Isolation, cultivation and preservation of microorganisms

Silvia Tabacchioni & Antonella del Fiore
ENEA-SSPT-BIOAG-SOQUAS

November 27, 2023
Training course SUS-MIRRI.it
ENEA CR Casaccia

It has been estimated that the fraction of already cultured bacterial species ranges between 0.1% and 0.001% (Overmann et al., 2017)

Why cultivating microorganisms?

- Pure cultures
- Preserve microbial diversity (Microbial Resource Centers)
- Biomass for biotechnological uses
- Reference strains
- New bacteria for new metabolites, enzymes

- **Culturing microorganisms**

The growth of microorganisms is controlled by a wide range of environmental variables, including pH, temperature, salinity, oxygen, light, osmotic pressure, nutrient availability….

- **Culture media**

Culture media are used to support the artificial growth of microrganisms in laboratory by providing the nutritional needs.

Minimal: precise amounts of highly purified chemicals

Complex: high amount of partially unknown chemical compounds

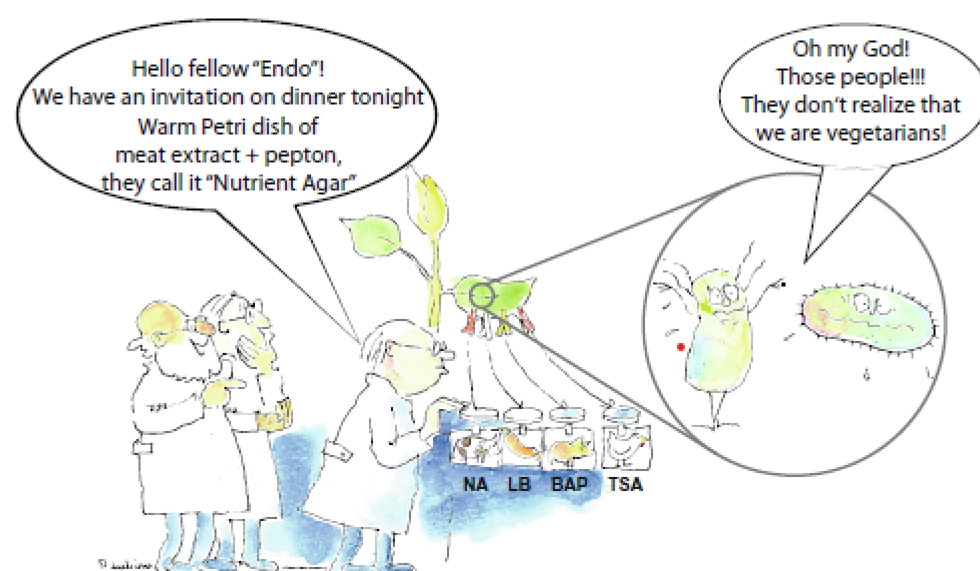
Selective: contains compounds that selectively promote the growth of a specific microorganism

Differential: contains indicator. It is used to distinguish one microorganism from another growing on the same medium

Culture media commonly used to isolate soil microorganisms

1/10 Reasoner's 2A agar (R2A)
1/100 Reasoner's 2A agar (R2A)
1/10 Tryptic soy agar (0.1×TSA)
1/100 Tryptic soy agar (0,01 TSA)
1/10 Nutrient agar (0.1×NA)
1/100 Nutrient agar (0,01 NA)

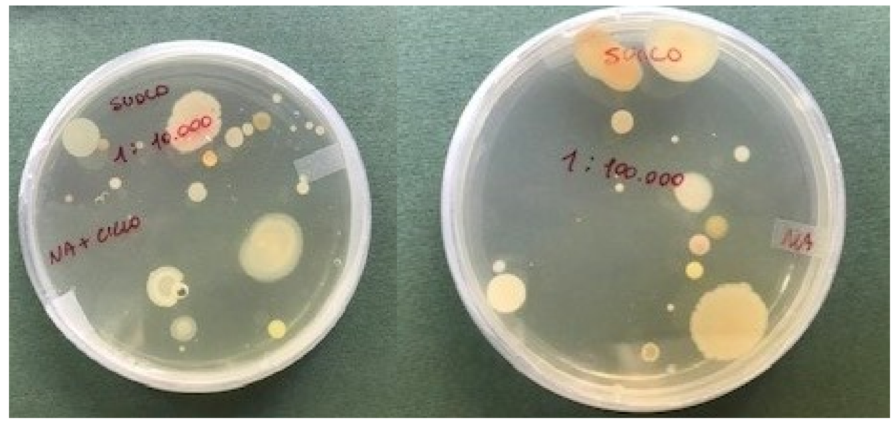
} Heterotrophic and oligotrophic bacteria



From: Saharan *et al*. J Adv Res (2019) | | https://doi.org/10.1016/j.jare.2019.04.002

Isolation of soil bacteria

1 grams of of soil = 10^{10} batteri -10^{3} - 10^{4} different species



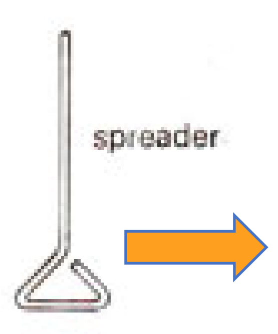
Rhizosphere soil

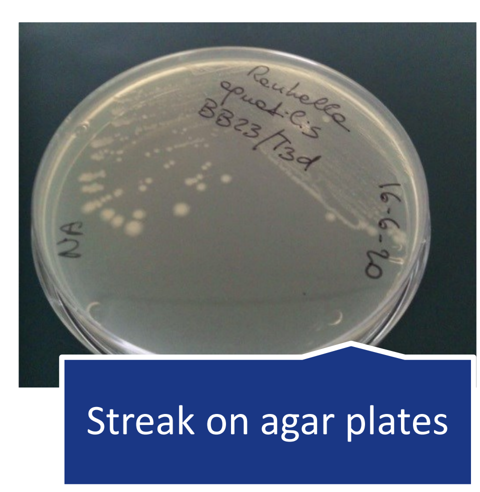
Bulk soil

Soil and rhizosphere samples recovered from sampling site must be processed as soon as possible to have a more reliable picture of the diversity of the microbial community

