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# Selected Case Studies on Fastidious Eukaryotic Microorganisms: Issues and Investigation Strategies

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**Abstract:** The concept of fastidious microorganisms currently found in scientific literature is mainly related to the difficulty of isolating/culturing/preserving bacteria. Eukaryotes are investigated much less in this respect, although they represent a fundamental part of the microbial world. Furthermore, not only isolation, but also identification and culturing (in the perspective of long-term preservation) should be considered key aspects often impacting on the study of fastidious microorganisms, especially in terms of preservation in culture collections and biotechnological exploitation. The present review aimed to investigate the current state of the art on fastidious eukaryotes, with special emphasis on the efforts to improve their isolation, identification, culturing and long-term preservation in culture collections practices. A few case studies focused on some fastidious eukaryotic microorganisms (including possible customized solutions to overcome specific issues) are also presented: isolation and preservation of slow-growing fungi, culturing of *Haematococcus lacustris*, isolation of unialgal strains of Cyanidiophytina (Rhodophyta), identification of *Metschnikowia pulcherrima* clade yeasts, isolation and preservation of *Pyricularia* species, preservation of *Halophytophtora* spp.

**Keywords:** slow-growing fungi; *Haematococcus*; *Cyanidium*; *Metschnikowia*; *Pyricularia*; *Halophytophtora*

## 1. Introduction

### 1.1. General Concept of Fastidious Microorganisms

Although an unequivocal definition of fastidious microorganisms is challenging to find in the literature, the conventional concept is often associated with the general difficulty of some microorganisms to be cultured under standard laboratory conditions [1]. Studies published to date reported, with great accuracy, the different meanings of the word "fastidious" in the microbial world. The number of articles (papers, book chapters and conference

proceedings) indexed in Google Scholar (June 2023, using "with the exact phrase" in Google Scholar advanced search mask excluding "including citations") and that exhibit specific contiguous couple of words is reported in Table 1. The word "fastidious" is largely associated with the word "bacteria" rather than the more generic one "microorganism" either in the title, keywords or text of the papers. Only 139 papers showed the presence of the couple of words "fastidious fungi" and only 12 showed "fastidious algae". Unexpectedly, the combination "fastidious eukaryotes" was not present in publications indexed in Google Scholar. These results showed how the association of the word "fastidious" with "eukaryotes" and, in particular, "fungi" and "algae" is neglected in comparison to the association with "bacteria". Using the combinations of "fastidious bacteria" + "pathogen" (present in the text but not necessarily next to "fastidious bacteria") or "fastidious bacteria" + "medium" (present in the text but not necessarily next to "fastidious bacteria"), a slightly lower number of publications were found. The combination "fastidious bacteria" + "environment" is less occurring, which demonstrates how the clinical and culture issues related to fastidious bacteria have been much more studied to date than environmental ones. Moreover, the very low number of publications showing the simultaneous presence of the words "fastidious bacteria" + "culture collections" demonstrates the need to implement the studies on the difficulties encountered by collections to successfully approach the preservation of fastidious microorganisms in their ordinary practices.

**Table 1.** Number of scientific papers indexed in Google Scholar that exhibit specific words either in the title, keywords or text.

| | | **Words Present Anywhere in the Articles** | | | |
|---|---|---|---|---|---|
| | | **+Pathogen** | **+Medium** | **+Environment** | **+Culture Collection** |
| Fastidious microorganisms | 3500 | 1600 | 2900 | 2120 | 390 |
| Fastidious bacteria | 10,100 | 7550 | 7610 | 6280 | 857 |
| Fastidious fungi | 139 | 113 | 132 | 128 | 31 |
| Fastidious algae | 12 | / | 1 | 1 | / |
| Fastidious eukaryotes | / | / | / | / | / |

Isolation and culturing are basic techniques of conventional microbiology. They are essential steps for the preservation of microbial diversity under viable form. Additionally, the correct identification of microbial strains at the species level is an essential condition for all microbiological studies. According to Kock's postulates, both steps are crucial prerequisites in clinical applications (where successful treatment of infectious diseases needs the correct species assignment of each pathogenic organism) and in testing the efficacy of different drugs for defining the correct antimicrobial therapy [2–4]. Due to the fundamental importance of the diagnosis of infections through culturing of microorganisms, new (or optimized) culture media have recently been developed to increase the chances of success in the culturability of fastidious pathogens [1].

From a biotechnological point of view, the correct identification of microbial strains is required for their patent coverage or for defining their biosafety level, which is a prerequisite for their use in food and feed chains (e.g., see guidelines of the European Food and Safety Authority [5].

