Deliverable 2.1

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for chemical characterization and sample preparatory techniques.

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Publishable Executive Summary

The SECRETed project will fully exploit the potential of Systems and Synthetic Biology toolboxes and their application within aquatic biotechnology to develop novel hybrid compounds for the agrochemical, pharmaceutical, cosmetic, and chemistry sectors. Biosynthetic pathways of marine and extremophilic microorganisms will be reverse engineered to infer the individual roles of their constituent genes, which will be further combined for the production of non-natural biosurfactants and siderophores with tailor-made properties. Biosurfactants are compounds with a surface-active nature tendency to adsorb at interfaces, while siderophores have the ability to chelate and transport Fe3+ ions. The amphiphilic nature of biosurfactants and marine siderophores provides an exciting opportunity to develop methods of biosynthesis that would enable the exchange of their hydrophobic and hydrophilic parts, among other structural changes. The development of hybrid molecules would allow the exploration of new-to-nature compounds endowed with the combination of their respective properties, to address new applications. Machine Learning algorithms, an inspection of databases, and new experimental and computational-based data will be employed to build a unique microbial amphiphilic compound chemical space to identify the desired genetic mechanisms. Detected genes will be reverse engineered to standardize and modularize associated metabolic elements, with a purpose to broaden their benefits by searching for Industrial-driven formulations based on suitable microbial hosts. The Design-Build-Test-Learn methodological steps will be used to produce new microbial strains that support the selected genetic elements and satisfy sustainable industrial processing solutions for the production of biosurfactants and siderophores. The SECRETed consortium is comprised of 15 partners across 9 European countries.

Deliverable 2.1 *'Handbook of protocols for chemical characterization and sample preparatory techniques',* provide guidelines on standardization and organization of protocols used for preparation and processing of samples within SECRETed for subsequent screening in WP2, subsequent sharing, and further analysis in downstream WPs. It defines the data/information to be associated with target compounds discovered and archived in the SECRETed project.

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1. Aim

The aim of the handbook is

1. to provide guidelines on standardization and organization of protocols used for preparation and processing of samples within SECRETed for subsequent screening in WP2, sharing and further analysis in downstream WPs,
2. to define the data/information to be associated with target compounds discovered and archived in the SECRETed project.

The" handbook" is a living document and added to as protocols are developed and established during the project. It includes information on the strain collection, selection of strains, and how it is best organized for an overview of data related to strains/samples selected, and the results of the primary screening.

The handbook will be reviewed in Month 12.

The handbook will be available for all consortium members and will facilitate further uploading into the SECRETed database. It can be used in downstream WPs, where additional information is added and used as a source of background information for possible future scientific investigations and commercial exploitation.

2. Strain collections and archiving

Strains are selected from proprietary diverse strain collections of four partners

PHM: 1500 marine microbial samples.
SZN: 450 psychrophilic and piezophilic strains;
MATIS: 200 thermophiles and marine microbes
USE: 50 model-targeted halophilic strains

The strains are selected on the basis of phylogenetic novelty and other relevant information on their properties. The four partners are working on physiologically very different microbes this will be reflected in the different cultivation media used and conditions. Protocols and media composition will mostly be relevant only to the specific physiological group. Samples from the strains will be made available for screening for the production of the target biomolecules siderophores and biosurfactants.
Genomes of microbes giving positive hits for the target compounds will subsequently be sequenced in order to identify the corresponding synthetic gene clusters.

***Archiving**:* The different groups have in-house cataloging systems of their proprietary strain collections that will be upheld as much as possible in the archiving of data in the SECRETed project. Data collected in SECRETEed for the different strain collections will be overseen and archived by the corresponding partners.

3. DATA collection

Data collected and registered for strains and samples are the following in broad outline

1. Sampling origin and isolation methods, (WP2)
2. Molecular characterization of strains (DNA fingerprint., 16S rRNA, etc.), WP2)
3. Cultivation procedures (WP2)
4. Extracts preparation (WP2)
5. Assay protocols (WP2)
6. Assay protocols (WP2)
7. Chemical de-replication protocols **(WP5)**
8. Compound identification **(WP5)**

Chemical dereplication protocols and compound identification will be collected in WP5 and added to the "Handbook table".

The collected data relating to individual strains will be archived in the SECRETed database (WP3) in association with positive screening results, along with additional data from downstream WPs.

4. Data types and Data points

The different groups have their own sample cataloguing systems that will be used in the project. However, certain data needs to be provided for the SECRETed archiving of positive samples. For clarification the MATIS sample cataloguing (a table) is shown. Informative protocols and media compositions will be supplied in appendixes if required and giving identifiers (short designations) so they can be referred to in the table (as data-points) under an appropriate column heading.

The subheadings and their numbers refer to data-types (headings of columns in the tables) and the Subheading number corresponds to the number of the column in the table (Data-table (excel).

Data-points corresponding to the datatypes, are in the column's cells underneath the data-type heading (Figure1).

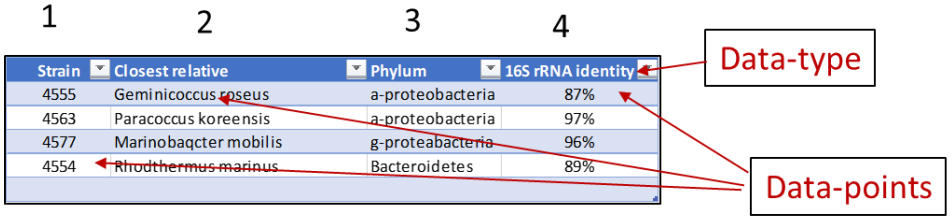

Figure 1.: Data-types and data-points

4.1 Strain

The datatype is a strains from the respective strain collection (of partners) that will be screened and datapoints are strain-numbers in column 1 of the table, below the datatype heading the column. In figure 1 this is the number of a specific strain from the ISCAR strain collection (of MATIS) and represents the sample screened in the project. If more than one strain is selected from the same species information about characteristics that distinguish between the strains can also be supplied and appended, if considered relevant. Distinguishing characteristics can be phenotypic or genotypic. e.g. sequence-based, and/or physiological-, biochemical-, morphological, or other phenotypic features, including differences in yield and type of the target biomolecules.

