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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 surface-active nature tendency to adsorb at interfaces, while siderophores have the ability to chelate and transport Fe^{3+} 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, 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 2.2 *'Protocols Handbook of screening procedures for extremophilic microorganisms',* provides guidelines on standardization and organization of protocols used for screening of microorganisms producing biosurfactants and siderophores within SECRETed for subsequent cultivation in WP5, subsequent sharing and further analysis in downstream WPs.

Table of contents

1. Aim

The aim of the handbook is

1. to provide guidelines on standardization and organization of protocols which will be used for screening of microorganisms producing biosurfactants and siderophores within SECRETed for subsequent cultivation in WP5, sharing and further analysis in downstream WPs.

The "handbook" is a living document and will be improved during the project. It includes already known methods which the SECRETED partners routinely use but that will be adapted to middle and high-throughput screening for the detection of biosurfactant and siderophore activities. The handbook is a starting point for the SECRETED screening available for all consortium members which will facilitate the selection of promising candidates for the following WPs.
Additional information will be added based on future scientific investigations.

2. Screening methods

Microorganisms can produce a wide range of biosurfactants and siderophores. In order to select the producing strains, the project aims to develop an integrated pipeline which will allow to explore the potential of marine microorganisms owned by different microbial collections.

The screening stage it has been "theoretically" divided in two main parts. The **first part** in which all partners (involved in the screening) defined and applied their own standard protocols and a **second part** where the Consortium partnes started to share their protocols and to introduce the challenges, requirements, problems encountered during the screening stage with the final aim to generate a critical discussion and exchange fruitful recomandations to develop an homogeneous and standardized SECRETED workflow.

In this document, the main protocols implied by the partners (and commonly used) for the screening of biosurfactants and siderophore molecules are generally described. The first stage was longer than expected (around 5 months) as most of the partners never collaborated together or met in person before the starting of the project. As the entire screening procedure will last 24 months future discussion will allow the selection of optimised protocols based on this methods collection. Microorganisms subjected to the screening procedures were initially cultivated based on growth requirements described in details in the D2.1

2.1. Screening methods for biosurfactants detection

CTAB-Methylene blue Agar Method

The CTAB-methylene blue agar [1] is a semi-quantitative assay that can reveal the presence of anionic biosurfactants in the in the cell-free supernatants. The anionic biosurfactants form an insoluble complex with cetyltrimethylammonium bromide (CTAB) and methylene blue, leading to the formation of a dark blue halo in the agar. The test is conducted making wells in the agar with the wide top of a sterile borosilicate Pasteur pipette, that are successively filled with 50 µL of the cell-free supernatant. 50 µL of non-inoculated medium is used as negative control, while 50 µL of 0.01% sodium dodecyl sulphate (SDS) is used as positive control. After 2 days at 4 °C, the samples containing anionic biosurfactants are selected by the presence of a dark blue halo around the wells. The halo diameter is directly proportional to the concentration of the surfactants [2, 3].

Blood agar method

This assay is based on the ability of most biosurfactants to cause lysis of erythrocytes. Wells are made in a sheep blood plate as described above. Then, 50 µL of cell-free supernatants (or extracts) are dispensed in wells and the plate is incubated for 2 days at 25 °C. Positive samples cause lysis of the red cells, exhibiting a colorless, transparent ring around the wells. 50 µL of 0.01% of SDS and 50 µL of non-inoculated medium, are utilized as positive and negative controls, respectively [4].

Emulsification Capacity Assay

Emulsifying activity [5]. A portion of culture is added to an equal volume of kerosene or other test-oil, and the mixture is vigorously vortexed for 2 min. After 1 min of stabilization, the emulsification ability is measured and calculated by dividing the height of the emulsion layer by the total height of the mixture, and multiplying by 100. Tween 20® (0.5% v/v) is used as positive control. E_{24} index can be determined after 24h of incubation at room temperature [6]. Moreover, the E_{24} is calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column.

$E_{24}(\%)=(\text{Emulsified layer height (mm)})/(\text{Total liquid height (mm)})\times 100$

Oil-Spreading Test

The test can reveal the presence of biosurfactants by the displacement of a thin layer of oil in water, leading to the formation of a clear zone into the oil layer [7]. The assay is conducted pouring 25 mL of distilled water in a Petri dish and gently adding 200 µL of (exhaust motor) oil on the water surface, creating a thin oil layer. 10 µL of cell-free supernatant is dispensed on the surface of the thin oil layer, and the diameter of the clear zone is measured immediately. 10 µL of 0.01% of SDS and 10 µL of non-inoculated medium, are utilized s positive and negative controls, respectively.