Finally, the correct identification of strains preserved inside culture collection should be the prerequisite for offering certified biological material to third parties.

Long-term preservation of microbial diversity maintaining high vitality is also a critical point. As the current techniques for preserving microbial biodiversity do not ensure the perpetuation of all sorts of microorganisms (including microbiomes), the risk of losing a more or less consistent part of ecologically and industrially relevant components of microbial community appears to be very high [6].

The erroneous idea that the issue related to fastidious microorganisms is a problem almost exclusively restricted to the difficulty of culturing bacteria (especially pathogenic species) under standard laboratory conditions may underestimate the true extent of the phenomenon.

Within this framework, the purpose of this review was to report on the state-of-the-art fastidious eukaryotes, with special emphasis on the efforts to improve their isolation, identification, culturing and long-term preservation in culture collections practices. Several case studies focused on different fastidious eukaryotic microorganisms describing possible customized solutions to overcome specific issues will also be presented.

### *1.2. General Concepts on Fastidious Bacteria*

According to the general description reported above, the word "fastidious" is typically related to some bacteria, whose complex species-dependent nutritional and physiological requirements (e.g., the presence of definite nutrients and/or buffering agents, as well as the need to grow them under specific ranges of temperature, pH, $O_2/CO_2$ concentration, osmotic conditions, pressure, etc.) make their growth difficult using standard laboratory conditions [7,8]. Some ecological limitations, e.g., the involvement in metabolic networks, as well as the failure to interact with other (more versatile) microbial species, apparently indicate that some fastidious bacteria may have adapted their life to extreme habitats characterized by a low degree of biological diversity [7,8].

From an ecological point of view, fastidious bacteria should not be considered "weak" organisms, because they sometimes exhibit an efficient growth within their specific niches, characterized by peculiar conditions of temperature, acidity/alkalinity, oligotrophy/eutrophy and, sometimes, deficit of biological competitors [5]. The pathogenic bacterial species *Treponema pallidum*, which causes syphilis in humans, is challenging to be cultured under standard laboratory conditions, essentially because it probably needs to find specific conditions simulating its own natural habitat [7,8].

Most of the bacteria defined as "fastidious" are pathogens responsible for serious infections. Therefore, early detection and monitoring are important steps for increasing the chances of their easy eradication through the definition of correct pharmacological therapies [2–5].

### *1.3. General Concepts on Fastidious Eukaryotic Microorganisms*

A number of fastidious organisms can also be found in the eukaryotic domain. Although a few protozoa are considered very fastidious in their growth requirements [9], most studies on fastidious eukaryotes have been focused on pathogenic fungi of clinical or phytopathological relevance, especially in relation to the efforts made by scientists worldwide to improve their isolation, culturing (e.g., the development of new culture media for both research and diagnosis), long-term cryopreservation and post-cryopreservation recovery [10–17].

Culturing of some fastidious fungi requires the supplementation of culture media with definite ingredients stimulating cell growth [18,19], e.g., the addition of oleic acid to conventional culture media significantly improves their efficiency in isolating the lipid-assimilating yeast *Malassezia* spp. [10]. As reported above, the difficult isolation of some fungal species from clinical and plant specimens even represents an obstacle capable of confuting Kock's postulates. Ali et al. [20] bypassed this problem by suggesting the concept of sequence-based identification of fastidious fungal pathogens through the determination of the transcriptome sequence of both symptomatic and asymptomatic hosts [21]. Furthermore, while the development of culture-independent molecular techniques (i.e., next generation sequencing) have simplified since early 2000s, the detection of fastidious (or even not-yet-cultured) fungal species [22–24], their cultivation and preservation still remains in most cases an unsolved problem.

## 2. Case Studies

As reported above, overcoming the problems given by fastidious eukaryotic microorganisms is a matter that cannot be addressed in a generalized way, because each taxon often needs customized solutions. This is, of course, determined by the general variability existing in the microbial world, not only at the domain/kingdom level but also among different genera/species, or even at the intraspecific level due to the high degree of phenotypic variability frequently found within taxa characterized by a high level of cosmopolitanism.

Long-term cryopreservation of fastidious eukaryotic microorganisms frequently failed [25,26]. Although a few studies [11,17] suggested that some modifications of standard protocols can improve the efficiency of post-cryopreservation recovery of some fungal species, a few authors [12,13] underlined the extreme difficulty in establishing a unique protocol allowing the optimal recovery for all fastidious fungi. The same authors also suggested that culture collections should establish custom-made suitable protocols to enhance the efficiency of long-term cryopreservation of their own fastidious strains. Gleason et al. [13] reported that many fastidious eukaryotic microorganisms failing to survive (or poorly surviving) to long-term cryopreservation also exhibit a common limited tolerance to deviations from their preferred growth conditions. Consequently, the knowledge of such tolerance mechanisms could increase their chances of post-cryopreservation recovery [13].