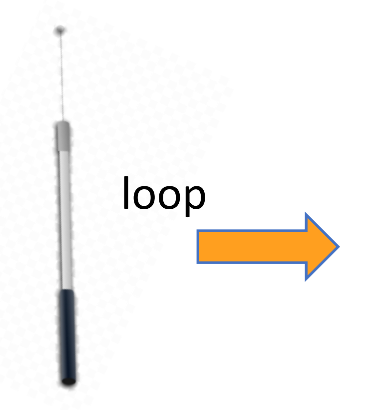
Isolating aerobic bacteria

Spread on the surface of agar plates



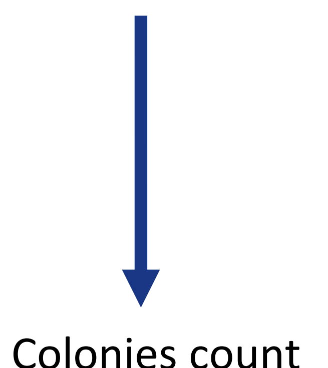


Streak on agar plates

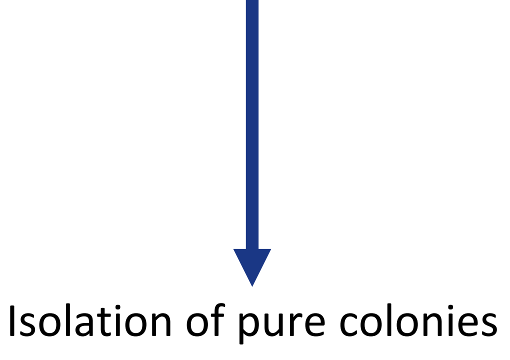


loop

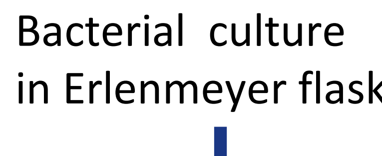
Bacterial culture in Erlenmeyer flask

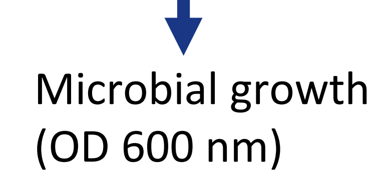


Colonies count



Isolation of pure colonies

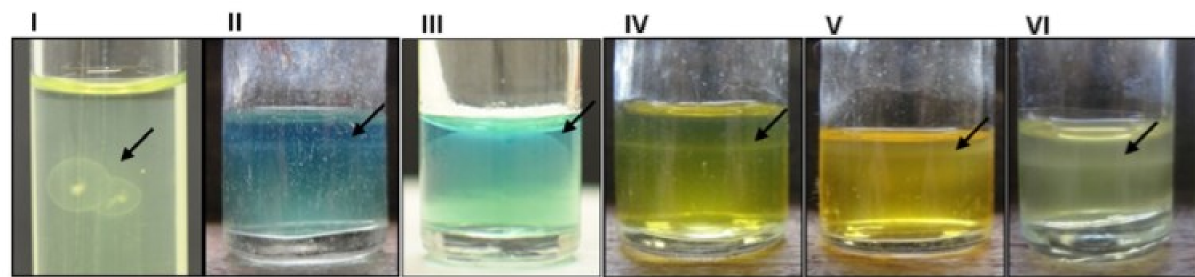




Microbial growth (OD 600 nm)

Isolating microaerophilic bacteria: semisolid media

Isolation of nitrogen-fixing bacteria on semisolid medium



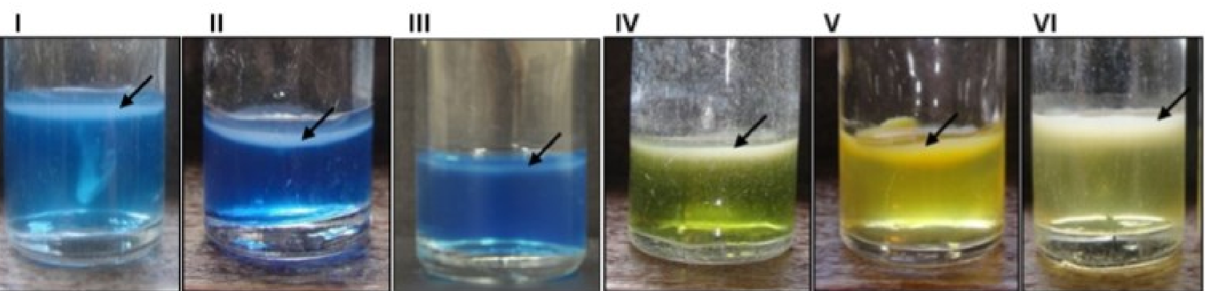
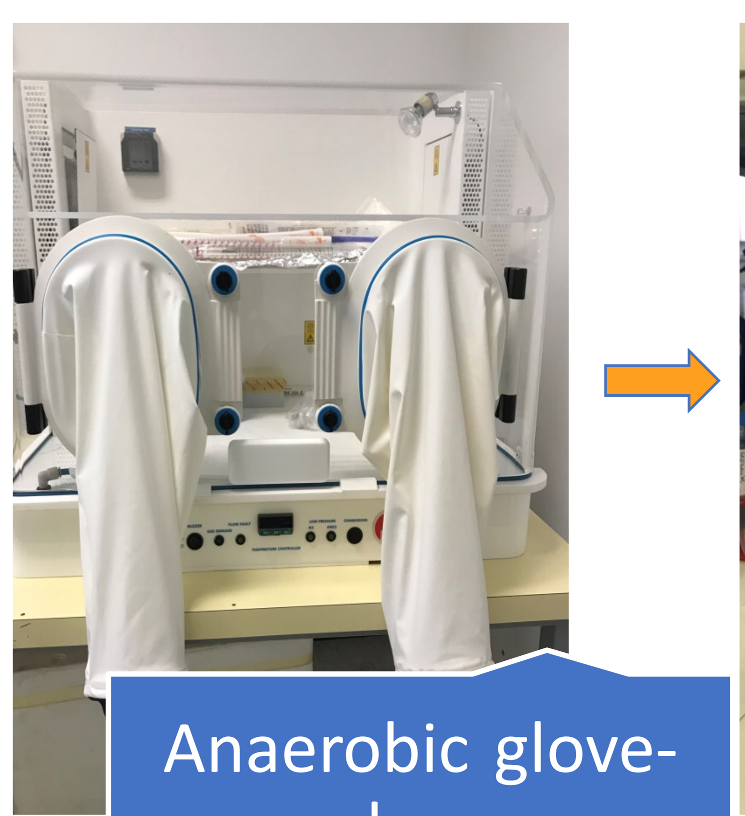