4.2 Closest relative of the strain

This is the species/strain in the public database (NCBI-genbank) that shows the closest relationship according to 16S rRNA analysis (sequence identity)

4.3 Phylum

This column (3) shows the phylum to which the is the species/belongs to.

4.4 16S rRNA identity to closest relative

16S rDNA of strains showing positive hits in the screening effort will be PCR-amplified using universal primers and at least partially sequenced (500-700 bases). The sequences will be compared with sequences in Genbank for identification of the species or the closest relative of the organism.

A separate file with 16S rRNA sequences of positive strains will be kept and will be available for uploading in fasta format for more detailed taxonomic positioning.

4.5 Sampling site

Data-points under this data-type (column 5) are sites where the strain/species originate from.

4.6 Providere of genetic resource

Public/Proprietary- *name of partner/*

4	5	6	7	8	9	10
16S rRNA identity	Isolation site	Genetic resource	Target biomole	Isolation mediu	Isolaton temp. C°	isolation salini
87%	Reykjanes, Iceland	Proprietary -MATIS	siderophore	Meidum A	50	3%
97%	Reykjanes, Iceland	Proprietary -MATIS	biosurfactant	Medium A	60	3%
96%	Reykjanes, Iceland	Proprietary -MATIS	biosurfactant	Medium A	60	3%
89%	Laugavík Iceland	Proprietary -MATIS	siderophore	Medium A	60	3%
99%	Yellowstone park	Public	siderophore	Medium B	37	0.1%

Refers to specific medium composition supplied in an appendix

16S rRNA sequence of strains with positive hits are kept in a separate file

Figure 2. Apart of the Data-table showing the data- types and column numbers, fo 16S rRNA identity to closest relative, isolation site, genetic resource, target biomolecules in the screening, isolation medium, isolation temperature, and isolation salinity.

4.7 Presence of target chemical

Data-type (column 7): Data-points: Target biomolecules (5) are of two kinds, siderophore, biosurfactant

4.8 Isolation medium and cultivation conditions,

Data points are media and cultivation conditions of strains when first isolated (column 8)

4.9 Isolation temperature

The incubation temperature was used when the strain was isolated.

4.10 Isolatioon salininty (or salininty range)

The salt concentration in the original isolation medium

4.11 Isolation pH

The temperature was used when the strain was isolated.

11	12	13
isolation pH	Primary screening medium	Optimized mediumm
7	Medium A	Medium AB
7	Medium A	Medium AC
7	Medium A	Medium AC
7	Medium A	Medium AB
6	Medium B	Medium BA

Isolation of the microbes in prior projects was carried out on various solid and liquid media. Standard plating techniques and variable conditions were used depending on the physiology of the different microbial groups. They may have included special carbon sources, i.e. complex polysaccharides or antibiotics with selective activity against certain microbial groups. These

data are available but the most important data points to be included in the (9), temperature (10), s,alinity (9) and (11) pH that distinguishes most distinctly between different physiological groups. DNA has been extracted from most of the bacteria.

4.12 Primary screening medium (standard medium)

This is the medium is used in primary screening for the target biomolecules in SECRETed. Often the same as the isolation medium but may have been modified. These media will get short designations such as *Medium-A*, *Medium-X,* or *Medium-Y*, to be used as data points in the table. The different media compositions are supplied in appendices.

4.13 Optimized medium for the production of target chemical

This medium has been developed to induce the production of the target biomolecules or to increase yield. May contain an inducing compound or specific conditions (iron scarcity). These media will get short designations such as *Medium-AB* or *Medium-AC*, to be used as data points data-points in the table. The initial media compositions are supplied in appendices.

13	14	15	16	
Optimized mediumm	Extraction protocol	Extract availability	Assay protocol	Patjhway identified
Medium AB	extr. 1	100 mg	Assay1	Path1
Medium AC	extr. 2	122mg	Assay2	path2
Medium AC	extr. 2	156mg	Assay2	Path3
Medium AB	extr. 1	120mg	Assay1	Path4
Medium BA	extr. 1	100mg	Asseya	Path5

4.14 Extraction protocol (sample preparation)

Sample preparation for screening will be standardized as much as possible. For siderophores, it is basically clarified (by centrifugation) of supernatant but may be condensed by evaporation, and/or filtration.

4.15 Extract availability

Protocols for extraction (12) of the target biomolecules from the culture supernatants will be supplied in appendices and given identifiers (short designations) e.g. Exract-1 – Extract-2, to be used as data-points in the table. Data-points for extract availability (will be = and 1, corresponding to absence and presence, respectively. The sample is stored freeze-dried.

4.16 Assay

Assay protocols for the detection of the target biomolecules will be supplied in appendices and referred to in the table (as data points) by short designations such as *Assay-A1* or *Assay-A2*. The assays will be different for the two target biomolecules e.g. cetyltrimethylammonium bromide, CTAB for biosurfactants and the chrome azurol S (CAS) assay for siderophores) The assays may have to be modified since reaction dyes or chelating agents may be altered by the presence of some salts in the samples. In those cases, cross-feeding assays with an indicator strain unable to grow if siderophores or biosurfactants are not provided can be used.