In the following paragraphs, some case studies focusing on the difficulties encountered in the isolation, culture and long-term preservation (including recovery) of some fastidious eukaryotic microorganisms, as well as the description of taxon-specific solutions to mitigate (or even overcome) some specific problems, are reported.

### *2.1. Case-Study 1: Isolation and Long-Term Preservation of Slow-Growing Fungi from Extreme Environments*

Slow fungal growth is often associated with the ability to survive under extreme conditions (high energy is required for adaptations). This ability is not always a mere consequence of the limitations imposed by extreme environments (e.g., both generalist or specialist fungi can exhibit the ability to slow their metabolic rates under unfavorable conditions), but may frequently be a true adaptive response (e.g., highly specialized guilds are often unable to reverse their growth rate upon restoration of the former physical–chemical conditions) [27,28]. Typical fastidious fungi exhibiting slow growth as a permanent feature (e.g., some obligate halophilic species, namely *Wallemia ichthyophaga* (Wallemiomycetes, Wallemiales,) or *Hortaea werneckii* (Dothideomycetes, Capnodiales), psychrophilic yeast-like species as *Aureobasidium subglaciale* (Dothideomycetes, Dothideales) or psychro-xerophilic rock-inhabiting Antarctic species as *Cryomyces antarcticus* (Dothideomycetes, incertae sedis) and *Friedmanniomyces endolithicus* (Dothideomycetes, Capnodiales) are specialist species which acquired the ability to express stress responses [28–31].

Inconveniences encountered in studying slow-growing fungi involve isolation, culturing and long-term preservation. Isolation may be difficult if specific procedures are not meticulously followed because slow-growing hyphae are easily outcompeted and overgrown by fast-growing (and more competitive) fungi, which are very difficult to eradicate, albeit still possible. A successful isolation of slow-growing fungi from environmental samples requires reproduction at the laboratory scale of a lot of physical-chemical stresses stimulating the development of a specific guild, as well as to prevent the growth of fast-growing fungi. The most recommended procedures basically imply:

(i) Repeated washings (when possible) of the environmental samples with sterile water added with 0.1 Tween 20 to remove potential contaminants [32];
(ii) Inoculation of a small portion (or dilution) of environmental samples on properly "poisoned" media (e.g., Rose–Bengal agar medium) to significantly deplete the growth of fast-growing fungi [33];
(iii) Incubation for a long time (even for months) through reproduction of the stress to which slow-growing fungi are adapted (e.g., low temperatures, i.e., 4–15 °C, for the isolation of psychrophilic fungi);

(iv) Continuous inspection of cultures to remove possible contaminants and even to transfer slow-growing colonies onto new media.

Some species (i.e., *Antarctolichenia onofrii* (Arthoniomycetes, Lichenostigmatales), *Leucosporidium creatinivorum* (Microbotryomycetes, Leucosporidiales)) characterized by peculiar nutritional requirements or by a close dependence from the metabolism of other microorganisms [34] may rapidly lose their viability if maintained in metabolically active forms. Therefore, customized long-term preservation protocols should be applied. *A. onofrii* can be successfully cryopreserved in 20% glycerol at −150 °C. Since highly costly processes are avoided under demanding conditions, these fungi basically reproduce asexually with minimal development of micronematous conidiophores in Ascomycota, which are often not observed at all. Sexual reproduction is instead often lost in some extremophilic fungi [35,36]. Therefore, the best option for long-term maintenance of slow-growing fungi is their cryopreservation at −80 °C or −150 °C.

### *2.2. Case-Study 2: Culturing of Haematococcus lacustris (Girod-Chantrans) Rostafinski*

*Haematococcus lacustris* (Girod-Chantrans) Rostafinski (Chlorophycae, Volvocales), is a unicellular freshwater microalga belonging to the class Chlorophyceae. In some publications, *H. lacustris* was called also *H. pluvialis* [37]. Its life cycle includes two phases that depends on the physiology of the microalga:

(i) Motile phase;
(ii) Non motile phase.

