphatases	7	44	25	25	0
Phytases	2	12	10	0	0

Figure 7: Five metagenomic libraries were screened for biotransformation activities including lipase, dioxygenase, cellulase, phosphatase and phytase activities**.**

Results of the screenings:

Metagenomic library 1: Rio Tinto Estuarine

- **7** clones exhibited phosphatase activity,
- **1** clone with lipase activity and **4** with esterase activity.
- **2** out of 7 clones phosphatase-positive exhibited phytase activity.

Metagenomic library 2: Salty underground waters polluted with hydrocarbons

- **44** clones exhibited phosphatase activity.
- **12** out of 44 clones phosphatase-positive, exhibited phytase activity.

Metagenomic library 3: Water sample from Motril harbour (Granada):

- **25** clones exhibited phosphatase activity,
- **10** out of 25 clones phosphatase-positive, exhibited phytase activity.

Encoded new proteins will be characterized with regard to their biochemical and physicochemical parameters.

Among all metagenomic libraries, Bio-Iliberis identified 76 phosphatases and 24 phytases. Phytases are more innovative enzymes and exhibit many interesting industrial applications, for this reason the company has focused on the characterization of clones with phytase activity.

A phytase (myo-inositol hexakisphosphate phosphohydrolase) is any type of phosphatase enzyme that catalyzes the hydrolysis of phytic acid (myo-inositol hexakisphosphate), an indigestible organic form of phosphorus that is found in grains and oil seeds; and releases a usable form of inorganic phosphorus. The table below shows the main application and desired properties for the industrial phytases.

Table 1: Properties of industrial phytases

APLICATION	MAIN EFFECT	DESIRED PROPERTIES
Phytase as feed additives	**Increased P utilisation and metal bioavailability; decreased P concentration in excrements.**	**Resistance to low pH and high temperatures.**
Inoculation of inorganic residues with phytase producing Bacteria for bioremediation.	Increased P availability decreased P pollution in water bodies.	Resistance to physiochemical changes during the remediation process capacity to utilize recalcitrant P forms.
Inoculation of roots with phytase producing Bacteria.	Increased uptake of P in organic forms by plants.	P liberation should be greater than the P requirement.
Improvement of mineral nutrition in Humans.	Increased uptake of mineral ions especially iron zinc and calcium.	Resistance to low pH peptidases and high temperatures.

To identify the variants with the highest catalytic activity, the most robust and temperature-resistant ones, the following determinations were made:

- Thermostability: The thermostability of the enzymes was determined by incubating the protein at various temperatures (10, 30, 50 and 70 °C) for 10 minutes, and then measuring the residual activity at 37 °C for 30 minutes.
- Optimum temperature: The effect of temperature on enzyme activity was measured at 10, 30, 50 y 70 °C at pH 5.5 for 30 minutes.
- Optimum pH: The pH optimum for the phytases activity was evaluated at 37ºC in Britton-Robinson buffer at pH values in the range from 2.0 to 8.0.

Results of enzymes characterization:

We have characterized **24 phytases** from the libraries constructed by the company. The characterization has been based on the parameters thermostability, optimum temperature and optimum pH. **4 clones** were selected to show a good enzymatic activity at high temperatures over a wide pH range.

Metagenomic library 1: Rio Tinto Estuarine

Two phytases clones were purified and sequenced and found to be the same protein (Figure 8). Bio-Iliberis determined of the thermostability and optimum temperature of 2 clones. None of the clones showed activity at 70°C. Currently we are studying the structure of the enzyme.