Drop collapse Method

A single drop of crude oil is set on a glass slide, and left to equilibrate for 24 hours at room temperature [8]. Bacterial cultures, after 48h growth are centrifuged at 12,000 g for 5 min to remove cells. Cell-free supernatants are transferred on the oil surface and drop collapse activity was observed after 1 minute by observing the drop size with the aid of a magnifying glass. A Tween 20 solution and a distilled water are used as positive and negative control, respectively.

2.2. Screening methods for siderophores detection

During the screening stage the partners will apply several methods for siderophores detection. The universal chemical assay for the detection and determination of siderophores developed by Schwyn and Neilands is mainly used as base for further assay optimization [9].

Siderophore detection from culture supernatants by CAS liquid assay

This protocol is used to detect and semiquantify siderophore in culture supernatants by a modified liquid CAS assay (Murakami et al., 2021). In this protocol, bacterial strains are grown in a siderophore-inducing medium. Samples of the culture (1ml) are taken at different times. Cells are eliminated by centrifugation at 12,000 rpm for 3 minutes and supernatants are stored at -20 °C until siderophore analysis. The components of the iron-chelated CAS reactive for liquid CAS assay are in higher concentrations than in the other assays. In this case, the stock solutions ("stock solution Fe III" and "stock CTAB") are prepared as follows:

- "Stock solution Fe III" is prepared with 1ml of $FeCl_3$ (50 mM) and 2 ml of distilled water.
- "Stock CTAB" is prepared with 39,7 mg of CTAB in 4 ml of distilled water.

The "stock solution I", which is the iron-chelated CAS reactive, is prepared dissolving 16.5 mg of CAS (Chrome Azurol S) in the "Stock solution Fe III", adding the total volum of "stock CTAB" and filling with distilled water until 10 ml. To prepare 50 ml of the final liquid CAS solution, 1.5g of PIPES are slowly dissolved in 45 ml of distilled water (using NaOH pellets). Then, 5 ml of "stock solution I" are added to the PIPES solution and the final mixture is filtered before storing it at 4 °C until use.
In order to quantify the siderophore concentration in a supernatant, the following protocol is followed: 100 µl of the samples are added to each well of a 96 well plate, followed by 70 µl of the CAS mixture. After 1 hour of incubation at room temperature, the absorbance is measured at 630nm (complex CAS-iron) and, additionally at 425nm (free CAS). The percent siderophore unit is calculated.

Siderophore detection from growing cells in solid medium by overlaid CAS (O-CAS) assay. (Developed by USE) [10]

This protocol is used to identify siderophore-producing bacteria by a modified CAS

assay which allows to grow halophilic bacteria in their optimum medium (Pérez-Miranda et al., 2007). In this protocol, bacterial strains are grown in their optimum medium at desired salinity, until mid-logarithmic phase is reached. One milliliter of culture samples are then centrifuged and resuspended in 30µl of fresh medium. Aliquots (10-20µl) are carefully spotted on plates with the appropriate media depending on the strain and incubated at its optimum temperature until the spot has grown. For the CAS reactive, a PIPES solution is prepared by slowly adding 3 g of PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)] to 90 ml of distilled water, using NaOH pellets to improve its solution (PIPES is not soluble with pH below 5). Final pH is adjusted to 6.6. The PIPES solution is poured into a flask containing 0.9 g of agarose and then, it is autoclaved. Once the PIPES solution has cooled down a bit and under aseptic conditions, 10 ml of the "stock solution I" are slowly added to the PIPES solution and this final mixture is poored over the solid medium where the bacteria has grown (20-25 ml per plate). CAS acts both as a chelating agent due to its low-iron binding affinity as well as an indicator. When higher-affinity chelating agents are present in the medium, its color turns from blue-green to orange and an orange halo is observed in positive strains.