Fig. 3 Inoculation of nitrogen-free semi-solid media and monitoring the pellicle formed in the media. **a.** Veil like pellicle formed 2 days after inoculation. **b.** Surface/subsurface pellicle formed 7 days after inoculation. The black arrows in figures indicate the characteristic pellicle of the diazotrophic bacteria during growth in different semi-solid media. I. *Azospirillum brasilense* in NFb 3x, II. *Herbaspirillum seropedicae* in JNFb, III. *Azoarcus olearius* in NFb 3x, IV *Azospirillum amazonense* in LGI, V. *Gluconacetobacter diazotrophicus* in LGI-P, VI *Burkholderia kururiensis* in JMV

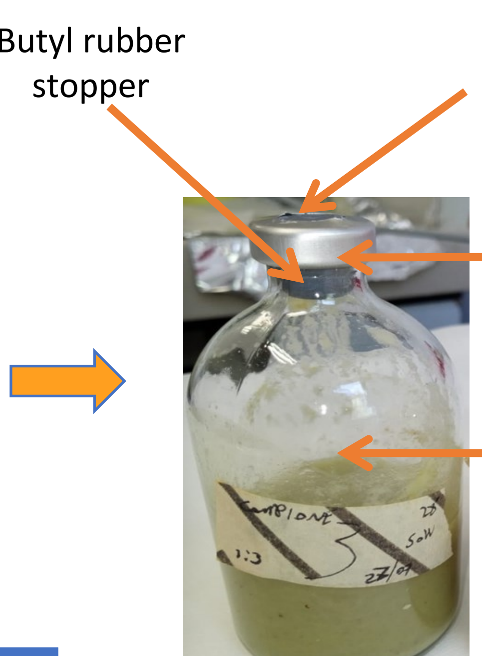
From: Baldani *et al*., (2014) Plant Soil | https://doi.org/ 10.1007/s11104-014-2186-6

Isolating anaerobic bacteria



Anaerobic glove-box

Anaerobic jar



Butyl rubber stopper

Insert syringe needle to flux gas and a needle to mantain the gas equilibrium

Aluminium screw cap with an opening

Headspace sparged with N_2 or gas **mixture**, 80% $\mathbf{N_2}$ and 20% $\mathbf{CO_2}$ to remove oxygen

Traditional and innovative culturing techniques

Soil sample

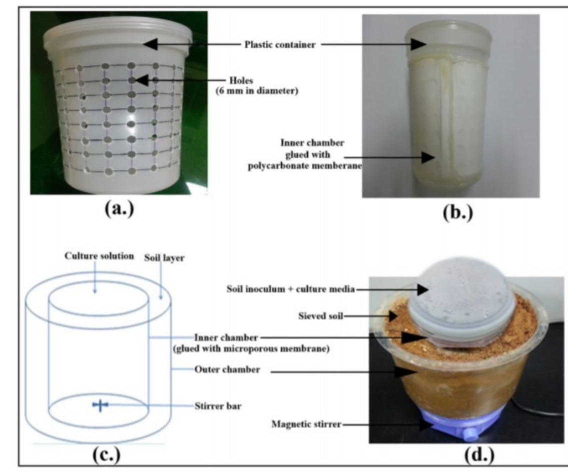


Figure 2. Design of the diffusion bioreactor for the cultivation of previously uncultured bacteria. (**a**) Plastic container perforated throughout with holes; (**b**) polycarbonate membrane glued inner chamber; (**c**) schematic diagram of the diffusion bioreactor; (**d**) overall experimental setup using the newly developed diffusion bioreactor.