4.17 Genome

Genomic sequencing will be done for pathway identification for the synthesis of the target chemicals. The sequences of identified synthesis clusters will be retrieved and designated an ID that will be used as data-points for the corresponding samples. A separate file with the

sequences will be kept and will be available for uploading to the consortium database for refining further predictions in the “mix and match” combinatorial approach. The genomes will be available for chassis evaluation and metabolic modelling in WP3.

5. Standard procedures for cultivation of microbes and sample preparation/extraction for screening of target biomolecules

5.1 Siderophore screening

5.1.1 Thermophiles: MATIS/LUND

The standard medium for primary screening of siderophore production in thermophiles. SWYE1.5

Primary screening medium: Having compared different media using O-cas plate assay to evaluate siderophore production, the best media was siderophore screening was **SWYE (1.5).** It was then used for the screening of the thermophile strain collection (appendix 2: 1.17). The medium is a complex medium originally used for the growth of halophilic bacteria and the recipe was obtained from USE. It is a saline medium that can be prepared at different final salt concentrations between 0,5 and 25% (w/v) by diluting a concentrated stock solution of salts (30% w/v) called **SW30** (Subow, 1931), which approximated the composition of seawater, together with the addition of 0.5% (w/v) of yeast extract. Depending on the final salt solution concentration, the medium is called e.g. **SWYE2** (2% w/v) or **SWYE10** (10% w/v) (Nieto et al., 1987), where X is the final salt solution concentration. For cultivating thermophiles for siderophore screening SWYE1.5 was used in both on solid and liquid media.

In order to prepare 1 L of **SW30** stock solution**,** the following salts are dissolved in 1L of distilled water: 234 g of NaCl, 39 g of $MgCl_2 \cdot 6H_2O$, 61 g of $MgSO_4 \cdot 7H_2O$, 6 g of KCl, 1g of $CaCl_2 \cdot 2H_2O$, 0.7 g of NaBr and 0.2 g of $NaHCO_3$. The final solution is filtered. Thus, SW30 maintains the relative proportions of seawater, containing only the major elements and with a slight decrease in the concentrations of $CaCl_2$ and $NaHCO_3$ to avoid their precipitation.

As an example, in order to prepare 100 ml of **SWYE2 or SWYE10**, the stock solution SW30 is added in the appropriate proportion to distilled water. Thus, 6.6 ml (SWYE2) or 33.3 ml (SWYE10) of SW30 are diluted in distilled water. In both cases, 0.5 g of yeast extract is added and pH is adjusted to 7.2 with KOH solution 1N. Finally, 2 g of agar (2% w/v) are added if necessary, and the solution is autoclaved.

Strains are precultured in standard medium and this culture is used to inoculate 20 ml of standard medium (1%). The cultures are then incubated overnight (approx.. 16 h) at the appropriate temperature of 40, 50, or 60°C depending on the original isolation temperature of the strain and then samples are assayed for the presence of siderophores.

Sample preparation.

The samples are prepared to detect production of siderophores in liquid cultures. After incuabation supernatant is clarified by centrifugation and cells removed. The presence of siderophore in (usually in 100ul volume) is then assayed with different assays (see Del2.2).

5.1.2 Halophiles: USE

Standard cultivation medium for primary siderophore screening of halophiles: SWYE (2,5,10) and M63mod (0.6 and 2.5)

final volume is adjusted with distilled water, pH is adjusted to 7.2 with KOH 1N and the medium is autoclaved. Finally, 1 ml of glucose 2 M sterile solution is added to the de medium in order to achieve a final concentration of 20 mM.

In order to prepare M63mod plates, the same volume of M63mod at 2X concentration from M63 10X stock solution and of distilled water with agar 4% (w/v) is prepared in separated flasks and autoclaved. Once the flasks have cooled to 60ºC, 1 ml of 2 M sterile carbon source solution is added to the M63 2x flask, to achieve a final concentration of 20 mM. Then this solution is carefully mixed with the sterile agar solution and poured into Petri dishes.

Strains are precultured in SWYE2 and this culture is used to inoculate 5 ml of SWYE-X or M63 with or without modifications. The cultures are incubated at 37°C during the appropriate time depending on the media and salinity, and samples are taken to analyse the presence of siderophores.

USE used both complex and minimal mediums at different salinities for primary siderophore screening of the halophilic strains.

Primary screening complex medium: As complex medium, USE used SWYE at different salinities (SWYE2, SWYE5 and SWYE10). This medium is described in 5.1.1.

Primary screening minimal medium: As minimal media, USE used M63 medium and M63 medium with modifications, both of them at different salinities (0.6 M NaCl and 2.5M NaCl).

M63 is a minimal medium that can be used for halophilic bacteria (Csonka, 1982). This medium is prepared by diluting 10-fold a concentrated stock of salts (M63 10X) and modifying this base composition with the appropriate NaCl concentration (e.g. 0.6 M or 2.5 M) and carbon source (20 mM) (e.g. glucose). In order to prepare 1L of stock solution M63 10X, these salts are dissolved in 1L of distilled water in the following order: 42 g of KOH, 136.1 g of KH_2PO_4, 20 g of $(NH_4)_2SO_4$ and 10 ml of $MgSO_4{\cdot}FeSO_4$ solution previously prepared (4 g of $MgSO_4{\cdot}7H_2O$ or 1.95 g of $MgSO_4$ and 0.05 g of $FeSO_4{\cdot}7H_2O$ in 100 ml of distilled water). The order in which the salts are incorporated into the solution is important to avoid undesired salt precipitation.

M63 medium may be **modified (M63mod)** by reducing the final concentration of $FeSO_4{\cdot}7H_2O$ (in order to induce siderophore production by the generation of iron limiting conditions) and KH_2PO_4 (because this compound is a weak iron chelator which interferes with some siderophore detection methods, giving false positives as a result). Thus, the final salts solution composition for 1L is as follows: 4.2 g of KOH; 1.0, 1.5 or 2.0 g of KH_2PO_4; 2 g of $(NH_4)_2SO_4$, and 1 ml of a $MgSO_4{\cdot}FeSO_4$ solution (4 g of $MgSO_4{\cdot}7H_2O$ or 1.95 g of $MgSO_4$ and 13.8 mg of $FeSO_4{\cdot}7H_2O$ in 100 ml H_2O).

