Fungal Biology

Vijai Kumar Gupta
Maria Tuohy *Editors*

Laboratory Protocols in Fungal Biology

Current Methods in Fungal Biology

Second Edition

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Fungal Biology

Series Editors

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About the Series

Fungal biology has an integral role to play in the development of the biotechnology and biomedical sectors. It has become a subject of increasing importance as new fungi and their associated biomolecules are identified. The interaction between fungi and their environment is central to many natural processes that occur in the biosphere. The hosts and habitats of these eukaryotic microorganisms are very diverse; fungi are present in every ecosystem on Earth. The fungal kingdom is equally diverse, consisting of seven different known phyla. Yet detailed knowledge is limited to relatively few species. The relationship between fungi and humans has been characterized by the juxtaposed viewpoints of fungi as infectious agents of much dread and their exploitation as highly versatile systems for a range of economically important biotechnological applications. Understanding the biology of different fungi in diverse ecosystems as well as their interactions with living and non-living is essential to underpin effective and innovative technological developments. This series will provide a detailed compendium of methods and information used to investigate different aspects of mycology, including fungal biology and biochemistry, genetics, phylogenetics, genomics, proteomics, molecular enzymology, and biotechnological applications in a manner that reflects the many recent developments of relevance to researchers and scientists investigating the Kingdom Fungi. Rapid screening techniques based on screening specific regions in the DNA of fungi have been used in species comparison and identification, and are now being extended across fungal phyla. The majorities of fungi are multicellular eukaryotic systems and therefore may be excellent model systems by which to answer fundamental biological questions. A greater understanding of the cell biology of these versatile eukaryotes will underpin efforts to engineer certain fungal species to provide novel cell factories for production of proteins for pharmaceutical applications. Renewed interest in all aspects of the biology and biotechnology of fungi may also enable the development of "one pot" microbial cell factories to meet consumer energy needs in the 21st century. To realize this potential and to truly understand the diversity and biology of these eukaryotes, continued development of scientific tools and techniques is essential. As a professional reference, this series will be very helpful to all people who work with fungi and should be useful both to academic institutions and research teams, as well as to teachers, and graduate and postgraduate students with its information on the continuous developments in fungal biology with the publication of each volume.

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Chapter 1
Various Methods of Long-Term Preservation of Fungal Cultures in All-Russian Collection of Microorganisms (VKM)

Svetlana M. Ozerskaya, Nataliya E. Ivanushkina, Galina A. Kochkina, Anastasya A. Danilogorskaya, Irina P. Pinchuk, and Alexander N. Vasilenko

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1.1 Introduction

Microorganisms are fundamental materials for scientific and practical studies. Culture collections (biological resource centers) play a primary role in the stable preservation and long-term storage of microbial resources and ensure regular access to well-documented strains after a long time from their isolation for scientific or biotechnological use [32, 33].

Various methods of preservation of fungal cultures have been reported [13, 25, 29]. Freeze-drying (lyophilization) and cryopreservation methods are utilized for thousands of fungal strains in microbial collections all over the world [7, 12, 27]. Nevertheless, it is clear that the fungal strains of different species vary in the ability to survive after long-time storage preservation under laboratory conditions. Some of them are very difficult to maintain *ex situ*, whereas others could be easily and successfully preserved alive by using almost any conservation technique.

Storage methods for filamentous fungi result from the type and degree of sporulation. Spore-forming strains (as opposed to nonsporulating strains) can be effectively freeze-dried. Both types can be frozen and stored for long periods in liquid nitrogen or in a low-temperature refrigerator. The experience of long-term preservation of fungal strains shows that the duration of storage directly depends not only on the choice of the method but also on the laboratory protocol and temperature of subsequent cultures storage.

This chapter presents the methods of cryopreservation, freeze-drying, drying on silica gel, and preservation in sterile soil that are utilized in VKM fungal collection, accompanied by data on maximal storage time registered. The methods take into consideration the special features of cultures preserved as well as the equipment used.

VKM fungal collection (All-Russian Collection of Microorganisms, Russia) was established in 1955 and has a long-term experience in the preservation and storage of fungal cultures. Collection of filamentous fungi is currently composed of approximately 7000 strains (590 genera, 1600 species) belonging to species of the kingdoms Chromista (*Oomycota*) and Fungi (zygomycetous, ascomycetous and basidiomycetous fungi).

All the information on preservation methods for each VKM fungal strain is presented in the MS Access database. It keeps curated data on the strain numbers, preservation dates as well as inspection dates in various methods, and other technical information. Fields in the database table are presented in Annex 1. For operational analysis of these data, we use MS Access requests – «FunPreservEnd», «FunPreserv_Times», «FunPreserv_MaxTimes». The maximal preservation time is calculated automatically; the latest results (25.11.2019) are presented in Annex 2.

Preserved for many years fungi of various taxa retain their ability to produce different substances suitable as a material for industry and medicine. For instance, the zygomycetous fungus *Cunninghamella japonica* VKM F-1204D was found to be a promising lipid producer for biodiesel production [22]. Fungi of the genus *Penicillium*, which are supported in the collection for more than 40 years (VKM

F-325, VKM F-691, VKM F-1823), are able to synthesize active compounds with diverse structures [10]. *Aspergillus brasiliensis* VKM F-1119, which was accepted by VKM 52 years ago, engaged in the vital process of biotransformation of artemisinin, uncial medicine for the treatment of tropical malaria [34]. Recently published data on the assessment of the effect of freeze-drying and long-term storage on the biotechnological potential of *Aspergillus* section Nigri strains show maintaining of biotechnological properties after preservation [19].

1.2 Cryopreservation of Filamentous Fungi

According to published data, the fast cooling rates followed by storage in liquid nitrogen at -196 °C allow secure and long-term preservation of some fungal cultures [21]. However, the ability to resist damage by freezing and warming differs considerably among genera/species and depends on their particular features (presence and type of sporulation, chemical composition of cytoplasmic membrane and cell wall, physiological state, etc.). Selection of optimal cryoprotectants, rates of cooling, and warming has enabled increasing the number and diversity of taxa preserved by this method [24, 28].

More than 75% filamentous fungi of VKM are stored using various cryopreservation protocols. Cultures with abundant sexual and nonsexual sporulation usually were preserved by using fast cooling rates followed by storage either in liquid nitrogen or in ultralow temperature freezers at −70 °C.

It was noticed that some cultures of zygomycetous fungi belonging to the genera *Mortierella*, *Basidiobolus*, *Coemansia*, and *Entomophthora* do not survive the ultrarapid freezing procedure even if they have abundant sporulation. Successful preservation of such strains was achieved by modification of the cryopreservation regime, for example, using slow programmed freezing. The same method was used either for nonsporulating fungi (basidiomycetous fungi) or zoosporeforming former fungi (*Chromista*, *Oomycota*).