Some unfavorable environmental conditions can lead to oxidative stress and to intensive light or nutrient deficiency and the formation of spores called haematocysts. This can determine an increased production of astaxanthin, which is accumulated during metabolic decline and transition towards haematocysts [38]. *H. lacustris* could be defined as a fastidious microalgal species due to its peculiar physiological and nutritional requirement, which make its culturing challenging under standard laboratory conditions. The fastidious growth of *H. lacustris* makes this species a good source of astaxanthin, but the difficulty lies in controlling the motile and non-motile growth phases and experimenting with the best unfavorable growth conditions for astaxanthin accumulation. As is well known, two-stage growth strategies are required to allow the growth of *H. lacustris* cells, which, under unfavorable conditions in the form of zoospores/aplanospores, increase astaxanthin content. However, one of the unresolved issues is high mortality of cells once grown under unfavorable conditions and astaxanthin accumulation [39]. *H. lacustris* is cultured on BG-11 (Blue-Green) medium containing a complex mixture of macro- and micronutrients [40,41]. The original composition of BG-11 medium included $NaNO_3$ (1.5 g/L), $K_2HPO_4$ (0.04 g/L), $MgSO_4 \cdot 7H_2O$ (0.075 g/L), $CaCl_2 \cdot 2H_2O$ (0.036 g/L), citric acid (0.006 g/L), ferric ammonium citrate (0.006 g/L), EDTA disodium magnesium salt (0.001 g/L), $Na_2CO_3$ (0.02 g/L) and micronutrient solution.

Under normal growth conditions, cells are in motile phase. They are characterized by a high motility due to isocont flagella. The motile cell can divide by mitosis during asexual reproduction to generate sporangium that can contain a maximum of eight zoospores/aplonospore within the cell wall [42].

When grown under adverse conditions, motile cells lose the two flagella and they transform into a non-motile cell whose size can be variable and which is initially green in color with a low secondary carotenoid content. The non-motile cells are able to reproduce by asexual division producing up to 20–32 aplanospores within each sporangium. When the condition is favorable, the aplonospore can become motile cells. On the other hand, under stressing conditions (e.g., an excessive light intensity and/or salinity, oligotrophy, excessive oxidative stress), the zoospores begin to thicken the cell wall until the formation of an ultra-resistant coating (composed by a trilaminar sheath and by a secondary wall, resistant to acetolysis), which allows them to survive under extreme chemical–physical conditions. The haematocysts (i.e., aplanospores) begins to accumulate large amounts of secondary carotenoids (under the form of esters of fatty acids) instead of the photosynthetic

apparatus shrinking, allowing the cell protection against damages caused by irradiation and oxidative stress [40,43,44].

Recent studies reported the culturing of *H. lacustris* in the BG-11 medium at 28 °C, pH 7.5–8.5 C, using the white light LED (4000 lux intensity, 100 mol/photons/$m^2$/s) with an air flow rate of 300 mL/min by using Packed Bed Reactors (PBRs) characterized by a volume/surface ratio of 56.5 L/$m^2$. Under such operative conditions, the motile phase was well-defined and the growth appeared consistent with several research studies [45]. Two different blue LED lights intensities (low and high: 55 µmol/s/$m^2$ = 500 lux and 280 µmol/s/$m^2$ = 2500 lux, respectively) were used to evaluate the effect of oxidative stress on *H. lacustris* cells. The effect of both high and low intensity of blue LED light in the terms of carotenoids and fatty acids content, including the switch to haematocysts form, is reported in Figure 1 [46,47].