In order to prepare 100 ml of M63mod medium (1x), 10 ml of M63mod 10X are dissolved in 80 ml of distilled water, together with e.g. 3.48 g of NaCl (M63 0.6 M NaCl) or 14.5 g of NaCl (M63 2.5 M NaCl). After that,

Sample preparation.

To detect the production of siderophores in liquid cultures, after incubation cultures are centrifuged at 12000 rpm for 10 min and the supernatant is collected to remove the cells and obtain clarified culture supernatants. Supernatant samples are stored at -20ºC for further use. The presence of siderophores (using different supernatant volumes depending on the assay) is analysed with different methods (see Del2.2).

5.1.3 Psychrophiles: SZN

The standard medium for primary siderophore screening of psychrophiles:

For culture maintenance
TYP: tryptone 6 g/L, yeast extract 16 g/L, NaCl 10 g/L, in distilled water
MB: 40 g/L marine broth (Condalab, Madrid, Spain) in distilled water

For siderophores production
ASG: Low-iron artificial seawater medium (ASG) containing 3 g/L Casamino acids, 15 g/L NaCl, 0.75 g/L KCl, 12 g/L $MgSO_4 \cdot 7H2O$, 3 g/L $CaCl_2 \cdot 2H_2O$, 1 g/L NH_4Cl, 0.05 g /L Glycerol phosphate and 2 mL 1.0 M $NaHCO_3$

Sample preparation.

Pre-inoculum from glycerol stock of selected producing microorganisms in a 96-multiwell plate containing TYP or MB and incubation for 2 days at 20 °C. Transfer 20 µL of microcultures in 96-deep-well plates containing different growth media to induce the production of biosurfactants and siderophores; experimental parameters (temperature, pH, agitation, incubation time, nutrients) can be adapted according to the microbial growth requirements. Centrifuge the deep-well plates and transfer supernatants in new 96 multi-well plates. Detection: use supernatants for CAS assays

5.1.4 Marine actinobacteria: PHM

PHM samples:

The samples from PHM were dried extract samples obtained from bacterial strains obtained and cultivated in previous projects and extracted with organic solvents. The isolated actinobacteria were cultured in 24 deep wells using two production media (2-7 and MP-12) Composition of the medium 2-7: Soybean flour (10g/L), Mannitol (50), Dextrin (14), $CaCl_2$ (16), CO_3Ca (14), ASW (artificial seawater 6g/L). Composition of MP-12: Soybean flour (12g/L), Peptone (1), malt extract (1), Dextrose (2.50), Dextrin (40), CO_3Ca (8), ASW (artificial seawater 20g/L). The plates were incubated in shakers at 220 rpm at 28ºC. The samples for screening were taken from 96h to 168h.

Sample preparation. 2 mL of whole broth was lyophilized and extracted with methanol/acetone/water (1/1/0.2), filtered and 0.5 mL of each organic phase was aliquoted into 1,2 mL tubes into 96 deep well plates. This was done in duplicate, one sample was tested in PharmaMar's screening platform to detect cytotoxic extracts. The other replicate was frozen and they were offered for Secreted project for siderophore screening. Samples and redissolved in 20% methanol and the presence of siderophores are analysed with ADL assay (see Del2.2).

5.2. Biosurfactant screening

5.2.1 Thermophiles: MATIS/LUND

The standard medium for primary screening of biosurfactant production in thermophiles

Two media are used for cultivation and subsequent screening of thermophilic strains for biosurfactants: **Marine broth** medium 210 for all strains except *Thermus* strains **where *Thermus* medium** strain was used. Sunflower oil was used as a substrate in all three media to enhance the production of biosurfactants.

Recipes of Marine Broth (Medium 210)

Marine broth (Medium 210) (used for most strains, when possible)	**Thermus rich medium**
1L H20	**1L H20**
18.7 gr Difco Marine Broth	4.g Yeast extract
2 g sunflower oil 1	8.g Tryptone
	2.0 NaCl
	2.g Sunflower oil

Sample preparation (extraction)

Sample preparation for rapid screening of biosurfactants

The culture broth from the cultivations is used directly for the rapid screening. The volumes applied depending on the assay used for three types of the assays described in deliverable D2.2 , CTAB, Emulsificaion and Crop collapse.

5.2.2 Halophiles: USE

Standard medium for primary screening of biosurfactant production in halophiles: BMB (3 and 6) and M63 (0.6 and 2.5)

Standard mediums for primary screening of biosurfactant production in halophiles:

Complex media, BMB and SWYE at 3 and 6% of salts; and the minimal medium M63-X (at 0.6 M NaCl and 2.5M NaCl were used by USE were used for the primary screening of biosurfactant

production in halophilic strains. SWYE and M63 media composition are described in 5.1.1. and 5.1.2.

Bacto marine broth (BMB) is a standard medium used for cultivating heterotrophic marine bacteria. In particular, this medium is described as the optimal medium for the majority of the moderately halophilic strains selected in the USE strain collection and thus, it was selected as a primary screening medium. Depending on the amount of NaCl requirements of each of the strains, the medium is designated as BMB-X, where X indicates the % of NaCl that needs to be added. For example, to prepare BMB-6, 60 g/100 mL needs to be added to the amount of NaCl indicated in the recipe.