Siderophore detection from culture supernatants by CAS agar diffusion (CASAD) assay. (Developed by USE)

This protocol is used to detect and semiquantify siderophore in culture supernatants by a modified CAS assay (Shin et al., 2001). In this protocol, bacterial strains are grown in a siderophore-inducing medium. Samples of the culture (1ml) are taken at different times. Cells are eliminated by centrifugation at 12,000 rpm for 3 minutes and supernatants are stored at -20 °C until siderophore analysis. To prepare CASAD plates, a PIPES solution is prepared following the same instructions indicated in 3.2. In this case, once the PIPES solution is prepared and its pH is adjusted at 6.6, it is poured in a flask containing 2 g of agar, and then, it is autoclaved. Once the PIPES solution has cooled down a bit and under aseptic conditions, 10 ml of the "stock solution I" are slowly added to the PIPES solution and this final mixture is poored into 120 mm^2 square Petri dishes (80 ml of the mixture is an appropriate amount for each plate). Once it is solid, 6 mm-diameter holes are made using the wide part of yellow pipette tips (2.0-200 µl). Twenty (4 x 5) holes could be done in each plate. Once the plates are prepared, 100 µl of each supernatant or control medium are added to each hole and the plate is incubated at 37 °C. Once these first volumes have spread, the same amount could be added as many times as desired in case the siderophore concentration is low. The plate is incubated 4 hours at 37ºC and, once this time has passed, the diameter of the orange halos generated could be related with the siderophore concentration, using desferroxamine mesylate (DFO) at different concentrations to represent the squared diameters (mm2) vs. DFO equivalents (µM).

SideroTec-Total AssayTM (general assay) (Developed by the ADL)

The SideroTec AssayTM is a colorimetric test for use in the detection of all types siderophores secreted by bacteria or fungi, or for the assessment of synthetic iron

chelators. The test can be used with liquid culture either directly or following filtration to remove microorganisms. The test may also be used with other aqueous based liquids. The SideroTec AssayTM is a universal assay that will react with all classes of siderophore regardless of chemistry or origin and so can be used for detection of a wider range of iron-binding compounds. The assay is based on the colour change that occurs as result of ferric iron transfer from the reagent complex to siderophore present in a sample. The key reagent is a complex of a dye, iron, and a detergent. The dye complex is initially blue but on removal of iron the dye changes colour to purple or pink colour depending on the amount of siderophore present in a sample. The test can be used as a qualitative indicator for siderophore detection or can be used as quantitative test to estimate the level of siderophore present in a sample. In the absence of any siderophore, the reaction colour remains blue. Samples (100µl) should be run with reagents (100µl), incubated for for 10 minutes at 25°C-37°C). at room temperature and then results could be record by reading visually, photographically, or on a microplate reader at 510 nm. For qualitative interpretation, either visual recording or plate reading can be used. Reaction of the dye complex with any iron-binding compound present will result in both a colour change and a reduction in optical density relative to the zero/blank control. For quantitative interpretation, results should be obtained by reading the microplate on a microplate reader. A standard curve should be prepared by plotting optical density of standards versus siderophore concentration (µg/ml). Alternatively results can be determined by plotting 1/OD vs Concentration to give a linear standard Sample concentration is then determined from the graph using the equation of the trendline plotted. Extrapolation from the reference curve will give an indication of the relative amount of siderophore in the sample. However, quantitative results should be interpreted with a degree of caution; different siderophores may react at a different rate with dye reagent.

SideroTec-Catecholate AssayTM (Developed by the ADL)

The Catecholates assay is a colorimetric test for use in the detection of Catecholate based Siderophores secreted predominantly by bacteria. The test can be used with liquid culture either directly or on cell-free supernatants. The test may also be used with other aqueous based liquids.

The assay allows to obtain both a qualitative and quantitative interpretation, by using visual recording or plate reading. Reaction of catecholate reagent with any catecholate siderophore present will result in an increase in optical density relative to the zero/blank control. Samples (50 ul) should be run with reagents (50 ul), incubated for 5 minutes at room temperature and then results could be recorded by reading visually, photographically, or on a microplate reader at 510 nm.

For qualitative interpretation, either visual recording or plate reading can be used. Reaction of catecholate reagent with any catecholate siderophore present will result in an increase in optical density relative to the zero/blank control. For quantitative interpretation, results should be obtained by reading the microplate on a microplate reader at 510 nM. A standard curve should be prepared by plotting optical density of standards vs concentration. Sample concentration is then determined from the graph using the equation of the trendline plotted.

It is recommended to test also base media or extracts to confirm if there is any interference and adapted as required.