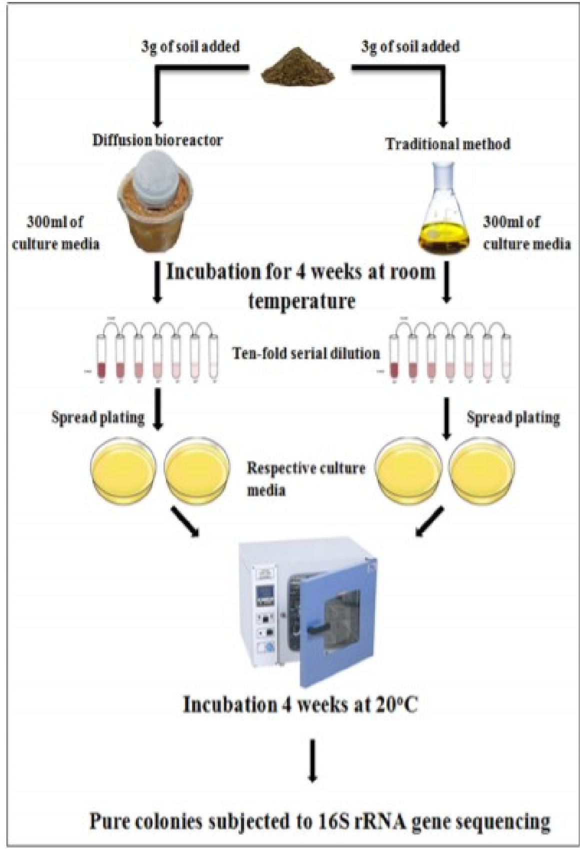
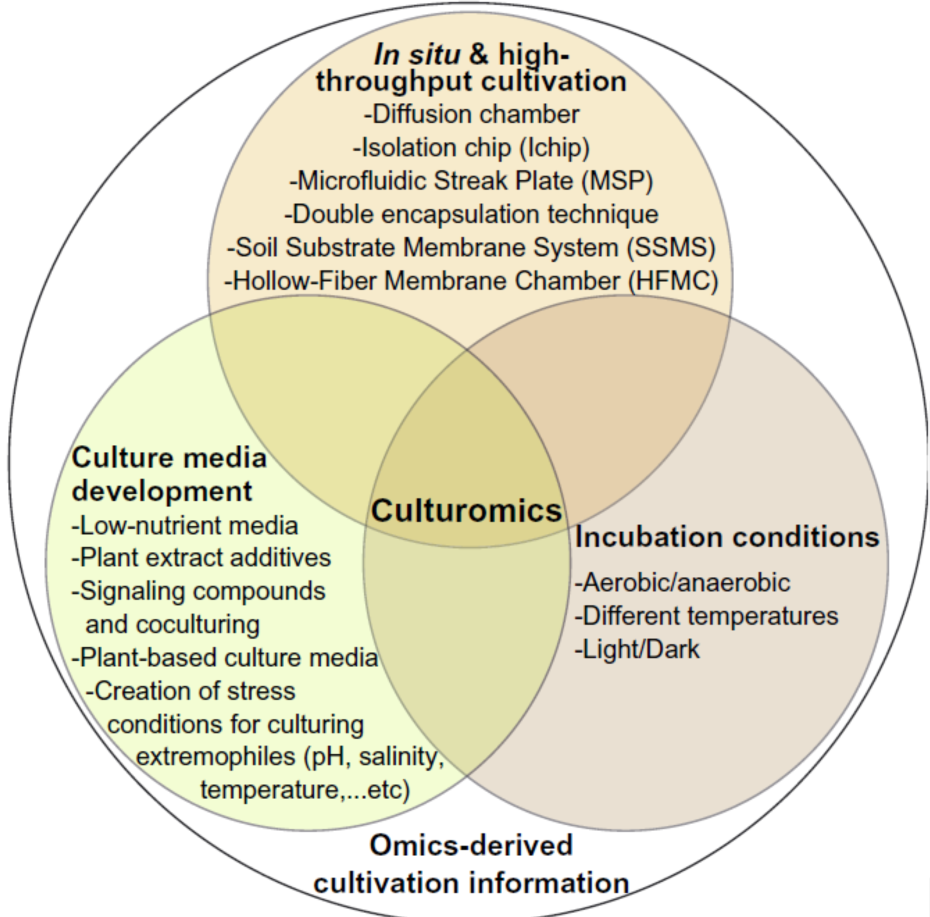


Figure 1. Scheme of the optimized protocol for the cultivation of previously uncultured microorganisms from forest soil.

From : Chaudary *et al*., (2019) Sci.Rep. | https://doi.org/10.1038/s41598-019-43182-x

Strategies to improve culturability of environmental microbiomes



From: Sahran et al., (2019) | https://doi.org/10.1016/j.jare.2019.04.002

Scheme of the culturomics approach

Steps | **Results**

Sample collection → Sample division → **Microbiological cultures**
- Different culture media
- Different supplements eg. metals, antibiotics, C, N sources etc.
- Different conditions (pH, temperature, oxygen sensitivity, salinity, acidity, pressure, etc.)

→ Selection of "clean" isolates (known & unknown species)

Taxonogenomics → MALDI–TOF identification —if unidentified bacteria→ Molecular identification 16S rRNA sequencing

Known and new species

→ Identification of known and new species

Biochemical characterization
Antimicrobial susceptibility profiles
Proteomics
Metabolomics

→ Characterization of known and new species

From: Nowrotek *et al*. (2019) | https://doi.org/ https://doi.org/10.1007/s11783-019-1121-8