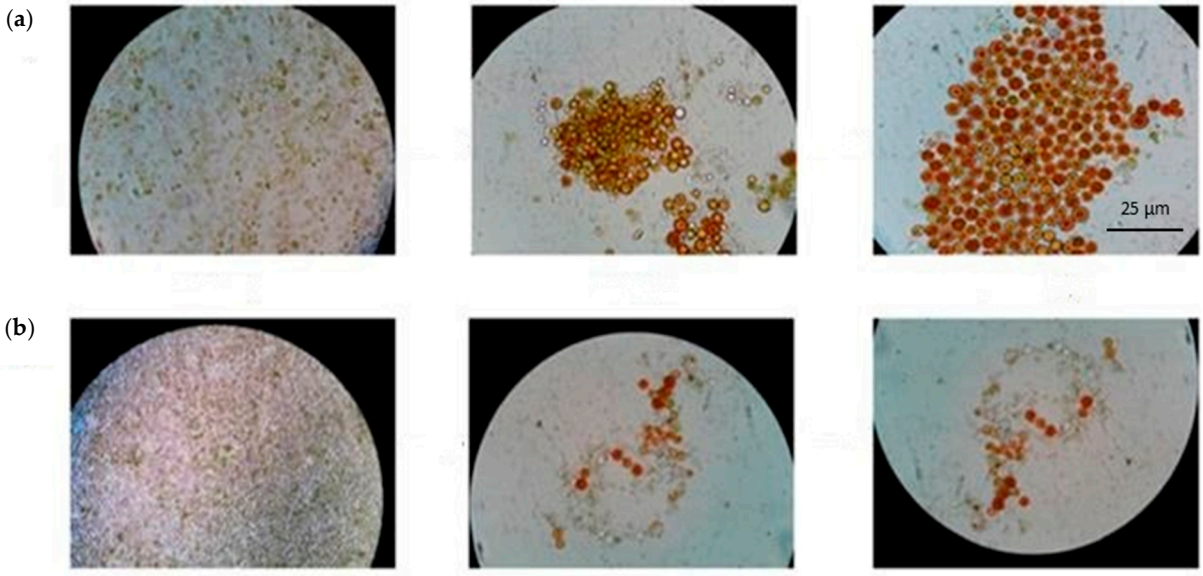


**Figure 1.** Microscope observation (400×) of morphological changes during the growth of the *H. lacustris* red phase: (**a**) a first switch from motile to non-motile phase at 2500 lux (280 mol/photons/s/$m^2$) of blue light; (**b**) a second switch from motile to non-motile phase at 500 lux (55 mol/photons/s/$m^2$) of blue light [40].

### *2.3. Case-Study 3: Isolation of Unialgal Strains from Mixed Populations of Cyanidiophytina (Rhodophyta)*

"If one has spent much time culturing and isolating blue-green algae, turning to *Cyanidium* is a refreshing experience. Here is an alga that lives in nature as a unialgal culture and that develops rapidly in enrichment cultures [48]". This idyllic statement seems to be far from the definition of "fastidious" that characterizes all the eukaryotic microorganisms included in this review. The landmarking studies of Brock and coworkers opened an avenue of research on extremophilic algae belonging to the genus *Cyanidium*, but after several years, it became clear that, under the appearance of a unialgal culture, the majority of field "*Cyanidium*" populations hide an unexpected diversity. At present, four different genera of unicellular Cyanidiophytina (Rhodophyta) have been described: *Cyanidium*, *Cyanidioschyzon*, *Cyanidiococcum* and *Galdieria* [49]. The species *Cyanidium caldarium*, *Galdieria sulphuraria* (both round-shaped, with a thick cell wall) and *Cyanidioschyzon merolae* (club-shaped, without cell wall) often live together, forming mats all along hydrothermal springs worldwide characterized by temperatures and pH values ranging from 30 to 50 °C and from 0.8 to 2.0, respectively [50].

Cyanidiophytina samples collected in the field have been cultured under laboratory conditions using a restricted number of culture media, all based on the inorganic liquid medium acidified with $H_2S0_4$ to pH 1.5–2.0 [51]. Other media elaborated afterward differed

from the above media in several aspects, namely the concentration and the formulation of some macroelements, especially N and Fe, and the presence of several microelements, such as V, Co and Cu [51–55].

The simultaneous presence of two species in the "*Cyanidium*" field population was described for the first time by De Luca and Taddei [56]. They provisionally identified them as *C. caldarium* form A and *C. caldarium* form B, which could apparently be isolated thanks to their relevant ecophysiological differences: *C. caldarium* form A exhibited an obligate autotrophic aptitude, whereas *C. caldarium* form B can also switch its metabolism to a heterotrophic ability and showed a pronounced bleaching when grown in the dark. Accordingly, the isolation of form A (the "true" *C. caldarium*) from B (subsequently named *G. sulphuraria*) [57] appeared simple and effective. Unfortunately, despite its obligate autotrophic aptitude, form A can survive for prolonged periods in the dark.

To make this frame even more complicated, De Luca et al. [58] reported the ability of the new species *Cyanidioschyzon merolae* to grow intermingled with both forms (A and B) in samples collected in European, American and Asian hot springs. The possibility to isolate pure cultures of each species from the above mixed populations has been struggled with using the traditional method of serial dilutions [59]. Anyway, although most pure cultures have been obtained in this way, recent developments of metagenomic techniques revealed that many of these isolates still contain traces of the other Cyanidiales species. Del Mondo [60] proposed a cytofluorimetric and sorting analysis to discriminate among these species and designed novel species-specific primers to confirm the identity of the sorted strains by polymerase chain reaction. Compared to the traditional time-consuming isolating techniques, this approach resulted relatively fast, but required facilities (both instruments and skilled personnel) that could not be available in any laboratory. Therefore, strains deposited in different culture collections should be checked using this approach to obtain pure Cyanidiales isolates. The case of the genus *Cyanidium* (and related genera) is probably not unpreceded. Algal collections, therefore, represent an untapped source of hidden diversity that deserve more attention and efforts to be fully explored.