SideroTec-Hydroxamate AssayTM (Developed by the ADL)

The Hydroxamate assay is a colorimetric test for use in the detection of Hydroxamate based Siderophores secreted by Bacteria and Fungi. The test can be used with liquid culture either directly or following filtration to remove microorganism. The test may also be used with other aqueous based liquids. The assay provides both qualitative and quantitative interpretation. Samples (100ul) should be run with reagents (100ul), incubated for 5 minutes at room temperature and then results could be record by reading visually, photographically, or on a microplate reader at 450 nm. For qualitative interpretation, either visual recording or plate reading can be used. Reaction of Hydroxamate reagent with any Hydroxamate siderophore present will result in an increase in optical density relative to the zero/blank control. For quantitative interpretation, results should be obtained by reading the microplate on a microplate reader. A standard curve should be prepared by plotting optical density of standards vs concentration. Sample concentration is then determined from the graph using the equation of the trendline plotted.

It is recommended to test also base media or extracts to confirm if there is any interference and adapted as required.

3. Multiwell screening methods for biosurfactants and siderophore detection

Because of the need to test a high number of samples, microorganisms and growth conditions, SZN set-up a screening system which combine micro-cultivation in deep/multiwell (using different media at same time to induce the production of target metabolites) and CTAB agar assay and liquid-based CAS assay to detect the presence of target molecules secreted in the supernatant of microbial growth.

In particular, selected strains are pre-inoculated in suitable medium (based on D2.1) in a 96 multiwell plate and incubated for 2 days at 20 °C under shaking condition. Then cultures are transferred using a multichannel pipette in 96 deepwell (800 µL final volume) containing different media to induce the production of biosurfactants and siderophores. Secretion of the metabolites of interest is detected by CTAB agar assay (for biosurfactants) and CAS assay (for siderophores). This methodology is easy to be applied and useful as primary screening to test a high number of samples in a relatively short time allowing for the selection of promising candidates. Taking into consideration the enormous variability of partners microbial collections, this approach could easily be adopted by LUND, MATIS and USE or adapted to the most advanced high-throughtput screening platforms, such as robotics.

The primary screening procedure has been preliminarly validated by SZN on already known biosurfactant/siderophore producers and can be summarized in 3 main activities:

Activity 1. Selection of strains which have shown to produce biosurfactants and/or siderophores (positive controls)

Activity 2. Set-up of a micro-cultivation system for metabolite production in 4 steps (see the figure 1 below):

1) Starting cultures: Pre-inoculum of selected producing microorganisms in a 96-multiwell plates and incubation for 2 days;
2) Metabolites production: Transfer 20 µL of microcultures in 96-deepwell plates (Thermo Scientific™ 278743) 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;
3) Cells separation: Centrifuge the deepwell plates and transfer supernatants in new 96 multiwell plates;
4) Detection: use supernatants for different assays (e.g. CTAB and CAS assays)

After 24/48h incubation at 4 °C (incubation time and temperature depend on the selected assay), bioactivity is revealed by observing a colour variation. The CTAB agar method reveals the presence of anionic biosurfactants by the arising of dark blue halos around the sample, while the CAS assay results in a color change from blue to orange in presence of siderophore molecules.

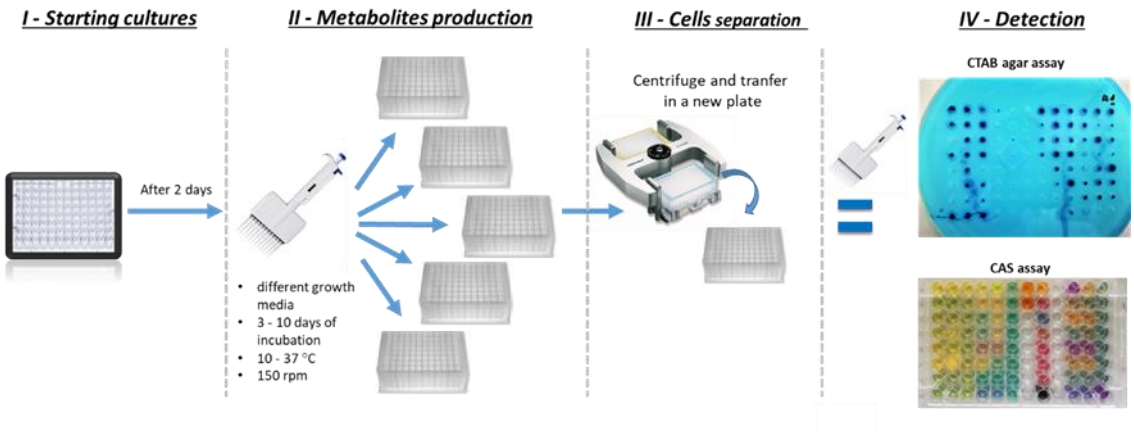

Figure 1: Multi/deepwell screening workflow

Activity 3. Bioactivity validation. Cultures of active strains selected from the primary screening, can be scaled-up to 25-50 mL to collect sufficient amount of supernatants for biosurfactant and siderophore activity assessment, following procedures reported in Par.2.