ARTICLE OPEN

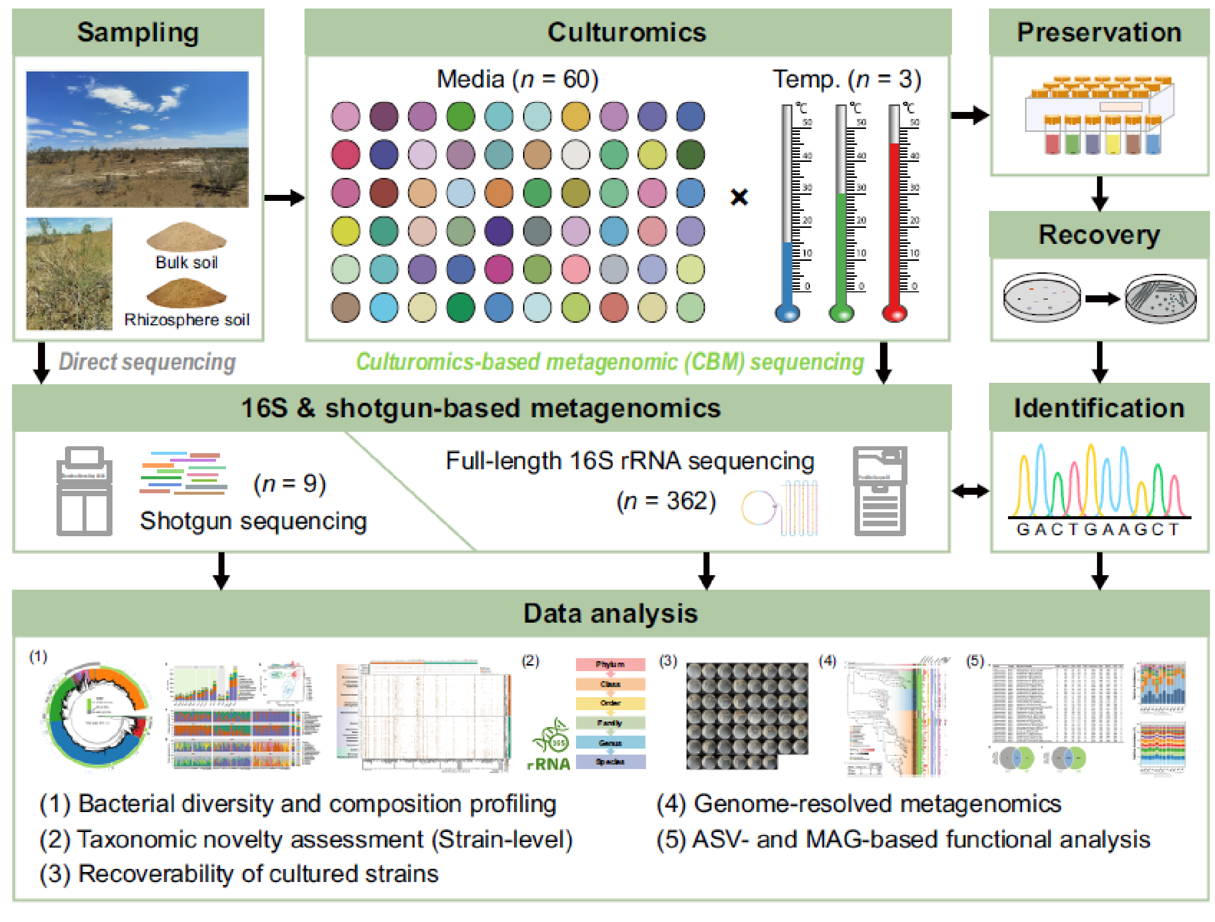
Capturing the microbial dark matter in desert soils using culturomics-based metagenomics and high-resolution analysis

Shuai Li[1,2], Wen-Hui Lian[1], Jia-Rui Han[1], Mukhtiar Ali[1], Zhi-Liang Lin[1], Yong-Hong Liu[3], Li Li[3], Dong-Ya Zhang[4], Xian-Zhi Jiang[4], Wen-Jun Li[1,3] ✉ and Lei Dong[1] ✉

Deserts occupy one-third of the Earth's terrestrial surface and represent a potentially significant reservoir of microbial biodiversity, yet the majority of desert microorganisms remain uncharacterized and are seen as "microbial dark matter". Here, we introduce a multi-omics strategy, culturomics-based metagenomics (CBM) that integrates large-scale cultivation, full-length 16S rRNA gene amplicon, and shotgun metagenomic sequencing. The results showed that CBM captured a significant amount of taxonomic and functional diversity missed in direct sequencing by increasing the recovery of amplicon sequence variants (ASVs) and high/medium-quality metagenome-assembled genomes (MAGs). Importantly, CBM allowed the post hoc recovery of microbes of interest (e.g., novel or specific taxa), even those with extremely low abundance in the culture. Furthermore, strain-level analyses based on CBM and direct sequencing revealed that the desert soils harbored a considerable number of novel bacterial candidates (1941, 51.4%), of which 1095 (from CBM) were culturable. However, CBM would not exactly reflect the relative abundance of true microbial composition and functional pathways in the in situ environment, and its use coupled with direct metagenomic sequencing could provide greater insight into desert microbiomes. Overall, this study exemplifies the CBM strategy with high-resolution is an ideal way to deeply explore the untapped novel bacterial resources in desert soils, and substantially expands our knowledge on the microbial dark matter hidden in the vast expanse of deserts.

npj Biofilms and Microbiomes (2023)9:67 ; https://doi.org/10.1038/s41522-023-00439-8

Culturomic-based metagenomic approach



Li *et al.*, Biofilms and Microbiomes (2023) | https://doi.org/10.1038/s41522-023-00439-8

Preservation of bacteria

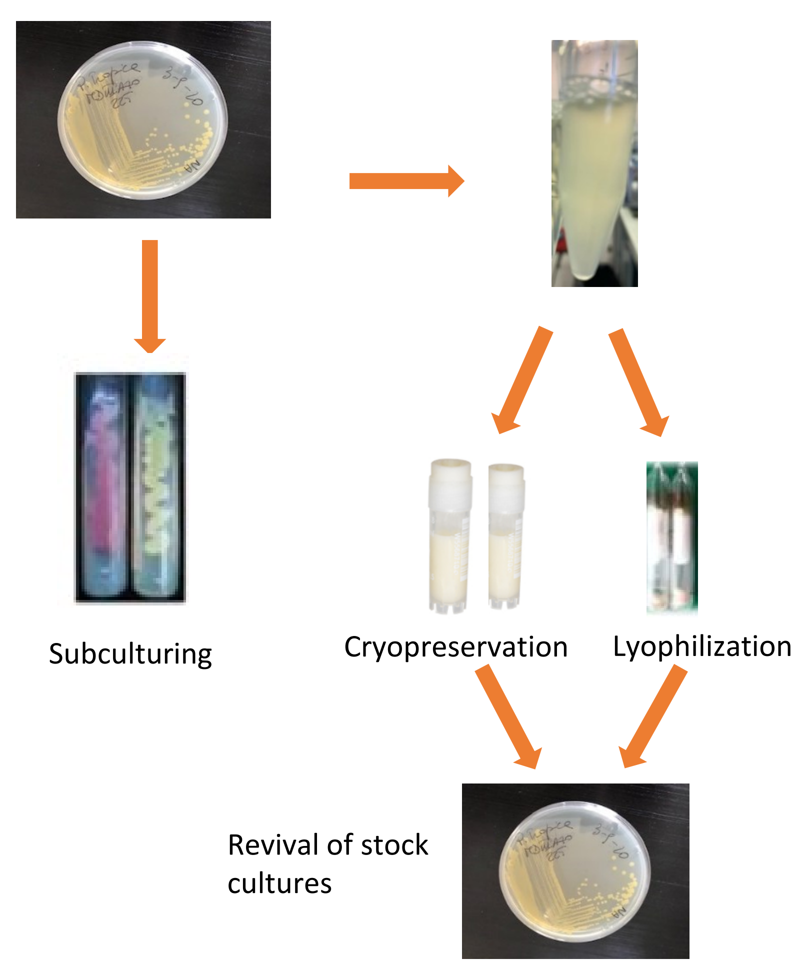
Storage method	Temperature	Storage time	Notes
Short term	Depend from the bacterium (room temperature, +4°C)	Individual property of the strain	Contamination, genetic drift
Medium term	-20°C, -30°C	Up to 1-2 years	Incomplete freezing (damage of cells)
Long term Cryopreservation	-80°C, -150°C	Up to 5-10 years	Expensive
Long term Freeze dry	+4°C, +8°C	Up to several decades	Standard method adopted by culture collections