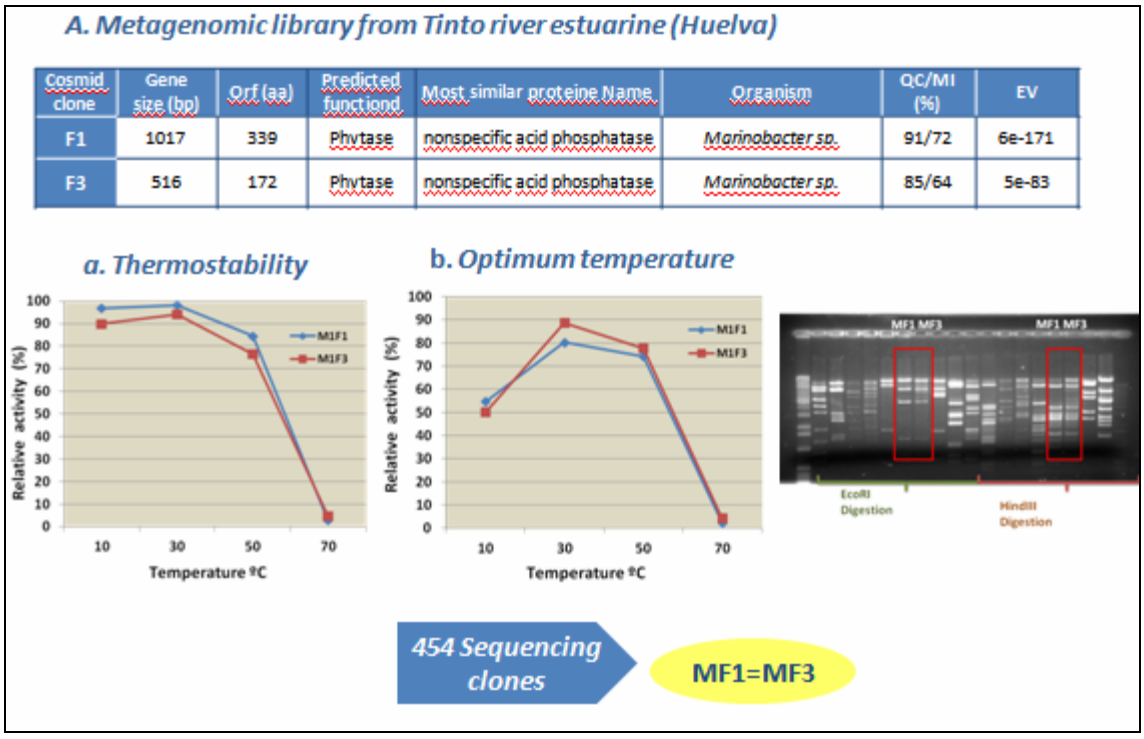


Figure 8: Two phytase clones identified from the Rio Tinto estuarine metagenomic library were found to encode the same protein

Metagenomic library 2: Salty underground waters polluted with hydrocarbons (Murcia)

Bio-Iliberis identified **12** metagenomic clones to be phytase-positive and determined the thermostability and optimum temperature of the 12 clones (Figure 9). Four clones, M2-32, M2-44, M2-49 and M2-62, have an enzyme activity of 25-50% at 70°C. These clones were digested with a restriction enzyme (SfiI) to compare restriction profile. Three clones showed the same pattern of restriction enzyme and found to be the same protein, M2-32, M2-.44 and M2-62. The clones **M2-32** and **M2-49** were selected for sequencing and further study. Currently the clones have been sent for sequencing.

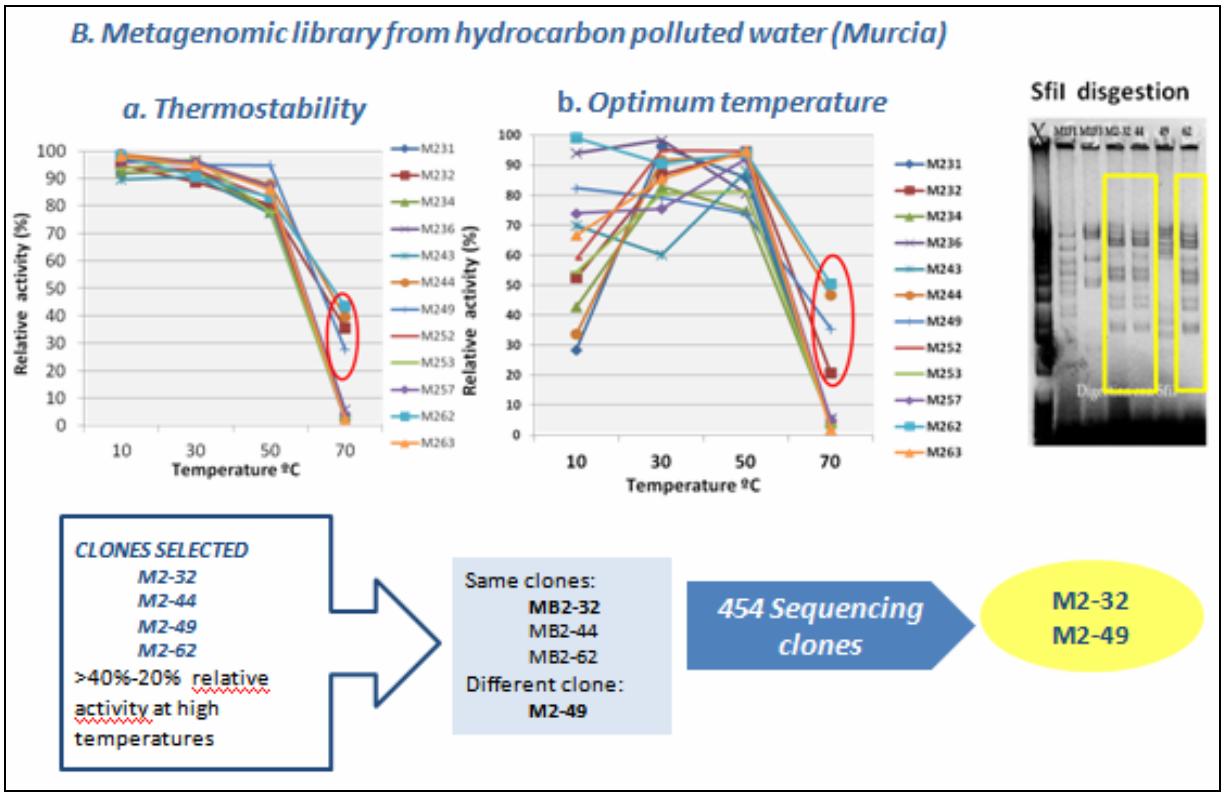


Figure 9: Characterisation of 12 phytase-positive clones from hydrocarbon polluted water metagenomic library