According to our data, some parts of strains of *Oomycota* (20%), basidiomycetous fungi (4%), zygomycetous fungi (1%), and ascomycetous fungi (1%) did not survive cryopreservation at all freezing regimes and modification applied [9]. The strains most difficult to maintain belong to genera *Dictyuchus* and *Phytophthora* and to some species of *Achlya* and *Saprolegnia*. Similar situations have also been seen with some species of basidiomycetous fungi (*Suillus*, *Amanita*, *Dictyophora*, *Mutinus*, etc.). They are usually maintained by subculturing and preservation under mineral oil.

It has been suggested that those microbial cultures that are able to survive the freezing and a short storage will permanently stay in the vital state after any length of storage [20]. According to our data, this is not quite true: some strains of *Achlya colorata*, *Achlya intricata*, *Clitocybe odora*, *Choanephora conjuncta*, *Conidiobolus thromboides*, *Kickxella alabastrina*, *Phanerochaete sanguinea*, *Rhodocollybia butyracea*, and *Saprolegnia terrestris* have lost their ability to grow after 5–7

years of storage in liquid nitrogen, although they were in the viable state after 24 h of storage. The reason is not yet known. Nevertheless, the viability test showed that representatives of 311 species of fungi remain alive after 20–30 years of storage (Annex 2).

The cooling equipment being used in VKM is storage tanks "Bioproducts-0.5" with a capacity of 500 liters of liquid nitrogen and ultralow temperature freezers (−80 °C, Sanyo, Japan).

1.3 Freeze-Drying of Filamentous Fungi

Currently, freeze-drying is used to preserve approximately 85% of filamentous fungi maintained in VKM. Fungi from different taxonomical groups (zygomycetous fungi, ascomycetous fungi – both teleo- and anamorph) able to produce dormant structures (spores, sclerotia, etc.) usually survive freeze-drying [11]. According to our data, about 90% of strains of these fungal groups remain alive in this method. We noticed that the freeze-dried strains of 817 species stored at 5 °C for more than 20 years were in a viable state, and cultures of 289 species have been sustained for even 40–50 years of storage. Some species did not survive freeze-drying even when the sporulation is abundant, those are *Conidiobolus coronatus*, *C. thromboides*, *Entomophthora thaxteriana*, *E. conica*, *E. dipterigena*, *Cunninghamella homothallica*, and *C. vesiculosa*. Species of genus *Botrytis* (*B. fabae* and *B. squamosa)*, forming only sclerotia as a dormant structure, remain in a vital state in freeze-drying only for rather a short time – less than 10 years [9].

Nonsporulating microorganisms from *Oomycota* and basidiomycetous fungi are not stored in VKM by freeze-drying, since sterile mycelia generally do not remain viable.

The equipment used in VKM for freeze-drying is the centrifugal freeze-dryer system Micromodulyo (Edwards, UK).

1.4 Drying in Sterile Soil of Filamentous Fungi

This simple and popular method for the preservation of fungi was applied at the beginning of the twentieth century [18]. Species of *Aspergillus* and *Penicillium* can be maintained by this way more effectively than other micromycetes. According to T.P. Suprun [31] who investigated the preservation of 78 *Penicillium* species (more than 1000 strains) in sterile soil for 7–10 years, the best preserved strains were representatives of *Assymmetrica* section. Less effectively preserved species were *Biverticillata-Symmetrica*, and the lowest effectiveness was observed with strains of the section *Monoverticillata*.

This method is also efficient for preservation of some human, animal, and plant pathogens with retaining their virulence [21]. For example, *Alternaria japonica* (syn.

A. raphani), *Fusarium oxysporum*, and the species of *Septoria* (*S. avenae*, *S. nodorum*, *S. passerinii*, *S. tritici*) have retained their ability to infect a plant host after 2–5 years of storage [2, 8, 23]. Some degraded strains of micromycetes partly recuperated their lost qualities after preservation in soil [30].

According to our data, fungal strains of 167 species stored by this method are able to maintain viability for more than 30 years, and cultures of 87 species have been sustained for even 40–55 years of storage.

1.5 Drying of Filamentous Fungi on Silica Gel

Immobilized cells of microorganisms retain viability and biological activity at action of different stressors, as a rule, better than free ones [4]. Therefore, the preliminary drying of the cells on the adsorbent allows the microorganisms to remain viable for a longer time. As an adsorbent on which a suspension of microorganisms is applied for subsequent drying, silica gel (a dried gel of polysilicic acid with numerous pores) is most often used [5]. Silica gel promotes the dehydration of microorganisms and helps them to survive a thermal stress [24]. Since the silica gel can prevent all fungal growth and metabolism, the risk of any morphological, physiological, and genetic changes could be minimized [1].

Using of anhydrous silica gel particles for maintaining stock cultures of *Neurospora crassa* was suggested by D. Perkins in 1962 [17]. This new method has proved consistently useful and effective over several years.

At present, this method is widely used in relation to different taxa and ecological groups of fungi. So, the method was effective for the storage of entomopathogenic fungi of the order of *Hypocreales* for 2 years [3] and, in particular, *Metarhizium anisopliae* [6], as well as for fungi of many other taxa, including the spores of obligate biotrophic parasite *Podosphaera fusca* [16] and rust fungi, which cannot be grown on agar media [1].

As a disadvantage of the silica gel preservation method, researchers note that the time of storage is quite short (between 2 and 4 years) [14, 26]. But it is clear that the features of the methodological protocols can be crucial for the fungi preservation by this method, wherein the temperature at which frozen fungi are stored affects how long they could be preserved while remaining viable.

The method of storage on silica gel was introduced in VKM in the middle of the 1980s [24]. Our experience has shown that several groups of fungi can be preserved by this method without losing vitality for many years (Table 1.1).

The viability of more than 300 strains of zygomycetous fungi with various types of sporogenous structures (6 classes, 6 orders, 15 families, 35 genera, and 118 species) and near 300 strains of dark-colored anamorphic ascomycetous fungi with different types of conidiogenesis (7 classes, 18 orders, 34 families, 79 genera, and 164 species) (Table 1.2) was assessed from 1 to near 30 years of preservation (Figs. 1.1 and 1.2).