### *2.4. Case-Study 4: Identification of Yeasts of the Metschnikowia pulcherrima Clade*

*Metschnikowia* is an ascomycetous yeast genus including either pathogenic or biotech-related species [61–63]. Among them, *Metschnikowia pulcherrima* is certainly the most exploited species due to its eclectic biotechnological potential [64,65]. Taxonomically speaking, the family Metschnikowiaceae belongs to order Serinales, while class is either uncertain in the subphylum Saccharomycotina (e.g., in Mycobank, taxon # 3147) or, more recently, it has been included into class Pichiomycetes on the basis of phylogenomic analysis [66] following the phylogenomic approach proposed for fungi [67]. In addition to the diverse class-level placements over time, the assignment at the species level is also challenging, and directly impacts the reliability of biological material provided by culture collection. Indeed, the scarcity of typical morphological and physiological features and the limited applicability of the standard molecular markers for species belonging to the *M. pulcherrima* clade (i.e., *M. andauensis*, *M. citriensis*, *M. fructicola*, *M. leonuri*, *M. persimmonesis*, *M. pulcherrima*, *M. rubicola*, *M. shanxiensis*, *M. sinensis*, *M. zizyphicola* and *M. chrysoperlae*) due to the presence of multiple copies of the primary (e.g., ITS segments and D1/D2 domain of 26S rRNA) and secondary (e.g., RPB2, TEF1α, ACT1 and EF2) barcodes, as well as their intragenomic sequence heterogeneity (comparable with or higher than the inter-species diversity), make clearly identifying *Metschnikowia* species extremely difficult (sometimes impossible) [61,68–70]. A recent study [71] revealed that all possible phylogenetic markers were present in multiple copies in the genome of *M. pulcherrima* strains, and the different copies exhibited a relatively high intragenomic variability. Therefore, these markers appear to fail when used for species identification within the *M. pulcherrima* clade. Due to the above-described limitations, Sipiczki [70] recently concluded that species of the *M. pulcherrima* clade cannot be, at present, distinguished from each other by the criteria of any of the phenotypic, phylogenetic and biological species concepts.

The whole-genome sequencing (including the free access to genome data) recently assumed increasing relevance as a current taxonomy tool for fungal identification [72–75]. Phylogenomic analysis may resolve problems related to the species identification, but this approach is still scarcely used in yeast taxonomy (especially if compared with bacterial one) and the limited availability of yeast genome sequences so far deposited in public databases appear to be the main bottleneck. Actually, only seven genomes of strains of the *M. pulcherrima* clade are publicly available [69,76–79]. Further data and the wider dissemination of genomic analysis could allow new insights into this complex species identification in the future.

As for cryopreservation, *Metschnikowia* pulcherrima-related strains are not particularly difficult to maintain in standard conditions of glycerol 25% (*v*/*v*) at −80 °C, while a study on a single strain showed that culture age and density, as well as type and concentration of protective agents, are relevant elements for a successful freeze-drying, a useful technique also for possible subsequent applications [80].

### *2.5. Case-Study 5: Isolation and Preservation of Pyricularia Species*

Rice blast is the major rice disease [81] caused by fungi of the genus *Pyricularia* (Sordariomycetes, Magnaporthales) belonging to the *Pyricularia grisea* complex [82,83]. This species complex provisionally accommodates the problematic status of *P. grisea sensu stricto* and *Pyricularia oryzae.*

Culture collections of *Pyricularia* species are fundamental for studying their inter- and intraspecific diversity, taxonomy and phylogeny [84]. Host infection caused by *P. oryzae* is neither easily inducible and reproducible in the greenhouse, nor in mesocosm, outside of standard conditions of temperature and humidity [85]. Accordingly, conservation in pure culture should be preferred. Anyhow, *Pyricularia* strains are difficult to isolate and to preserve as pure cultures, due to their low and short-termed survival rate under laboratory standard media, at least under the form of monosporic cultures.

*Pyricularia* species exhibit a hemibiotrophic aptitude, which is strongly shifted towards biotrophism [86,87]. Consequently, the isolation of strains is very difficult. A protocol reported by Valent et al. [88] still constitutes the "gold standard" of successful methods for long-term preservation, despite it only relying on sterile mycelium instead of conidia. Conidia are hardly produced in culture, although this phenomenon is probably strain-dependent [89,90].