To prepare 1 L of BMB medium the following components are dissolved in distilled water: 5 g of Bacto peptone, 1 g of Bacto yeast, 0.10 g of Fe(III) citrate, 19.45 g of NaCl, 5.90 of $MgCl_2$ (anhydrous), 3.24 g of Na_2SO_4, 1.8 g of $CaCl_2$, 0.55 g of KCl, 0.16 g of $NaHCO_3$, 0.08 g of KBr, 34 mg of $SrCl_2$, 22 mg of H_3BO_3, 4 mg of Na-silicate, 2.40 mg of NaF, 1.60 mg of $(NH_4)NO_3$ and 8 mg of Na_2HPO_4. Final pH should be adjusted to 7.6 ± 0.2 at 25^{o}C. If using the complete medium from Difco add 37.40 g to 1 liter of distilled water.

Strains are precultured in SWYE2 and this culture is used to inoculate 5 ml of SWYE-X or M63. When using BMB, cells are precultured in the same medium. The cultures are incubated at 37°C during the appropriate time depending on the media and salinity, and samples are taken to analyse the presence of biosurfactants.

Sample preparation (extraction)

After incubation, liquid cultures are centrifuged at 12000 rpm for 10 min and the supernatant is collected. Supernatant samples are stored at -20ºC for further use. Biosurfactant production is then assessed by the methods described in Deliverable 2.2.

5.2.2 Psychrophiles: SZN

Standard medium for primary screening of biosurfactant production in sychrophiles

For culture maintenance
TYP: tryptone 6 g/L, yeast extract 16 g/L, NaCl 10 g/L, in distilled water
MB: 40 g/L marine broth (Condalab, Madrid, Spain) in distilled water

For biosurfactants production (used also for siderophores)
SV2 SW: glucose 15 g/L, peptone 15 g/L, glycerol 15 g/L, CaCO3 1 g/L, in sea water

Sample preparation (extraction)

Pre-inoculum from glycerol stock of selected producing microorganisms in a 96-multiwell plates containing TYP or MB and incubation for 2 days at 20 °C. Transfer 20 µL of microcultures in 96-deepwell plates containing different growth media to induce the production of biosurfactants and siderophores; experimental parameters (temperature, pH, agitation,

incubation time, nutrients) can be adapted according to the microbial growth requirements. Centrifuge the deep-well plates and transfer supernatants in new 96 multi-well plates. Detection: use supernatants for CTAB assays

6. Protocols harmonization strategy

Deliverable, D2.1 (month 6), corresponds to Task 2.1 having the aim to harmonize collection of data to be used downstream WPs, WP3 and WP5. This involved defining the types and format of data to be collected for archiving, and for bioinformatic analysis in WP3; and to test and standardize cultivation procedures/media and sample preparation protocols for the primary screening in Task 2.3, and for optimization subsequent functional and structural investigations in WP5.

The strain collections contain physiologically very different strains, and this is reflected in the different protocols and media for cultivation and sample preparation, tested and selected by the partners. In contrast to psychrophiles, cultivation protocols for the thermophiles and halophiles needed to be modified and adapted for use for screening. The capability of different media to “induce” production of the target biomolecules was tested and compared using representative strains from these collections.

The interaction was between T2.1 and T2.2 was very important in selection of media and composition and choosing cultivation procedures (liquid vs. solid plate cultivation) and which assay to use for detection and evaluation of different media. This is exemplified by the testing and subsequent selection of the SWYE 1,5 medium by MATIS for the screening of thermophiles, after consulting with USE: And generally to use of solid medium plate cultivation for testing of target-miolecules. The assays for detection and the evaluation of the different media were selected on the basis work in the parallel, Task T2.2 (reported in D2.2). The results of T2.1 and T2.2 directs the cultivation and sample preparation in the primary screening effort in Task 2.3 which is ongoing:

This screening pipeline adopted for primariy screening in T2.3 is based on results from T2.1 and T2.2 is following :

1. Detection of production (primary screening): Appropriate medium (depending on strain collection (T2.1)) and solid plate screening (see D2.2)
2. Production in liquid culture using appropriate assay from T&D2.2.
3. Yield evaluation Appropriate cultivation assay from T&D2.2

Samples were also sent to ADL for evaluation of media but production in liquid cultures could not be detected for thermophiles and not in all halophiles. This suggests, medium composition, cell density and even quorum sensing mechanisms are important factors for the production. Further modification of media and conditions is therefore needed and will and will be carried out in Task2.3. Especially for the samples from -PHM which were dried solvent extracts from prior projects. They have been assayed by USE and ADL and shown to be difficult. Therefore, fresh cultivation samples from selectged strain will be produced based on prior structural analysis of the metabolites produced.

Appendix 1. Strain collections to be screened

SZN. SZN has approximately 450 bacteria isolated from different areas around the World in the framework of EU programmes or SZN expeditions, in agreement with the rules of Nagoya protocol (Table 1). The collected marine samples (both sediments and water) were used to isolate microorganisms by serial dilution followed by spreading on agar plates using different growth media. Pure cultures were stored at -80 °C in presence of 20% glycerol. Two techniques have been applied for the identification of microorganisms: classical 16S rRNA amplification and MALDI-TOF MS, which is as a more rapid and cost-effective technique. Approximately, half of the SZN collection is composed of marine psychrophilic bacteria isolated from shallow Antarctic water, and Deep-sea Antarctic and Mediterranean sediments. 100 strains have been isolated from Sarno river mouth, a highly polluted (heavy metals and polycyclic aromatic hydrocarbons) coastal environment close to Naples, while other mesophilic marine strains have been isolated from Piran (Slovenia), Faro (Portugal) and from the volcanic island of Ischia (Italy), an environment dominated by the presence of CO_2 submerged vents. All these strains will be screened during the project implementation to detect biosurfactants and siderophores producers. In previous published works, SZN already demonstrated the potential of some bacteria belonging to this collection to produce a wide range of new biosurfactants (rhamnolipids, trehalolipids, aminolipids, surfactins). These strains will be used to set-up the screening procedure described in D2.2.