Table 1.1 Drying of VKM fungal cultures on silica gel (storage time)

No.	Name of species	Number of strains	Storage time at different temperature (years)		
			5 °C	−12 °C	−70 °C
1	*Absidia caerulea* Bainier 1889	4	13,98	8,84	29,95
2	*Absidia cuneospora* G.F. Orr et Plunkett 1959	1	1,21	1,21	30,57
3	*Absidia cylindrospora* Hagem 1908	2	16,15	11,79	30,02
4	*Absidia glauca* Hagem 1908	4	10,77	7,78	29,95
5	*Absidia repens* van Tieghem 1878	1	1,07	1,07	29,44
6	*Absidia spinosa* Lendner 1907	1	1,66	1,09	30,00
7	*Acrophialophora fusispora* (S.B. Saksena 1953) Samson 1970	1	32,24	22,34	32,24
8	*Actinomucor elegans* (Eidam 1884) C.R. Benjamin et Hesseltine 1957	7	15,52	11,34	30,04
9	*Albifimbria verrucaria* (Albertini et Schweinitz 1805) L. Lombard et Crous 2016	1	11,05	11,05	32,41
10	*Alternaria alternata* (Fries 1832) Keissler 1912	5	10,98	8,66	31,61
11	*Alternaria atra* (Preuss 1852) Woudenberg et Crous 2013	4	31,81	13,31	31,87
12	*Alternaria botrytis* (Preuss 1851) Woudenberg et Crous 2013	9	25,40	11,32	32,11
13	*Alternaria brassicicola* (Schweinitz 1832) Wiltshire 1947	1	21,75	10,36	31,68
14	*Alternaria chartarum* Preuss 1848	4	26,64	10,07	31,97
15	*Alternaria consortialis* (Thuemen 1876) Groves et Hughes 1953	3	9,63	8,25	32,25
16	*Alternaria japonica* Yoshii 1941	1	6,96	3,04	32,26
17	*Alternaria macrospora* Zimmermann 1904	2	5,47	1,63	31,97
18	*Alternaria multirostrata* E.G. Simmons et C.R. Jackson 1968	1	1,30	6,18	31,56
19	*Alternaria oudemansii* (E.G. Simmons 1967) Woudenberg et Crous 2013	1	1,00	4,89	31,26
20	*Alternaria radicina* Meier et al. 1922	1	5,88	2,84	32,32
21	*Alternaria solani* Sorauer 1896	1	1,19	3,21	31,91
22	*Alternaria tenuissima* (Kunze 1818) Wiltshire 1933	1	22,35	10,91	32,26
23	*Amerosporium concinnum* Petrak 1953	1	31,26	9,91	31,26
24	*Ampelomyces artemisiae* (Voglino 1905) Rudakov 1979	1	10,61	6,64	31,95
25	*Ampelomyces heraclei* (Dejeva 1967) Rudakov 1979	1	10,61	10,61	31,97
26	*Ampelomyces humuli* (Fautrey 1890) Rudakov 1979	1	31,99	10,61	31,95
27	*Ampelomyces polygoni* (Potebnia 1907) Rudakov 1979	1	0,72	2,51	22,56
28	*Ampelomyces ulicis* (Adams 1907) Rudakov 1979	1	22,03	10,61	31,95
29	*Ampelomyces uncinulae* (Fautrey 1893) Rudakov 1979	1	21,69	1,35	31,62

(continued)

Table 1.1 (continued)

No.	Name of species	Number of strains	Storage time at different temperature (years)		
			5 °C	−12 °C	−70 °C
30	*Apenidiella strumelloidea* (Milko et Dunaev 1986) W. Quaedvlieg et P.W. Crous 2014	1	0,96	3,09	32,48
31	*Aposphaeria caespitosa* (Fuckel 1869) Jaczewski 1917	1	10,64	3,30	32,05
32	*Arthrinium arundinis* (Corda 1838) Dyko et Sutton 1981	1	10,28	10,30	31,62
33	*Arthrinium sphaerospermum* Fuckel 1874	1	21,99	10,57	31,91
34	*Ascochyta malvicola* Saccardo 1878	1	2,17	2,17	31,62
35	*Aureobasidium melanogenum* (Hermanides-Nijhof 1977) Zalar et al. 2014	6	28,72	8,05	32,33
36	*Aureobasidium microstictum* (Bubak 1907) W.B. Cooke 1962	1	31,99	6,68	31,99
37	*Aureobasidium pullulans* (de Bary 1866) G. Arnaud 1918	7	24,50	9,05	32,09
38	*Backusella circina* J.J. Ellis et Hesseltine 1969	1	4,30	7,25	29,98
39	*Backusella indica* (Baijal et B.S.Mehrotra 1965) G. Walther et de Hoog 2013	1	4,42	4,42	30,49
40	*Backusella lamprospora* (Lendner 1908) Benny et R.K. Benjamin 1975	3	6,55	4,25	29,93
41	*Backusella oblongielliptica* (H. Naganishi et al. ex Pidoplichko et Milko 1971) G. Walther et de Hoog 2013	1	1,33	1,33	29,67
42	*Backusella recurva* (E.E. Butler 1952) G. Walther et de Hoog 2013	1	4,45	7,42	29,84
43	*Backusella tuberculispora* (Schipper 1978) G. Walther et de Hoog 2013	1	4,11	6,66	28,58
44	*Backusella variabilis* (A.K. Sarbhoy 1965) G. Walther et de Hoog 2013	1	7,42	4,45	29,84
45	*Beauveria brongniartii* (Saccardo 1892) Petch 1926	1	2,98	6,02	32,37
46	*Benjaminiella poitrasii* (R.K. Benjamin 1960) Arx 1981	1	16,01	7,33	29,84
47	*Berkeleyomyces basicola* (Berkeley et Broome 1850) W.J. Nel et al. 2017	1	3,24	3,24	31,99
48	*Bipolaris australiensis* (M.B. Ellis 1971) Tsuda et Ueyama 1981	4	19,07	5,70	31,58
49	*Bipolaris cynodontis* (Marignoni 1909) Shoemaker 1959	1	21,62	10,21	31,56
50	*Bipolaris sorokiniana* (Saccardo 1890) Shoemaker 1959	2	8,51	8,45	31,82
51	*Bipolaris victoriae* (F. Meehan et H.C. Murphy 1946) Shoemaker 1959	1	0,96	0,96	21,23
52	*Bispora antennata* (Persoon 1801) E.W. Mason 1953	1	4,79	1,70	31,19

(continued)

Table 1.1 (continued)