- *It is good practice to preserve strains that you work with for long term storage as repeated subculturing can leave these cultures vulnerable to contamination and genetic drift*

- *A cryoprotectant must be used for medium and long term storage*

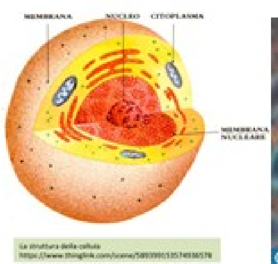
Main steps of bacterial preservation

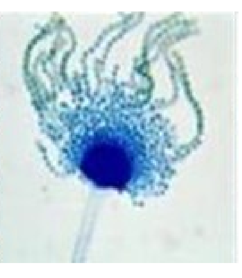
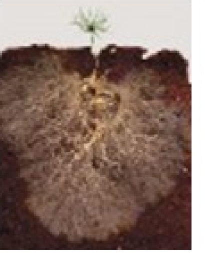
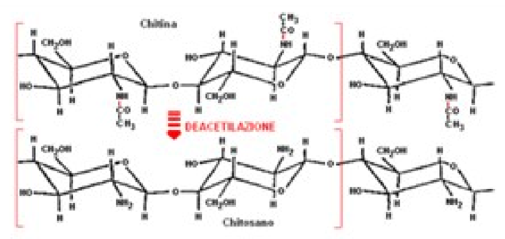
- *Check for the the purity*
- *Do not use a single cell to preserve a bacterial culture*
- *Growth the bacterium at the optimal conditions (T, culture medium, atmosphere)*
- *Cryopreservation or freeze-dry*
- *Revival of the preserved bacterial culture at regular intervals*



FUNGI

- ✓ Eukaryotic, macroscopic or microscopic organisms
- ✓ Single-cell such (yeasts), multicellular microscopic organisms (filamentous molds)
- ✓ Saprotrophs/ Symbionts/ Parasites
- ✓ Asexual or sexual reproduction by spores
- ✓ Cell wall (mainly containing chitin as well as other components including polysaccharides, glucans, proteins and melanins



Ubiquitous in terrestrial and aquatic habitat, universal spread (Maharachchikumbura et al. 2016)

✓ Fungi and agrifood System:

FOOD SPOILAGE (e.g. altherative yeasts/molds

FOOD BOORNE OUTBREAKS (e.g. toxigenic molds)

Aflatoxin B_1

SUSTAINABLE AGRICULTURE (e.g Biostimulants PGPMs Plant Growth-Promoting Microbs)

AGRO- INDUSTRIAL PROCESSES (e.g. pro-technological yeasts and molds)

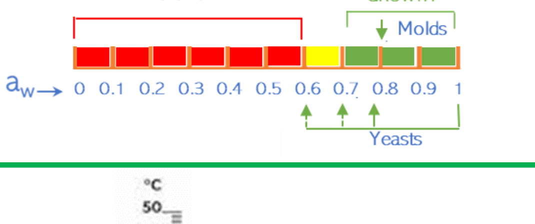
High adaptability to different environmental and host-related factors

Main parameters affecting fungal growth

✓ **Activity/metabolic water (a_w)**

Fungal colonization can take place at $a_w > 0.80$

Xerophilic molds and osmophilic yeasts are viable ≤0.70



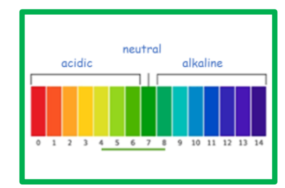
✓ **Temperature**

Growth in the range 15 - 35 °C. There are also some thermophilic, psychrophilic or psychrotolerant fungal species/strains

✓ **pH**

Fungi grow at pH between 4 and 8

Some species are able to colonize their host even in unfavorable acid or alcaline conditions

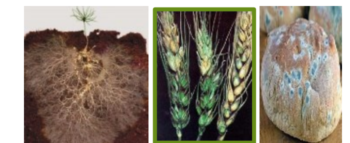


✓ **Oxygen**

Generally obligate aerobes; several species can also grow in microaerophilic or anaerobic conditions

FUNGI ISOLATION, WHY?