USE. USE will be screening approx. 50 phylogenetically diverse halophilic bacteria isolated from different areas around the World that are found in public strain selected for their potential in producing siderophore and/or biosurfactants based on literature/database search and/or genomic pathway analysis of those genomes (Figure 1). Their genome sequences are deposited in NCBI database.

PHM. PHM has an approx. 1500 taxonomically diverse marine bacteria isolated and extracted in prior projectswsuch as the Eureopean FPL7 projects Micro3B and MaCumbaþ Including deep sea bacteria (80% actinobacteria). 76 genera in total (Figure 2). These strains have been anlysed in prior projedts and a subset showing metabolites of promising structural characteristisc, indicating sierophore properties Will be screened in T2.3.

MATIS

MATIS will be screening approx. 200 phylogenetically diverse marine bacteria isolated around Iceland and including 50 novel species and genera from coastal hot springs from the Fpl7 EU project SeaBiotech. (Figure 3).

Appendix 2. - Media

1. Cultivation media

1.1. BMB(-X): BACTO MARINE BROTH OR DIFCO 2216

Marine broth is used for cultivating heterotrophic marine bacteria. This medium contains minerals that nearly duplicate the mineral composition of seawater (Lyman and Fleming, 1940). Additionally, peptone and yeast extract provide a good source of nutrients.

To prepare 1L of BMB medium the following components are dissolved in distilled water: 5 g of Bacto peptone, 1 g of Bacto yeast, 0.10 g of Fe(III) citrate, 19.45 g of NaCl, 5.90 of $MgCl_2$ (anhydrous), 3.24 g of Na_2SO_4, 1.8 g of $CaCl_2$, 0.55 g of KCl, 0.16 g of $NaHCO_3$, 0.08 g of KBr, 34 mg of $SrCl_2$, 22 mg of H_3BO_3, 4 mg of Na-silicate, 2.40 mg of NaF, 1.60 mg of $(NH_4)NO_3$ and 8 mg of Na_2HPO_4. Final pH should be adjusted to 7.6 ± 0.2 at 25°C. If using the complete medium from Difco add 37.40 g to 1 liter of distilled water.

The amount of NaCl can be adjusted depending on the requirements of each strain. Depending on the amount of NaCl that needs to be added, the medium is designated as BMB-X, where X indicates the % of NaCl that needs to be added. For example, to prepare BMB-6, 60 g/100 mL needs to be added to the amount of NaCl indicated in the recipe.

*For strain DSM 12178, add 1% of Na acetate.

**For strain DSM 22530 add 10 mg/L $MgSO_4$.

***For strain DSM 22784, add 10 mg/mL $MnSO_4$.

1.2. CM+YE: CM + YE medium

To prepare 1L of CM + YE medium, the following components are dissolved in distilled water: 7.5 g of casamino acids Difco (vitamin free), 10 g of yeast extract, 20 g of $MgSO_4 \cdot 7H_2O$, 3 g of Na_3Citrate, 2 g of KCl, 200 g of NaCl, 10 ppm of Fe ([a]). Final pH should be adjusted to 7.4 with NaOH. When required, agar is added at a concentration of 15 g/L for solid media.

([a]) To prepare 4.98% $FeSO_4 \cdot 7H_2O$ dissolve 10 g of $FeSO_4 \cdot 7H_2O$ in 1L 0.001 M HCl; add 1 mL per liter medium.

1.3. HA: HORIKOSHI ALKALINE MEDIUM

To prepare Horikoshi alkaline medium, the following components are added: 10 g of D-glucose, 5 g of peptone, 5 g of yeast extract, 1 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7\ H_2O$ and 5 g of Na_2CO_3 in 1 L of distilled water. Final pH is adjusted to 9.0. When required, agar is added at a concentration of 15 g/L for solid media.

1.4. HBM10: HALOBACTERIA MEDIUM (10% NaCl)

To prepare 1L of *Halobacteria* medium, the following components are dissolved in distilled water: 5 g of yeast extract, 5 g of casamino acids, 1 g of Na-glutamate, 2 g of KCl, 3 g of Na_3-citrate, 20 g of $MgSO_4 \cdot 7H_2O$, 100 g of NaCl, 36 mg of $FeCl_2 \cdot 4H_2O$, 0.36 mg of $MnCl_2 \cdot 4H_2O$.

Final pH should be adjusted to 7.0-7.2. When required, agar is added at a concentration of 20 g/L for solid media.

1.5. HBSM: HALOBACILLUS MEDIUM

To prepare 1L of *Halobacillus* medium, the following components are dissolved in distilled water: 100 g of NaCl, 5 g of $MgSO_4 \cdot 7H_2O$, 5 g of peptone casein digest and 3 g of yeast extract. Final pH should be adjusted to 7.5. When required, agar is added at a concentration of 15-20 g/L for solid media.

1.6. HVM: HALOVIBRIO MEDIUM

Halovibrio medium is prepared by mixing the following components in distilled water: 120 g of NaCl, 3 g of K_2HPO4, 0.5 g of NH_4Cl, 1mL of trace elements and 2,38 g of HEPES. Adjust pH to 7.5. Dispense the medium into sealed bottles and autoclave. After sterilization, add the following amounts from sterile stock solutions: 2 mL/L of $MgSO_4 \cdot 7H_2O$ (1 M) , 10 mL/L of sodium acetate (2 M), 2 mL/L of yeast extract (10%).