No.	Name of species	Number of strains	Storage time at different temperature (years)		
			5 °C	−12 °C	−70 °C
53	*Bispora betulina* (Corda 1838) S. Hughes 1958	1	3,38	3,38	3,38
54	*Bispora effusa* Peck 1891	1	6,24	2,17	10,30
55	*Blakeslea trispora* Thaxter 1914	8	16,69	12,33	29,84
56	*Botryotrichum piluliferum* Saccardo et Marchal 1885	4	17,10	6,78	25,82
57	*Botrytis aclada* Fresenius 1850	2	10,87	6,84	32,22
58	*Botrytis anthophila* Bondartsev 1913	1	5,82	2,72	32,20
59	*Botrytis cinerea* Persoon 1794	8	1,30	1,65	15,34
60	*Botrytis convoluta* Whetzel et Drayton 1932	2	5,23	6,26	32,11
61	*Botrytis elliptica* (Berkeley 1881) Cooke 1901	1	1,02	1,02	1,02
62	*Botrytis galanthina* (Berkeley et Broome 1873) Saccardo 1886	1	1,02	1,02	1,02
63	*Botrytis gladiolorum* Timmermans 1941	2	2,11	4,48	17,66
64	*Botrytis tulipae* (Libert 1830) Lind 1913	1	0,08	0,08	2,90
65	*Cadophora fastigiata* Lagerberg et Melin 1928	1	10,76	3,35	32,14
66	*Cadophora malorum* (Kidd et Beaumont 1924) W. Gams 2000	4	8,82	6,40	32,16
67	*Cadophora melinii* Nannfeldt 1934	1	3,31	8,34	32,07
68	*Cephalotrichum gorgonifer* (Bainier 1907) Sandoval-Denis et al. 2016	1	3,02	7,00	32,24
69	*Cephalotrichum purpureofuscum* (Schweinitz 1832) S. Hughes	1	0,98	0,98	31,24
70	*Cephalotrichum stemonitis* (Persoon 1801) Nees 1812	3	3,21	4,22	23,24
71	*Chaetocladium brefeldii* van Tieghem et G. Le Monnier 1873	2	23,8	12,77	30,4
72	*Chaetocytostroma* sp.	1	0,01	1,02	21,34
73	*Chloridium caesium* (Nees et T. Nees 1818) Réblová et Seifert 2016	1	1,32	1,32	31,58
74	*Chloridium virescens* (Persoon 1797) W. Gams et Holubova-Jechova 1976 var. *caudigerum* (Hoehnel 1903) W. Gams et Holubova-Jechova 1976	1	1,13	1,13	31,39
75	*Choanephora infundibulifera* (Currey 1873) Saccardo 1891	1	17,56	7,35	29,88
76	*Circinella muscae* (Sorokin 1870) Berlese et de Toni 1888	3	21,11	5,88	30,11
77	*Circinella umbellata* van Tieghem et G. Le Monnier 1873	1	15,27	6,34	28,33
78	*Cladophialophora chaetospira* (Grove 1886) Crous et Arzanlou 2007	1	10,57	3,21	31,91
79	*Cladosporium aecidiicola* Thuemen 1876	1	1,01	4,82	31,24

(continued)

Table 1.1 (continued)

No.	Name of species	Number of strains	Storage time at different temperature (years)		
			5 °C	−12 °C	−70 °C
80	*Cladosporium brevicompactum* Pidoplichko et Deniak 1941	2	6,23	6,21	31,65
81	*Cladosporium cladosporioides* (Fresenius 1850) G.A. de Vries 1952	2	9,08	5,15	32,41
82	*Cladosporium colocasiae* Sawada 1916	1	1,04	2,94	32,15
83	*Cladosporium cucumerinum* Ellis et Arthur 1889	1	1,07	1,07	32,07
84	*Cladosporium elegantulum* Pidoplichko et Deniak 1938	2	10,51	10,53	31,86
85	*Cladosporium gossypiicola* Pidoplichko et Deniak 1941	2	16,32	6,72	32,08
86	*Cladosporium herbarum* (Persoon 1794) Link 1816	15	9,14	7,05	29,98
87	*Cladosporium lycoperdinum* Cooke 1883	1	4,82	4,82	31,24
88	*Cladosporium macrocarpum* Preuss 1848	3	3,32	4,61	31,68
89	*Cladosporium pseudocladosporioides* Bensch et al. 2010	1	31,22	9,87	31,22
90	*Cladosporium sphaerospermum* Penzig 1882	5	10,02	7,61	30,28
91	*Cladosporium straminicola* Pidoplichko et Deniak 1938	1	3,09	3,09	32,48
92	*Cladosporium transchelii* Pidoplichko et Deniak 1938	1	11,13	3,09	32,48
93	*Cokeromyces recurvatus* Poitras 1950	2	16,73	7,31	29,45
94	*Colletotrichum gloeosporioides* (Penzig 1882) Penzig et Saccardo 1884	2	13,94	2,12	20,95
95	*Colletotrichum musae* (Berkeley et M.A. Curtis 1874) Arx 1957	1	9,94	1,00	31,26
96	*Conidiobolus coronatus* (Costantin 1897) Batko 1964	1	1,74	1,74	30,37
97	*Coniothyrium concentricum* (Desmazieres 1840) Saccardo 1878	1	31,62	21,69	31,62
98	*Coniothyrium hellebori* Cooke et Massee 1886	1	10,96	1,05	10,96
99	*Coniothyrium rosarum* Cooke et Harkness 1882	2	10,49	6,52	31,83
100	*Coniothyrium wernsdorffiae* Laubert 1905	1	1,28	2,15	2,15
101	*Cunninghamella blakesleeana* Lendner 1927	1	7,80	7,80	30,16
102	*Cunninghamella echinulata* (Thaxter 1891) Thaxter ex Blakeslee1905	11	17,54	6,51	30,00
103	*Cunninghamella japonica* (Saito 1905) Pidoplichko et Milko 1971	7	10,65	7,76	29,56
104	*Curvularia comoriensis* Bouriquet et Jauffret 1955 ex M.B. Ellis 1966	1	3,17	10,59	31,93
105	*Curvularia geniculata* (Tracy et Earle 1896) Boedijn 1933	2	5,44	3,45	16,72

(continued)

Table 1.1 (continued)

No.	Name of species	Number of strains	Storage time at different temperature (years)		
			5 °C	−12 °C	−70 °C
106	*Curvularia inaequalis* (Shear 1907) Boedijn 1933	1	31,91	6,96	32,01
107	*Curvularia lunata* (Wakker 1898) Boedijn 1933	2	5,93	3,35	32,11
108	*Dematioscypha delicata* (Berkeley et Broome 1859) Hosoya 2014	1	0,00	1,94	4,99
109	*Dicyma ampullifera* Boulanger 1897	1	6,20	6,20	6,20
110	*Dicyma olivacea* (Emoto et Tubaki 1970) Arx 1982	1	0,80	5,90	5,90
111	*Didymella glomerata* (Corda 1840) Q. Chen et L. Cai 2015	6	32,92	8,97	31,92
112	*Didymella pomorum* (Thümen 1879) Q. Chen et L. Cai 2015	2	16,31	6,67	31,95
113	*Dinemasporium strigosum* (Persoon 1801) Saccardo 1881	1	1,30	1,30	31,56
114	*Discula brunneotingens* E.I. Meyer 1953	1	0,00	0,96	0,96
115	*Discula pinicola* (Naumov 1926) Petrak 1927 var. *mammosa* Lagerberg et al. 1927	1	5,83	5,83	31,28
116	*Dothiora prunorum* (Dennis et Buhagiar 1973) Crous 2016	1	31,97	10,62	31,97
117	*Entomophthora conica* Nowakowski 1883	1	1,33	6,86	29,67
118	*Entomophthora thaxteriana* I.M. Hall et J. Bell 1963	1	0,10	0,10	30,7
119	*Epicoccum nigrum* Link 1815	2	2,20	2,20	31,98
120	*Exophiala castellanii* Iwatsu et al. 1984	1	0,00	2,92	32,33
121	*Exophiala salmonis* J.W. Carmichael 1966	1	5,99	5,99	31,42
122	*Fennellomyces linderi* (Hesseltine et Fennell 1955) Benny et R.K. Benjamin 1975	1	16,94	7,34	29,39
123	*Fonsecaea pedrosoi* (Brumpt 1922) Negroni 1936	1	1,07	1,07	29,34
124	*Fulvia fulva* (Cooke 1883) Ciferri 1954	1	0,00	0,00	1,32
125	*Geomyces pannorum* (Link 1824) Sigler et J.W. Carmichael 1976	1	5,82	5,82	10,79
126	*Gilbertella persicaria* (E.D. Eddy 1925) Hesseltine 1960	1	16,72	16,72	30,37
127	*Gliocephalotrichum bulbilium* J.J. Ellis et Hesseltine 1962	1	1,04	1,04	32,24
128	*Gliomastix murorum* (Corda 1838) S. Hughes 1958 var. *murorum*	1	6,23	6,23	31,66
129	*Gongronella butleri* (Lendner 1926) Peyronel et Dal Vesko 1955	5	0,90	3,34	24,40
130	*Gonytrichum macrocladum* (Saccardo 1880) S. Hughes 1951	1	22,34	22,34	32,24
131	*Hansfordia pulvinata* (Berkeley et M.A. Curtis 1875) S. Hughes 1958	1	0,98	5,83	31,24
132	*Harzia acremonioides* (Harz 1871) Costantin 1888	3	6,29	6,31	31,46