Metagenomic library 3: Water sample from Motril harbor (Granada)

Ten phytase-positive clones were identified. Bio-Iliberis determined the thermostability and optimum temperature of the 10 clones. All clones were digested with two restriction enzymes (EcoRI and HindIII) for comparing the restriction profile. Only two clones showed the same restriction profile with both enzymes.

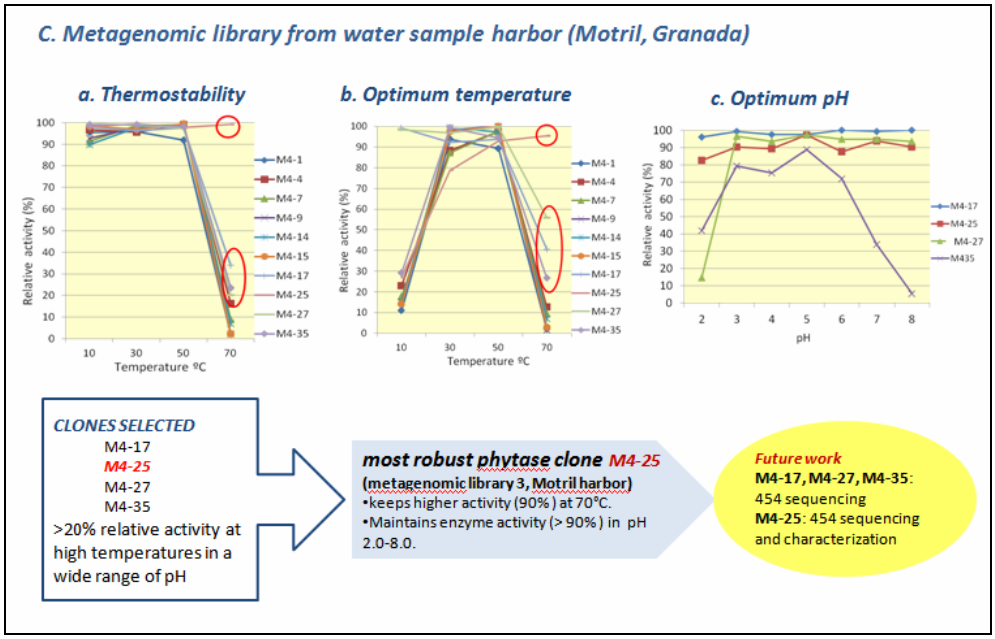


Figure 10: Phytase positive clones identified from the water sample metagenomic library from Motril harbour

Four clones, M4-17, M4-25, M4-27 and M4-35 clones showed an enzyme activity of 20-90% at 70 °C (Figure 10).

- **M4-25** clone shows 90% activity at 70 °C. It also maintains more than 90% of the enzyme activity in a pH range of 2.0-8.0.
- **M4-17**, **M4-27** and **M4-35** clones have an enzyme activity of 20-40% at 70 °C. The clone M4-27 has no activity at pH 2, however, has more than 90% of enzyme activity between pH 3 and 8. Clone M4-35 shows more than 70% enzyme activity in a pH range of 3.0 to 6.0 and the M4-27 clone has over 90% of enzyme activity at all pH values tested.

According to these preliminary results, the clone **M4-25** from the **metagenomic library 3** (Water sample from Motril harbour) is the best candidate to be studied and characterized. For future work the most robust phytase clone, **M4-25**, will be sequenced and characterized.

Screening of metagenomic libraries constructed by other partners

Bio-iliberis has performed functional screenings of 2 metagenomic libraries constructed by the partners; Bangor University and Matís Ltd.. The search has been focused on dioxygenase and oxygenase, lipase, cellulase and phytase activities.

Figure 11: Collaborative work scheme for phosphatase and phytases screens for different metagenomic libraries.

Bio-Iliberis has performed the functional screenings for:

- Phosphatases: Phosphatase screening using LB agar with X-phosphate as substrate. We found 25 Phosphatase-positive clones.
- Phytases: Phytate mineralization on Sodium Phytate medium agar. No Phytase positive clones were obtained.

- Phosphatases: Phosphatase screening using LB agar with X-phosphate as substrate. No Phosphatase positive clones were obtained.
- Phytases: Phytate mineralization on Sodium Phytate medium agar. No Phytase positive clones were obtained.

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