The trace element solution (Pfennig and Lippert, 1966) for this medium is prepared as follows: 5 g of EDTA, 2.2 g of $FeSO_4 \cdot 7H_2O$, 0.10 g of $ZnSO_4 \cdot 7H_2O$, 0.03 g of $MnCl_2 \cdot 4H_2O$, 0.03 g of H_3BO, 0.20 g of $CoCl_2 \cdot 6H_2O$, 0.3 g of $CuCl_2 \cdot 2H_2O$, 0.3 g of $NiCl_2 \cdot 6H_2O$, 0.03 g of $Na_2MoO_4 \cdot 2H_2O$ are added to 1l of distilled water. Final pH should be adjusted to 3.0-4.0.

When required, agar is added at a concentration of 15-20 g/L for solid media.

1.7. MAM: METHYLOPHAGA ALCALICA MEDIUM

Methylophaga alcalica medium is specific to cultivate this moderately halophilic and obligate methylotrophic bacteria.

In order to prepare 1L of MAM medium the following components are dissolved in distilled water: 1 g of KH_2PO_4, 1 g of KNO_3, 0.22 g of$MgSO_4 \cdot 7H_2O$, 30 g of NaCl and 1 mL of trace elements. Final pH must be adjusted to 9.5.

To prepare the trace elements solution the following salts are mixed in distilled water: 30 mg of ferric citrate, 30 mg of $CaCl_2 \cdot 2H_2O$, 5 mg of $MgCl_2 \cdot 4H_2O$, 5 mg of $ZnSO_4 \cdot 7H_2O$ and 0.5 g of $CuSO_4 \cdot 5H_2O$. The final volume is adjusted to 1L with distilled water.

This medium is initially prepared without the Na_2CO_3 (5 g/L), which can be sterilized separately by autoclaving. Then, 10 mL/L of sterile methanol is added to the cooled medium. When preparing liquid media, the mineral salts solution and Na_2CO_3 have to be cooled to room temperature before mixing.

To prepare solid media, 20 g/L agar is added to the mineral salts solution and autoclaved. Cool the Na_2CO_3 stock solution and agar to 50-55^oC before mixing.

1.8. MHM: MH MEDIUM

To prepare 1L of MH medium, the following components are dissolved in distilled water: 60.7 g of NaCl, 15 g of $MgCl_2 \cdot 6H_2O$, 7.4 g of $MgSO_4 \cdot 7H_2O$, 0.27 g of $CaCl_2$, 1.5 g of KCl, 0.045 g of

$NaHCO_3$, 0.019 g of NaBr, 5 g of proteose peptone N°3, 10 g of yeast extract and 1 g of glucose. Final pH should be adjusted to 7.2. When required, agar is added at a concentration of 15-20 g/L for solid media.

1.9. 15MHM: 15% MH MEDIUM

Marinobacter lipolyticus is cultivated in 15% MH medium. To prepare 1L of 15% MH medium, the following components are dissolved in distilled water: 121.5 g of NaCl, 10.5 g of $MgCl_2$, 14.4 g of $MgSO_4$, 0.540 g of $CaCl_2$, 3 g of KCl, 0.039 g of NaBr, 5 g of proteose peptone N° 3, 10 g of yeast extract and 1 g glucose.

Final pH should be adjusted to 7.5. $NaHCO_3$ is added to the medium (to a final concentration of 0.09 g/L) once the medium is cooled. When required, agar is added at a concentration of 20 g/L for solid media.

1.10. NA(-X): NUTRIENT AGAR

To prepare 1L of nutrient agar, 5 g of peptone, 3 g of meat extract are dissolved in distilled water, and the volume is adjusted to 1 liter of volume. Adjust pH to 7.0. When required, agar is added at a concentration of 15 g/L for solid media.

The amount of NaCl can be adjusted depending on the requirements of each strain. Therefore, the medium is designated as NA-X, where X indicates the % of NaCl that needs to be added. For example, to prepare NA-10, 10 g/100 mL are added to the amount of NaCl indicated in the recipe.

*For *Virgibacillus halodenitrificans* DSM10037, 50% of soil extract is also added.

1.11. PSGM3: PAYNE, SEGHAL & GIBBONS MEDIUM (3% NaCl)

To prepare 1L of Payne, Seghal & Gibbons medium, the following components are dissolved in distilled water: 7.5 g of casamino acids, 10 g of yeast extract, 3 g of trisodium citrate, 2 g of KCl, 20 g of $MgSO_4 \cdot 7H_2O$, 36 mg of $FeCl_2 \cdot 4H_2O$, 0.36 mg of $MnCl_2 \cdot 4H_2O$, 30 g of NaCl. Final pH should be adjusted to 7.4. When required, agar is added at a concentration of 20 g/L for solid media.

1.12. RM: RICH MEDIUM

Rich Medium (RM) is prepared by mixing the following components in distilled water: 10 g of Bacto peptone, 5 g of yeast extract, 5 g of casamino acids, 2 g of meat extract, 5 g of malt extract, 2 g of glycerol, 1 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of Tween 80 0.05 g. Final pH is adjusted to 7.2 and distilled water is added up to 1 liter of volume. When required, agar is added at a concentration of 20 g/L for solid media.

1.13. SDOM: SDO MEDIUM

To prepare 1L of SDO medium, the following components are dissolved in distilled water: 100 g of NaCl, 3.45 g of $MgSO_4 \cdot 7H_2O$, 3 g of $MgCl_2 \cdot 6H_2O$, 0.25 g of NH_4Cl, 0.14 g of $CaCl_2 \cdot 2H_2O$, 0.25

g of $CaSO_4 \cdot 2H_2O$, 0.14 g of KH_2PO_4, 1 g of $NaHCO_3$, 0.1 g of yeast extract, 1 mL of trace elements [(a)], 1 mL of vitamin solution [(b)]. Final pH should be adjusted to 6.0 with HCl.