(continued)

Table 1.1 (continued)

No.	Name of species	Number of strains	Storage time at different temperature (years)		
			5 °C	−12 °C	−70 °C
133	*Helicostylum elegans* Corda 1842	1	18,23	18,23	30,33
134	*Helicostylum pulchrum* (Preuss 1851) Pidoplichko et Milko 1971	2	10,5	10,5	30,36
135	*Hesseltinella vesiculosa* H.P. Upadhyay 1970	1	0,10	0,10	0,98
136	*Hormoconis resinae* (Lindau 1906) Arx et G.A. de Vries 1973	8	17,31	13,39	32,08
137	*Hormonema macrosporum* L. Voronin 1986	1	3,21	10,60	31,97
138	*Humicola fuscoatra* Traaen 1914	2	16,11	2,02	31,73
139	*Hyphopichia burtonii* (Boidin et al. 1964) Arx et Van der Walt 1976	1	10,98	3,11	32,33
140	*Kickxella alabastrina* Coemans 1862	1	8,30	8,30	17,34
141	*Lecythophora decumbens* (J.F.H. Beyma 1942) E. Weber et al. 2002	1	11,05	11,05	32,41
142	*Lecythophora fasciculata* (J.F.H. Beyma 1939) E. Weber et al. 2002	1	31,99	10,62	31,99
143	*Lecythophora hoffmannii* (J.F.H. Beyma 1939) W. Gams et McGinnis 1983	2	27,12	10,72	32,08
144	*Lecythophora mutabilis* (J.F.H. Beyma 1944) W. Gams et McGinnis 1983	1	32,18	2,98	32,18
145	*Lichtheimia blakesleeana* (Lendner 1924) Kerst. Hoffmann et al. 2009	3	16,79	11,03	30,38
146	*Lichtheimia corymbifera* (Cohn 1884) Vuillemin 1903	11	15,65	7,93	29,86
147	*Lichtheimia hyalospora* (Saito 1906) Kerst. Hoffmann et al. 2009	1	16,72	11,88	30,37
148	*Linderina pennispora* Raper et Fennell 1952	1	1,00	1,00	30,16
149	*Macrophoma mantegazziana* (Penzig 1882) Berlese et Voglino 1886	1	1,19	1,19	31,91
150	*Memnoniella echinata* (Rivolta 1884) Galloway 1933	2	12,21	6,52	31,57
151	*Menispora ciliata* Corda 1837	1	0,96	0,96	31,15
152	*Microsphaeropsis olivacea* (Bonorden 1869) Höhnell 1917	1	22,13	10,73	32,03
153	*Monodictys paradoxa* (Corda 1938) S. Hughes 1958	1	32,47	11,05	32,41
154	*Mortierella alpina* Peyronel 1913	1	7,10	4,51	28,99
155	*Mortierella beljakovae* Milko 1973	1	0,10	0,10	29,63
156	*Mortierella capitata* Marchal 1891	1	22,67	7,03	29,8
157	*Mortierella dichotoma* Linnemann 1936 ex W. Gams 1977	1	1,33	4,35	29,67
158	*Mortierella exigua* Linnemann 1941	1	6,87	1,29	29,63
159	*Mortierella gemmifera* M. Ellis 1940	1	6,90	4,34	29,63

(continued)

Table 1.1 (continued)

No.	Name of species	Number of strains	Storage time at different temperature (years)		
			5 °C	−12 °C	−70 °C
160	*Mortierella globulifera* O. Rostrup 1916	1	1,33	1,33	29,67
161	*Mortierella hyalina* (Harz 1871) W. Gams 1970 var. *hyalina*	3	2,24	3,24	29,47
162	*Mortierella jenkinii* (A.L. Smith 1898) Naumov 1935	1	1,15	1,15	7,06
163	*Mortierella lignicola* (G.W. Martin 1937) W. Gams et R. Moreau 1959	1	15,82	7,06	28,99
164	*Mortierella mutabilis* Linnemann 1941	1	0,10	15,44	28,38
165	*Mortierella parvispora* Linnemann 1941	4	2,43	2,43	18,19
166	*Mortierella polycephala* Coemans 1863	1	2,12	6,20	28,16
167	*Mortierella pusilla* Oudemans 1902	1	4,78	1,29	29,63
168	*Mortierella reticulata* van Tieghem et G. Le Monnier 1873	1	0,42	0,42	1,29
169	*Mortierella stylospora* Dixon-Stewart 1932	1	7,16	7,16	7,16
170	*Mortierella verticillata* Linnemann 1941	5	2,12	3,32	29,52
171	*Mortierella zychae* Linnemann 1941	1	0,10	0,75	3,05
172	*Mucor aligarensis* B.S. Mehrotra et B.R. Mehrotra 1969	1	1,24	2,40	29,27
173	*Mucor bainieri* B.S. Mehrotra et Baijal 1963	1	6,27	6,27	29,10
174	*Mucor circinelloides* van Tieghem 1875 var. *circinelloides*	9	20,75	6,18	29,34
175	*Mucor circinelloides* van Tieghem 1875 var. *janssenii* (Lendner 1907) Schipper 1976	7	21,2	11,56	30,09
176	*Mucor circinelloides* van Tieghem 1875 var. *lusitanicus* (Bruderlein 1916) Schipper 1976	6	12,73	5,61	30,02
177	*Mucor durus* G. Walther et de Hoog 2013	1	15,4	6,43	29,27
178	*Mucor exponens* (Burgeff 1924) G. Walther et de Hoog 2013	4	7,54	2,87	29,95
179	*Mucor flavus* Bainier 1903	13	10,27	7,5	30,04
180	*Mucor fuscus* Bainier 1903	3	5,72	4,15	30,19
181	*Mucor genevensis* Lendner 1908	3	3,02	2,95	28,98
182	*Mucor griseocyanus* Hagem 1908	2	16,08	4,08	29,88
183	*Mucor guilliermondii* Nadson et Philippow 1925	1	7,27	4,39	29,78
184	*Mucor heterogamus* Vuillemin 1903	1	4,62	4,62	30,16
185	*Mucor hiemalis* Wehmer 1903 var. *corticolus* (Hagem 1910) Schipper 1973	2	12,46	12,2	30,24
186	*Mucor hiemalis* Wehmer 1903 var. *hiemalis*	13	10,78	7,06	29,94
187	*Mucor hiemalis* Wehmer 1903 var. *silvaticus* (Hagem 1908) Schipper 1973	3	2,30	2,30	29,78
188	*Mucor indicus* Lendner 1930	2	16,86	12,54	30,29
189	*Mucor laxorrhizus* Y. Ling 1930	5	4,24	5,36	25,11