- ✓ Fungi are in soil, water and food as (saprophytes/pathogenic/symbiontes) components of mixed fungal-yeast-bacterial) microbial communities

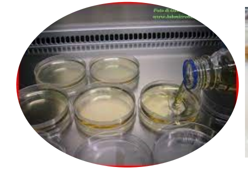


- ✓ The species of interest must therefore be isolated from the microbial community to which it belongs and grown in ***genetically pure culture***

  - Useful for morphological identification
  - To produce fungal biomass for DNA extraction and for molecular characterization/identification
  - For Microbial Culture Collection
  - For industrial applications

CULTURE MEDIA FOR FUNGI

- ✓ Sintetic media ensuring easily nutrients and suitable moisture, **favoring both the growth and sporulation of fungi.** Enrichment media **Potato Dextrose Agar (PDA) (Potato Dextrose Broth- PDB is its liquid form)** is the most used

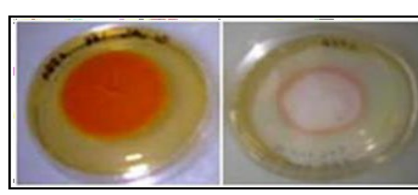


- ✓ **Czapek Dox Agar (CDA) or Czapek Dox Broth (CDB)**, having sodium nitrate as the only source of nitrogen, and sucrose as the only carbon source, is instead **a selective medium,** widely used for saprophytic fungi (particularly soil fungi) able to use inorganic forms of nitrogen

- ✓ Other commercial selective/ differential media (e.g. Aspergillus flavus-parasiticus agar selective medium)...

.

Aspergillus flavus **on PDA growth medium after 7 days of incubation at 27ºC.**



Aspergillus flavus **on AFPA selective/colorimetric growth medium after 7 days of incubation at 27ºC.**

ISOLATION METHODS

Direct plating

✓ **To detect, enumerate and isolate fungi from particle samples (g.e. seeds, cereal…..).**

1. Put particles often after surface disinfection (e.g. 0.4% chlorine solution) essential for example to isolate endophytic fungi, directly on PDA plates containing antibiotic (Streptomycin 300 ppm and Neomycin 150 ppm) to avoid bacterial growth and then incubate in thermostatic chamber at 25 -30 °C in darkness for .7 days.

2. .Results usually expressed as percentage of particles with fungal growth

Direct plating on PDA growth medium of maize kernels
Incubation at 25°C for 7 days.

2. Isolate fungi of interest by subculturing into new media picking up a small agar plug of each mycelium (approx. 5mm diameter), putting it on fresh PDA plates and then incubating at 25 -30 °C in darkness. This operation must be replicated for several generations until to obtain the pure strains of fungal isolate .

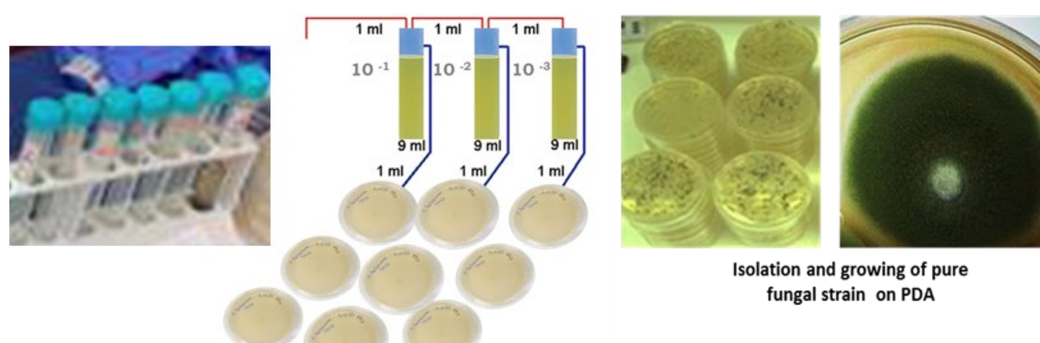
ISOLATION METHODS

Dilution plating

- ✓ For liquid, complex matrices or powdered samples

1. Dilute/resuspended in sterile aqueous solution (e.g. physiological saline solution 0.9% NaCl or 0,01% Triton x- 100) and then blended/homogenized. Prepare Serial dilutions, plate on PDA plates containing antibiotic (Streptomycin 300 ppm and Neomycin 150 ppm) to avoid bacterial growth and then incubate in thermostatic chamber at 25 -30 °C in darkness for 7 days.
2. Pick up colonies of interest and reinoculate on PDA plates until pure colonies are obtained

Tomato Sauce dilution with sterile 0.9% NaCl physiological solution

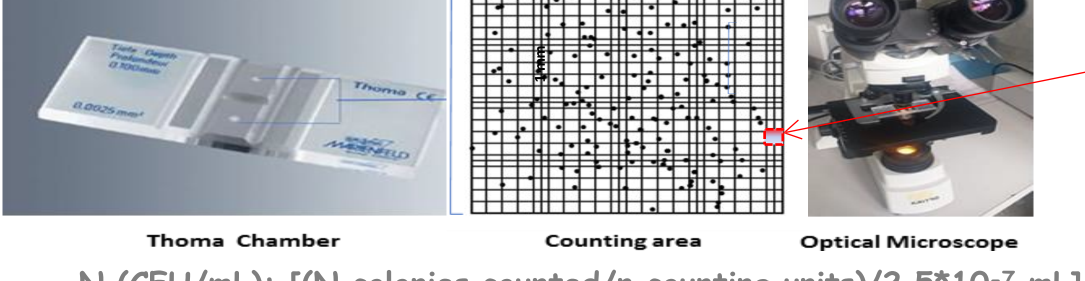


Preparing Serial dilutions, plating on PDA plates and incubation in thermostatic chamber at 25 -30 °C in darkness for 7 days