In addition to the possible presence of microbial contaminants, the need of having monosporic cultures (resulting from the germination of a single macroconidium) is one more issue to be taken into consideration. Even in pure and (expectedly) haploid cultures, mycelia from different individuals may be casually mixed in the same sample. Therefore, the simultaneous presence of different individuals may finally induce parasexual phenomena and increase the difficulty in the characterization of the single strain. Monosporic culture should be consequently preferred as a way to certify strain quality. The protocol for obtaining monosporic cultures can be summarized as follows:

(i) Storage of portions of infected plants at −20 °C;
(ii) Washing of samples in water [91], disinfection by 3% NaClO, re-washing in sterile water and plate spreading in tap water agar (TWA) solid medium as a humid chamber [92,93];
(iii) Incubation of unsealed plates at room temperature and under light;
(iv) Periodical checking of plates to detect the presence of conidiophores bearing macroconidia [94];
(v) Isolation of mycelia in potato dextrose agar (PDA) solid medium for the production of macroconidia;
(vi) Transfer of single macroconidia in TWA solid medium; spatial set to obtain monosporic cultures is shown in Figure 2;
(vii) Checking of conidia germination after 24 h;

(viii) Transfer of new colonies in modified rice–meal–yeast extract agar (RYA) solid medium [84], where colonization typically takes 15–25 days to give ripe conidiophores.

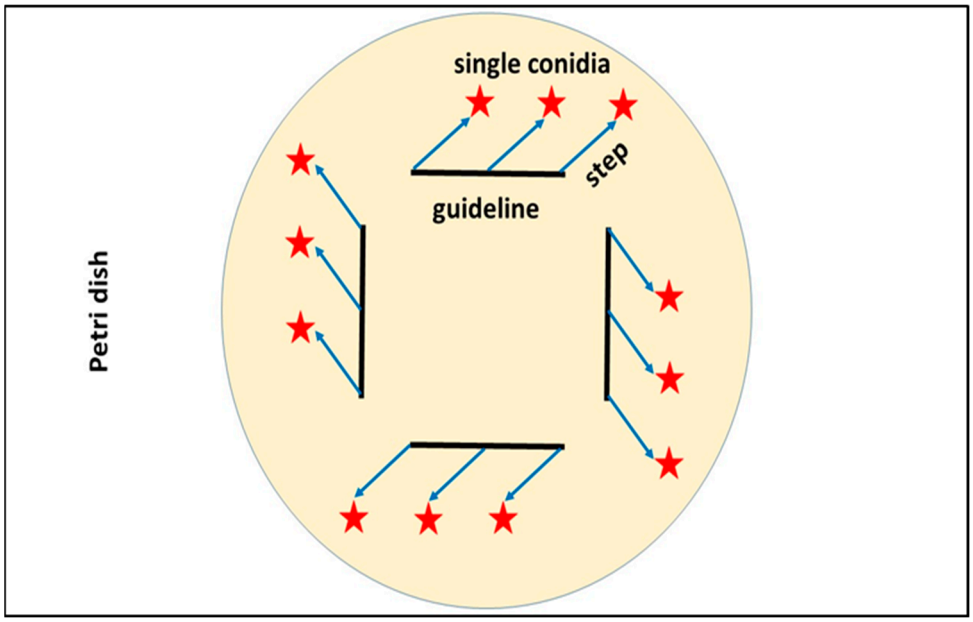


**Figure 2.** Basic scheme for monosporic cultures of *Pyricularia* species. Guidelines are cut into the medium to facilitate the equidistant inoculation of conidia as well as to easily track them when checking the early stages of germination; stars represent single macroconidia inoculated.

RYA supports short-term conservation only. Paper discs were aseptically placed onto the colony mat grown for 14 days on RYA at room temperature. After full mycelial colonization, discs were aseptically drawn from the plate, dried at 37 °C for 48 h, and stored at −20 °C [88]. Periodical inocula in fresh media and viability check on PDA or RYA are suggested.

### *2.6. Case-Study 6: Preservation of Halophytophtora spp. (Peronosporomycetes) from Marine Environment*

*Halophytophthora* is a sister genus of *Phytophthora* (Peronosporomycetes, Peronosporales) mainly found in marine, mangrove and estuarine habitats. Although its saprotrophic aptitude was considered an established evidence for decades [95,96], some studies suggested that this genus might be involved in the widespread decline of the seagrass *Zostera marina* [97,98]. The long-term preservation protocols for pathogenic Peronosporomycetes mainly include cryopreservation, storage under mineral or paraffin oil, or storage in sterile water at room temperature [16]. However, the viability and/or genetic stability of these organisms are generally difficult to maintain for long periods of time [99]. Indeed, a number of marine *Halophytophthora* strains cannot be maintained in conditions of active growth for a long time, nor can they be preserved using standard long-term cryopreservation protocols.