(continued)

Table 1.1 (continued)

No.	Name of species	Number of strains	Storage time at different temperature (years)		
			5 °C	−12 °C	−70 °C
190	*Mucor luteus* Linnemann 1936	2	0,70	0,70	30,14
191	*Mucor microsporus* Namyslowski 1910	1	0,71	0,71	28,16
192	*Mucor moelleri* (Vuillemin 1903) Lendner 1908	4	12,54	9,00	29,84
193	*Mucor mousanensis* Baijal et B.S. Mehrotra 1966	1	30,38	8,33	30,38
194	*Mucor mucedo* Linnaeus 1753	6	6,99	6,70	29,71
195	*Mucor odoratus* Treschew 1940	2	1,46	3,22	29,02
196	*Mucor piriformis* A. Fischer 1892	3	8,27	5,37	29,02
197	*Mucor plasmaticus* van Tieghem 1875	1	0,99	0,99	29,88
198	*Mucor plumbeus* Bonorden 1864	10	16,68	9,50	28,71
199	*Mucor psychrophilus* Milko 1971	1	17,19	7,02	29,75
200	*Mucor racemosus* Fresenius 1850 var. *racemosus*	17	17,53	10,28	30,10
201	*Mucor racemosus* Fresenius 1850 var. *sphaerosporus* (Hagem 1908) Schipper 1970	1	16,79	30,07	30,07
202	*Mucor ramosissimus* Samoutsevitch 1927	1	15,23	6,27	29,10
203	*Mucor saturninus* Hagem 1910	1	4,47	17,10	30,55
204	*Mucor sinensis* Milko et Beliakova 1971	1	16,57	7,95	30,29
205	*Mucor strictus* Hagem 1908	1	7,32	7,32	29,84
206	*Mucor zonatus* Milko 1967	2	16,42	7,75	29,97
207	*Mucor zychae* Baijal et B.S. Mehrotra 1965 var. *zychae*	1	0,10	0,10	30,49
208	*Mycogone cervina* Ditmar 1817	1	31,58	5,33	31,58
209	*Mycogone nigra* (Morgan 1895) C.N. Jensen 1912	3	15,45	7,84	31,88
210	*Mycogone rosea* Link 1809	4	0,87	1,23	31,30
211	*Mycosticta cytosporicola* Frolov 1968	2	5,76	2,06	21,25
212	*Mycotypha africana* R.O. Novak et Backus 1963	1	18,05	18,05	30,51
213	*Myrothecium* sp.	2	0,94	0,94	31,12
214	*Neocamarosporium betae* (Berlese 1888) Ariyawansa et K.D. Hyde 2015	1	11,01	11,01	32,37
215	*Neottiospora caricina* (Desmazieres 1836) Hoehnel 1924	1	4,76	4,76	31,17
216	*Nigrospora gorlenkoana* Novobranova 1972	2	6,00	6,00	31,78
217	*Nigrospora gossypii* Jaczewski 1929	1	5,91	5,96	31,24
218	*Nigrospora oryzae* (Berkeley et Broome 1873) Petch 1924	2	10,55	4,13	31,90
219	*Nodulisporium verrucosum* (J.F.H. Beyma 1929) G. Smith 1954	1	5,82	2,94	29,15
220	*Ochrocladosporium elatum* (Harz 1871) Crous et U. Braun 2007	1	22,58	11,13	32,48
221	*Oidiodendron cereale* (Thuemen 1880) G.L. Barron 1962	1	9,92	9,92	31,24

(continued)

Table 1.1 (continued)

No.	Name of species	Number of strains	Storage time at different temperature (years)		
			5 °C	−12 °C	−70 °C
222	*Paraconiothyrium fuckelii* (Saccardo 1878) Verkley et Gruyter 2012	1	10,28	21,69	31,62
223	*Paraconiothyrium sporulosum* (W. Gams et Domsch 1969) Verkley 2004	2	7,00	6,78	32,05
224	*Paramyrothecium roridum* (Tode 1790) L. Lombard et Crous 2016	1	31,81	10,46	31,81
225	*Parasitella parasitica* (Bainier 1884) Sydow 1903	1	6,31	6,31	28,33
226	*Pestalotia pezizoides* de Notaris 1841	1	21,69	9,27	30,59
227	*Phialophora atrovirens* (J.F.H. Beyma 1935) Schol-Schwarz 1970	1	2,94	1,04	32,15
228	*Phialophora bubakii* (Laxa 1930) Schol-Schwarz 1970	1	10,83	22,82	32,18
229	*Phialophora lagerbergii* (Melin et Nannfeldt 1934) Conant 1937	1	0,98	3,22	31,99
230	*Phialophora verrucosa* Medlar 1915	1	8,27	8,27	31,99
231	*Phycomyces blakesleeanus* Burgeff 1925	4	4,75	5,50	23,91
232	*Phycomyces nitens* (C. Agardh 1823) Kunze 1823	2	7,47	5,91	29,44
233	*Phyllosticta pucciniospila* C. Massalongo 1900	1	3,19	3,19	31,95
234	*Pilaira anomala* (Cesati 1851) J. Schroeter 1886	1	4,39	4,39	29,78
235	*Pilaira caucasica* Milko 1970	1	6,92	17,12	29,75
236	*Pirella circinans* Bainier 1882 var. *volgogradensis* (Milko 1974) Benny et Schipper 1988	1	6,29	6,29	29,1
237	*Pirella naumovii* (Milko 1970) Benny et Schipper 1992	1	8,05	8,05	30,33
238	*Pleotrichocladium opacum* (Corda 1837) Hernández-Restrepo et al. 2017	1	22,09	10,64	31,99
239	*Pleurophoma cava* (Schulzer 1871) Boerema 1996	3	18,57	7,63	21,54
240	*Pyrenophora biseptata* (Saccardo et Roumeguere 1881) Crous 2013	1	9,89	0,98	31,24
241	*Radiomyces spectabilis* Embree 1959	1	15,95	7,16	29,98
242	*Rhinocladiella atrovirens* Nannfeldt 1934	1	22,34	6,95	32,24
243	*Rhizomucor miehei* (Cooney et R. Emerson 1964) Schipper 1978	1	17,04	8,54	30,49
244	*Rhizomucor pusillus* (Lindt 1886) Schipper 1978	3	16,98	16,65	30,10
245	*Rhizomucor tauricus* (Milko et Schkurenko 1970) Schipper 1978	1	16,52	7,92	30,24
246	*Rhizopus arrhizus* A. Fischer 1892	8	17,71	8,60	29,74
247	*Rhizopus microsporus* van Tieghem 1875 var. *chinensis* (Saito 1904) Schipper et Stalpers 1984	2	16,67	10,37	30,17
248	*Rhizopus microsporus* van Tieghem 1875 var. *microsporus*	4	15,88	6,76	29,38