4. Protocols harmonization strategy

Due to the numerous assays available for the early detection of biosurfactants and siderophores, continuous discussion among the partners is needed, in order to revise and select the best combination of screening tests and approach for the SECRETED project. The consortium has worked (and will contine for all the duration of the screening phase) to assess the feasibility of using common methods. Several issues and limits have been observed already in the first six months of project and will be solved during the following months improving the screening procedure.

In some cases divergent results have beed detected if using solid or liquid based-assay when testing siderophores. Growth media composition, oxygenation, cell density, vehicle employed for extracts solubilisation, may affect the assay outcomes. USE evaluated the influence of high salt concentration suggesting that siderophore production is dependent on salinity, however NaCl concentration does not affect siderophore chelating capacity. These information will be useful for the Consortium during the screening stage and molecule-production phase (WP5) as several partners have marine strains in their collection which can be halophiles. ADL shared its results about solvent interferences with the liquid-based CAS assay, thus allowing to improve sensitivity and reduce false positives and/or ambigous results in the siderophore assays which will be performed by SZN, USE and LUND. Based on ACL and USE experience in siderophores testing and production, they will receive samples (supernatants or extratcs) from the other partners, including PHM. SZN and LUND have experience in the identification of microbial biosurfactants and in previous works have successfully applied and validated the wide range of assays described in Par. 2.1. Their experience will guide the other partnes in the detection of promising new biosurfactans. CTAB agar assay is commonly used and SZN has shown that it can be easily scaled-up to increase the number of tested samples. However, this assay is used to detect mainly anionic biosurfactants, so further assays are needed to identify different classes of biosurfactants. As for siderophores, partners will exchange knowledge and samples. LUND will receive samples from MATIS in order to identify promising candidates. Appliyng the multi/deepwell method (described in Par. 3 and figure 1), SZN will be able to explore multiple growth requirements supporting the other partners in the selection of the best conditions. The figure 2 below provides a general overview about the connection among partners which will be adopted during the project. The preliminary indications obtained during the first six months of SECRETED will provide useful indications for future optimization of the screening procedure.

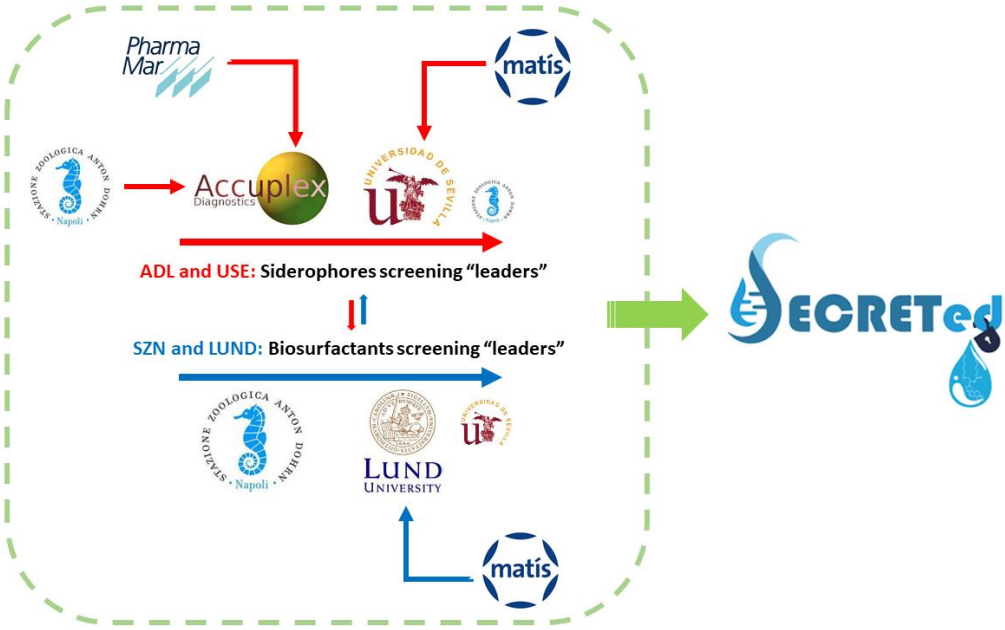

Figure 2: Network diagram showing interactions among SECRETED partners during the screening stage

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