Isolation and growing of pure fungal strain on PDA

EVALUATION AND QUANTIFICATION OF FUNGAL GROW

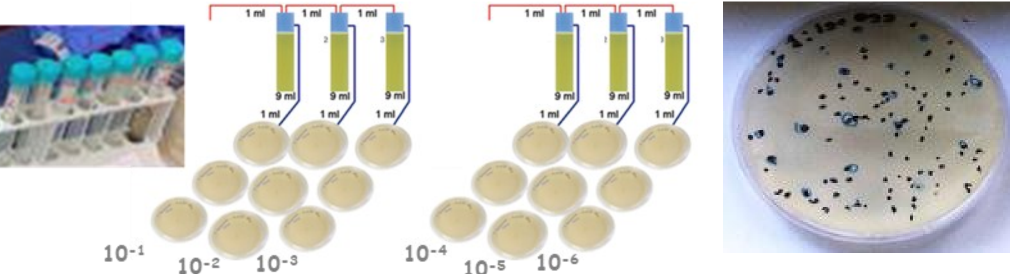
- ✓ **Direct count (CFU/ml)** by **Thoma cell counting chamber** and **optical microscope;**



Counting unit Area: 0.0025 mm^2
Volume: 0.00025 $mm^3 = 2.5*10^{-7}$ mL

N (CFU/mL): [(N colonies counted/n counting units)/$2.5*10^{-7}$ mL]

- ✓ **Colonies count on PDA agar plates (viable fungi) (N CFU/mL)**



Preparing Serial dilutions, plating on PDA plates and incubation in thermostatic chamber at 25 -30 °C for 7 days

PDA PLATE fungal colonies counted

EVALUATION AND QUANTIFICATION OF FUNGAL GROW

FILAMENTOUS FUNGI- MOLDS

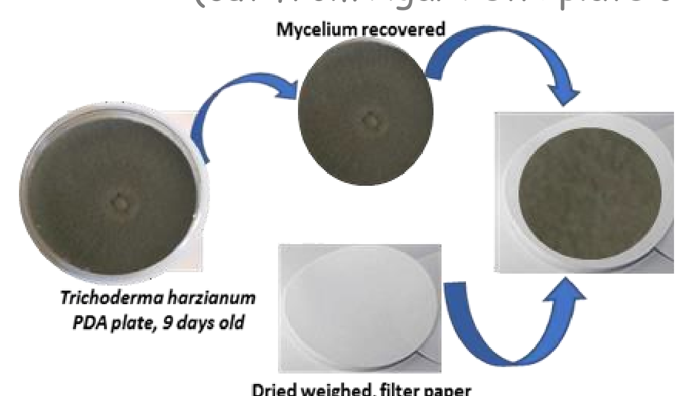
✓ Critical Issues

1. **Fungal Hyphal filaments**: more difficult quantification by traditional microbiological counting techniques
2. **Spores production**: high increasing of viable counts, low correlation with fungal biomass.

EVALUATION AND QUANTIFICATION OF FUNGAL GROW

✓ **Mycelium dry weight (mg)**

(cut from Agar Petri plate or recovered from liquid culture medium)



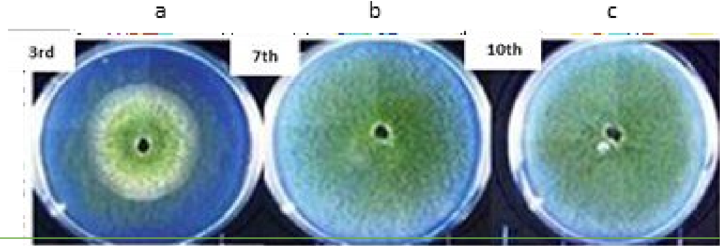
✓ Oven-drying at 80°C for 18 h;

✓ Dry weight mycelium calculated by difference

✓ **Colonies diameter (mm) measurement**

Evaluation and quantification of fungal growth

(linear correlation between colony diameter and growth rate)



PDA Petri plates *Aspergillus flavus 3357*, 3(a), 7(b) and 10(c) days after inoculation. Incubation a 25°C. Plates Diameter 9 mm.

Chemical assays

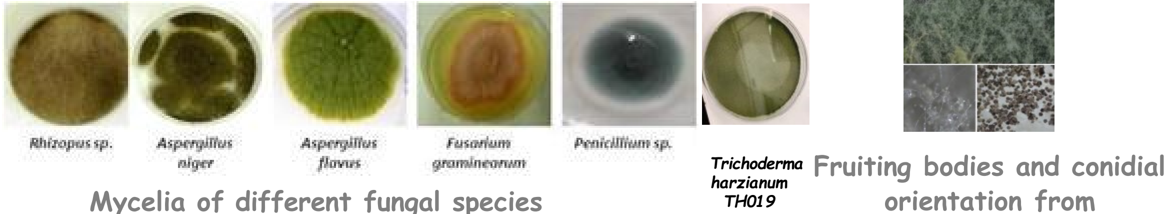
✓ <u>HPLC determination of ERGOSTEROL</u>.

Prevalent/ sterol in most fungi , not significant extent in plants, animals or bacteria. Marker for fungal biomass.

Fungi identification

- ✓ The most common traditional method used for fungi identification is the classification based on phenotypic characters For the identification of the fungal species **macromorphological** and **micromorphological** criteria are evaluated such as color of the mycelia, diameter of mycelia and conidia, production of conidiophores, production of sclerotia, etc,

- ✓ Analysis of macromorphological characters is performed with Stereomicroscope (magnification of 10-100 times)



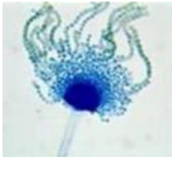
Trichoderma harzianum TH019

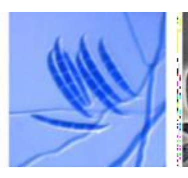
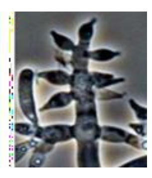
Mycelia of different fungal species

Fruiting bodies and conidial orientation from stereomicroscope

- ✓ **Micromorphological analysis** of sexual and asexual reproductive structures can be performed with Optical Microscope at increasing magnifications (up to 100X) .

Conidiophores



A. Flavus *A. Parasiticus* *A. Carbonariuus* *F. graminearum* *Trichoderma harzianum*

Optical Microscope

Thank you for your attention!

silvia.tabacchioni@enea.it
antonella.delfiore@enea.it