(continued)

Table 1.1 (continued)

No.	Name of species	Number of strains	Storage time at different temperature (years)		
			5 °C	−12 °C	−70 °C
249	*Rhizopus stolonifer* (Ehrenberg 1818) Vuillemin 1902 var. *stolonifer*	13	19,86	9,71	29,17
250	*Scopulariopsis brevicaulis* (Saccardo 1882) Bainier 1907	1	31,22	9,87	31,22
251	*Spadicesporium acrosporum* V.N. Borisova et Dvoinos 1982	1	21,69	10,32	31,64
252	*Spadicesporium acrosporum-majus* V.N. Borisova et Dvoinos 1982	1	6,22	6,22	31,64
253	*Spadicesporium bifurcatum* V.N. Borisova et Dvoinos 1982	1	6,22	6,22	31,64
254	*Spadicesporium bifurcatum-majus* V.N. Borisova et Dvoinos 1982	1	21,69	6,22	31,64
255	*Spadicesporium copiosum* V.N. Borisova et Dvoinos 1982	1	10,32	6,22	31,64
256	*Spadicesporium persistens* V.N. Borisova et Dvoinos 1982	1	10,28	2,14	31,60
257	*Spadicesporium ramosum* V.N. Borisova et Dvoinos 1982	1	21,69	10,30	31,64
258	*Sphaerostilbella penicillioides* (Corda 1840) Rossman et al. 2015	2	9,88	5,80	31,22
259	*Stachybotrys chartarum* (Ehrenberg 1818) S. Hughes 1958	9	18,27	5,33	31,88
260	*Stachybotrys cylindrospora* C.N. Jensen 1912	1	31,95	10,57	31,91
261	*Stemphyliomma* sp.	1	10,57	5,52	31,91
262	*Stemphylium botryosum* Wallroth 1833	1	10,59	10,59	31,93
263	*Stemphylium sarciniforme* (Cavara 1890) Wiltshire 1938	1	3,17	6,67	31,93
264	*Striaticonidium brachysporum* (Nicot 1961) L. Lombard et Crous 2016	1	10,37	6,31	31,70
265	*Striaticonidium cinctum* (Corda 1842) L. Lombard et Crous 2016	1	0,01	2,35	5,16
266	*Syncephalastrum racemosum* Cohn ex J. Schroeter 1886	6	18,97	8,06	30,08
267	*Syncephalis cornu* van Tieghem et G. Le Monnier 1873	1	18,62	18,62	30,48
268	*Thamnidium elegans* Link 1809	2	19,05	6,77	29,2
269	*Thamnostylum piriforme* (Bainier 1880) Arx et H.P. Upadhyay 1970	2	16,7	23,5	30,35
270	*Thysanophora canadensis* Stolk et Hennebert 1968	1	0,90	3,50	32,26
271	*Thysanophora penicillioides* (Roumeguere 1890) W.B. Kendrick 1961	4	0,61	2,13	18,34
272	*Torula ligniperda* (Willkomm 1866) Saccardo 1906	1	31,91	1,28	31,91

(continued)

Table 1.1 (continued)

No.	Name of species	Number of strains	Storage time at different temperature (years)		
			5 °C	−12 °C	−70 °C
273	*Trichocladium asperum* Harz 1871	1	32,13	3,31	32,07
274	*Trichocladium griseum* (Traaen 1914) X. Wei Wang et Houbraken 2018	2	15,71	1,31	26,17
275	*Trichocladium nigrospermum* (Schweinitz 1832) X. Wei Wang et Houbraken 2018	1	10,83	10,83	32,18
276	*Trichoderma deliquescens* (Sopp 1912) Jaklitsch 2011	1	31,15	9,79	31,15
277	*Truncatella angustata* (Persoon 1801) S. Hughes 1958	1	1,05	1,05	31,68
278	*Umbelopsis isabellina* (Oudemans 1902) W. Gams 2003	6	16,65	6,59	29,24
279	*Umbelopsis longicollis* (Dixon-Stewart 1932) Y.N. Wang et al. 2015	3	20,11	5,82	28,9
280	*Umbelopsis nana* (Linnemann 1941) Arx 1984	2	7,07	4,29	29,78
281	*Umbelopsis ramanniana* (Moeller 1903) W. Gams 2003	6	14,18	8,67	29,29
282	*Umbelopsis vinacea* (Dixon-Stewart 1932) Arx 1984	1	4,34	3,34	29,41

The analysis of the results testifies that this method has proved very successful for the storage of most of the investigated fungi within 3–7 years (Table 1.2).

Where it is desired to keep and constantly to renew cultures within 1–2 years, a temperature of 5 °C is perfectly applicable. More than 97% of the studied zygomycetous fungi and 94% of ascomycetous fungi were viable after storage. For long-term (more than 10 years) storage, however, this temperature is not reliable, since the viability of fungi in both groups is reduced to 57% and 55 % respectively.

A temperature of −12 °C is least favorable for long storage. Only 60% of zygomycetous fungi and 35% of ascomycetous fungi stored at such a temperature were viable after 10 years. After 17–20 years viability decreased to 10–13% and 4%, respectively (Fig. 1.1). Among zygomycetous fungi representatives of the classes *Mortierellomycetes*, *Entomophthoromycetes*, and *Kickxellomycetes* lost their vitality most rapidly at these temperatures. After 17 years of storage, their viability decreased to 0–4%. In contrast, the strains from the psychrotolerant species *Helicostylum elegans* and *Thamnostylum piriforme* and thermotolerant species *Rhizomucor pusillus* remained steady. Among dark-colored anamorphic ascomycetous fungi the best viability at temperature −12 °C after 20 years was found in strains of the genera *Acrophialophora*, *Alternaria*, *Coniothyrium*, *Gonytrichum*, *Hormoconis*, *Paraconiothyrium*, *and Phialophora*. After 30 years, only 1 strain (*Hormoconis resinae)* was viable.