A recent study on the isolation of *Halophytophthora* strains revealed that, after the first sub-culturing on both corn meal agar (CMA) supplemented with pimaricin, ampicillin and rifampicin, and PDA, about 40% of isolates were found to be no longer viable [90]. The protocol for improving long-term cryopreservation of *Halophytophthora* strains can be summarized as follows:

(i) Collection of 2 mm mycelium plugs from colonies pre-grown on PDA in a 10% glycerol solution;
(ii) Fast controlled with the glycerol solution;
(iii) As an alternative, freezing (−1 °C/minute up to −80 °C and then transfer at −152 °C) of the material collected in the cryovials direct submersion of colonies in 10% glycerol

solution followed by an overnight incubation at 5 °C before freezing (to overcome the difficulties encountered in collecting mycelium plugs).

Post cryopreservation recovery proved to fail in some of these Peronosporomycetes, with a survival rate of less than 10%. To overcome this problem, the following protocol could be suggested (Figure 3):

(i) Inoculum of *H. lusitanica* strains on PDA plates and incubation at their optimal temperature for growth (15 °C) for two weeks;
(ii) Addition of sterile wheat grains (about 30 for each strain) to the surface of actively growing colonies and incubation at 15 °C for about 3 weeks;
(iii) Transfer of wheat grains colonized by *H. lusitanica* strains into cryotubes containing 10% glycerol aqueous solution;
(iv) Six months cryopreservation at both −80 °C and −152 °C.

An excellent post cryopreservation recovery (survival rate = 100%) was observed.

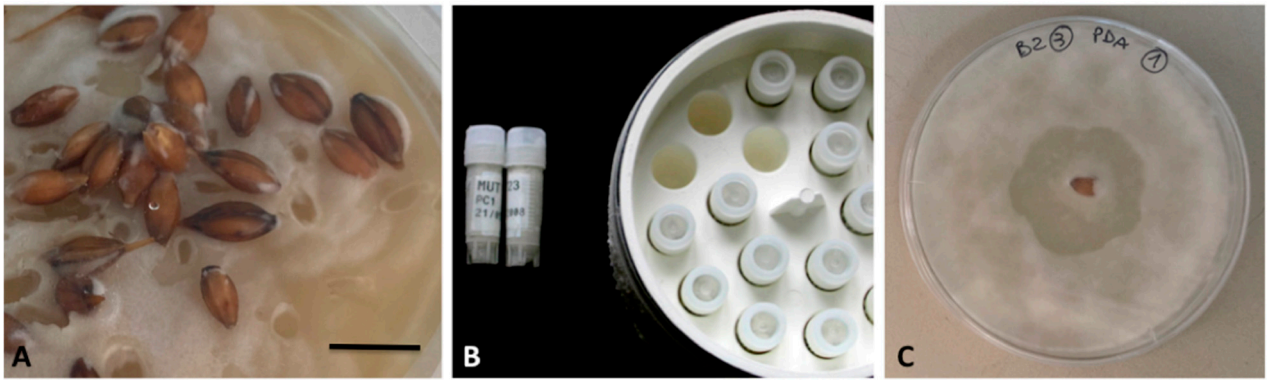


**Figure 3.** (**A**) wheat grains colonized by *Halophytophthora lusitanica* (bar, 10 mm); (**B**) cryovials containing grains; (**C**) actively growing colony after revitalization (plate diameter, 90 mm).

## 3. Conclusions

Although little considered in the current literature, fastidious eukaryotes may represent a problem in the routine work inside worldwide microbiological laboratories. This aspect is much more remarkable in culture collections where a large diversity of species (needing sometimes customized techniques of cultivation, identification, long-term preservation and revitalization) is preserved [100].

The present review underlined that using standard models and protocols to overcome the above issues is challenging because the single genus/species exhibits peculiarities limiting any generalization. On the other hand, the continuous research in this field and the publication of new successful techniques (or the upgrade of the existing ones) are crucial to share the knowledge suggesting similar solutions to the global scientific body.

In recent years, with the development of cheaper and simpler genome sequencing, metagenomic and proteomic studies, the understanding of microbial community structure, microbial guilts, and also peculiar needs of single species, became possible also without any isolation (culture independent molecular approach). Recent studies revealed that over >90% of eukaryotic diversity is not yet cultured [101,102], although it is recognized to be responsible of ecological, clinical and industrial implications. Along with the development of "OMICS" techniques, the importance of isolation, identification, cultivation and long-term preservation in culture collections of eukaryotes (including fastidious ones) should always be a clear landmark for all microbial studies through the development of new and customized tools.

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