(a) To prepare 1L of trace elements, the following components are dissolved in distilled water: 0.1 g of $ZnSO_4 \cdot 7H_2O$, 0.03 g of $MnCl_2 \cdot 4H_2O$, 0.3 of H_3BO_3, 0.2 g of $CoCl_2 \cdot 6H_2O$, 0.01 g of $CuCl_2 \cdot 2H_2O$, 0.02 g of $NiCl_2 \cdot 6H_2O$ and 0.03 g of $Na_2MoO_4 \cdot 2H_2O$.
(b) To prepare 1L of vitamin solution, the following components are dissolved in distilled water: 2 mg of biotin, 2 mg of folic acid, 10 mg of pyridoxine-HCl, 5 mg of thiamine-$HCl \cdot 2H_2O$, 5 mg of riboflavin, 5 mg of nicotinic acid, 5 mg of D-Ca-pantothenate, 0.1 mg of vitamin B_{12}, 5 mg of p-aminobenzoic acid and 5 mg of lipoic acid.

When required, agar is added at a concentration of 15-20 g/L for solid media.

1.14. SNM: STARCH NITRATE MEDIUM

To prepare starch nitrate medium, the following components are dissolved in distilled water: 20 g of starch, 1 g of K_2HPO_4, 2 g of KNO_3 , 0.5 g of $MgSO_4$, 3 g of $CaCO_3$, 100 g of NaCl, 1 mL of Trace salt solution (see below).

Trace salt solution is prepared by dissolving the following salts in 100 mL of distilled water: 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $MnCl_2 \cdot 4H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$.

When required, agar is added at a concentration of 20 g/L for solid media.

1.15. SSM: SALINIVIBRIO SHARMENSIS MEDIUM

To prepare 1L of *Salinivibrio sharmensis* Medium (SSM) the following compounds are dissolved in distilled water: 100 g of NaCl, 3 g of Tri-Na citrate, 1 g of $MgSO_4 \cdot 7H_2O$, 2 g of KCl, 1 mL of $MnCl_2 \cdot 4H_2O$ (0.36 g/L), 1 mL of $FeSO_4 \cdot 7H_2O$ (50 g/L), 10 g of yeast extract. Final pH should be adjusted to 9.0. The medium is autoclaved and Na_2CO_3 is added (final concentration 3 g/L) from a sterilized stock solution when the medium has been cooled to 55°C. When required, agar is added at a concentration of 20 g/L for solid media.

1.16. STMS(-X): STARCH-MINERAL SALT-AGAR MEDIUM

To prepare 1L of STMS medium, the following components are dissolved in distilled water: 10 g of soluble starch, 2 g of $(NH_4)_2SO_4$, 1 g of K_2HPO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 1 g of NaCl, 2 g of $CaCO_3$, 1 mL trace element solution. Final pH should be adjusted to 7.2. When required, agar is added at a concentration of 15 g/L for solid media.

Trace element solution for this medium is prepared as follows: 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $MnCl_2 \cdot 4H_2O$ and 0.1 g of $ZnSO_4 \cdot 7H_2O$ in 100 mL distilled water.

The amount of NaCl can be adjusted depending on the requirements of each strain. Therefore the medium is designated as STMS-X, where X indicates the % of NaCl that needs to be added. For example, to prepare BMB-10, 10 g/100 mL is added to the amount of NaCl indicated in the recipe.

1.17. SWYE(X) medium

SWYE is a complex medium used for the growth of halophilic bacteria. It is a saline medium which can be prepared at different final salt concentrations between 0,5 and 25% (w/v) by diluting a concentrated stock solution of salts (30% w/v) called **SW30** (Subow, 1931), which approximated the composition of sea water, together with the addition of 0.5% (w/v) of yeast extract. Depending on the final salt solution concentration, the medium is called e.g. **SWYE2** (2% w/v) or **SWYE10** (10% w/v) (Nieto et al., 1987), where X is the final salt solution concentration.

In order to prepare 1 L of **SW30** stock solution**,** the following salts are dissolved in 1L of distilled water: 234 g of NaCl, 39 g of $MgCl_2 \cdot 6H_2O$, 61 g of $MgSO_4 \cdot 7H_2O$, 6 g of KCl, 1g of $CaCl_2 \cdot 2H_2O$, 0.7 g of NaBr and 0.2 g of $NaHCO_3$. The final solution is filtered. Thus, SW30 maintains the relative proportions of sea water, containing only the major elements and with a slight decrease in the concentrations of $CaCl_2$ and $NaHCO_3$ to avoid their precipitation.

As an example, in order to prepare 100 ml of **SWYE2 or SWYE10**, the stock solution SW30 is added in the appropriate proportion to distilled water. Thus, 6.6 ml (SWYE2) or 33.3 ml (SWYE10) of SW30 are diluted in distilled water. In both cases, 0.5 g of yeast extract is added and pH is adjusted to 7.2 with KOH solution 1N. Finally, 2 g of agar (2% w/v) are added if necessary, and the solution is autoclaved.

1.18. TSB-5:TRYPTONE SOYA BROTH + 5% NaCl

To prepare 1L of TSB with additional sodium chloride, the following components are dissolved in distilled water: 17 g of peptone from casein, 3 g of peptone from soymeal, 2.5 g of D(+)-glucose, 55 g of NaCl, 2.5 g of KH_2PO_4. Final pH should be adjusted to 7.2-7.5. When required, agar is added at a concentration of 15 g/L for solid media.

1.19. YIM10: YIM MEDIUM + 10% NaCl

To prepare 1L of YIM Medium + 10% NaCl, the following components are dissolved in distilled water: 1 g of glucose, 2 g of yeast extract, 0.5 g of tryptone, 1 g of $CaCO_3$, 100 g of NaCl. Final pH should be adjusted to 7.2. When required, agar is added at a concentration of 20 g/L for solid media.