Table 1.2 Viability (%) of different taxa of VKM fungi after long-term preservation (30 years) at various temperatures on silica gel

Subkindom	Division	Class	Viability (%) after long-term preservation at different temperature (°C)														
			1–2 year			3–7 year			Near 10 year			Near 20 year			Near 30 yearr		
			5	−12	−70	5	−12	−70	5	−12	−70	5	−12	−70	5	−12	−70
Dikarya	*Ascomycota*	*Dothideomycetes* (27 genera, 77 species, 144 strains)	98	99	100	83	86	97	64	41	96	38	4	95	23	0	93
		Eurotiomycetes (7 genera, 12 species, 22 strains)	91	96	100	65	74	100	39	35	91	22	13	91	9	4	91
		Insertae Sedis (6 genera, 9 species, 9 strains)	89	100	100	67	56	100	22	11	89	11	11	67	11	0	56
		Leotiomycetes (5 genera, 13 species, 27 strains)	81	89	93	48	63	85	30	19	67	4	0	63	4	0	63
		Ascomycetes (1 genus, 1 species, 1 strains)	100	100	100	100	100	100	100	100	100	0	0	100	0	0	100
		Saccharomycetes (1 genus, 1 species, 1 strain)	100	100	100	100	100	100	100	0	100	0	0	100	0	0	100
		Sordariomycetes (32 genera, 51 species, 87 strains)	94	100	100	75	68	99	55	34	93	39	1	92	23	0	91
Mucoromyceta	*Mortierellomycota*	*Mortierellomycetes* (1 genus, 18 species, 27 strains)	78	86	100	41	53	96	17	16	94	4	4	74	0	0	74
	Mucoromycota	*Mucoromycetes* (28 genera, 89 species, 280 strains)	99	97	100	89	92	100	79	68	99	63	63	98	7	1	98

(continued)

Table 1.2 (continued)

Subkindom	Division	Class	Viability (%) after long-term preservation at different temperature (°C)													
			1–2 year			3–7 year			Near 10 year			Near 20 year			Near 30 yearr		
			5	−12	−70	5	−12	−70	5	−12	−70	5	−12	−70	5	−12	−70
		Umbelopsidomycetes (1 genus, 5 species, 18 strains)	100	100	100	100	96	100	94	61	100	74	74	100	11	6	100
Zoopagomyceta	*Entomophthoro-mycota*	*Entomophthoromycetes* (2 genera, 3 species, 3 strains)	67	67	67	22	33	67	0	22	67	0	0	67	0	0	67
	Kickxellomycota	*Kickxellomycetes* (2 genera, 2 species, 2 strains)	100	100	100	50	50	100	17	17	100	0	0	100	0	0	50
	Zoopagomycota	*Zoopagomycetes* (1 genus, 1 species, 1 strain)	100	100	100	100	100	100	100	100	100	100	100	100	0	0	100

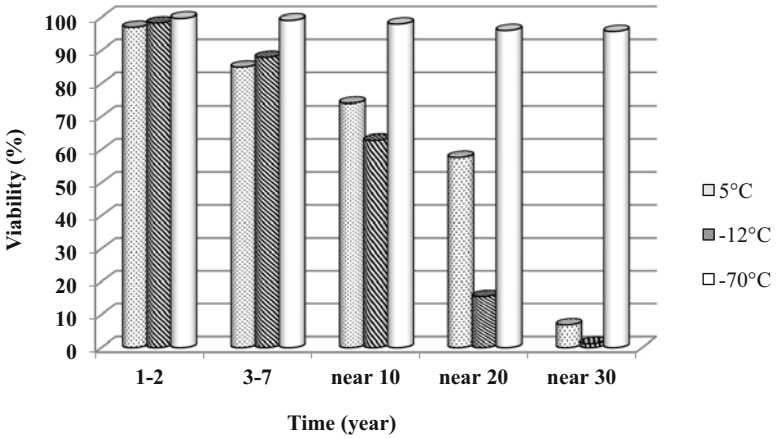

Fig. 1.1 The long-term preservation of zygomycetous fungi on silica gel at different temperatures

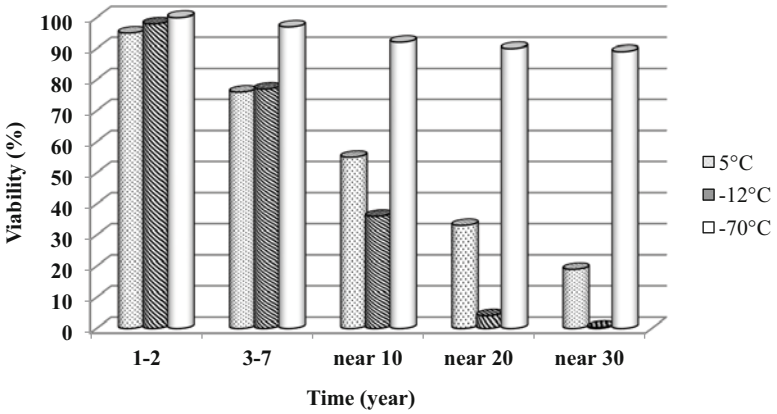

Fig. 1.2 The long-term preservation of ascomycetous fungi on silica gel at different temperatures

The most acceptable temperature for the storage of mycelial fungi is the temperature −70 °C. In these conditions after 30 years of storage, 90% of strains were viable (Fig. 1.1).

The advantages of storing mycelial fungi at different temperatures on silica gel are obvious. On one hand, this method is so simple that the storage at 5 °C and −12 °C can be carried out for the most part in poorly equipped laboratories. On the other hand, the presence of a low-temperature refrigerator (−70 °C) means it is possible to support large numbers of cultures in a small area. The advantages of this method are also a minimum of preparatory work, the rapid reconstituted part of the stored

material by transferring a few granules on appropriate culture medium, as well as the possibility of using the same vial without defrosting for a long time.

The cooling equipment being used in VKM is ultralow temperature freezers (−70–80 °C, Sanyo, Japan) and household refrigerators (5 and −12 °C).

1.6 Protocols

Protocols of cryopreservation, freeze-drying, and drying in sterile soil were described earlier [15].

1.7 Protocol of Drying on Silica Gel

1.7.1 Preparation of Sterile Silica Gel and Ampoules

- Silica gel is pre-dried and sterilized by dry heat for 3 h at a temperature of 160 °C, conducting careful control of sterility.
- Plastic ampoules (Nunc) (3 for each culture) are labeled and sterilized by autoclaving, at 121 °C for 20 min.
- Sterile silica gel that has been washed with a concentration of cobalt chloride is placed in the ampoules to indicate the humidity. The cobalt chloride is deep blue when dry and turns pink when wet.
- A sterile cotton ball is placed on top of the indicator.

1.7.2 Preparation of Cryoprotectant: 10% (v/v) Glycerol

- Pour 5 mL of 10% glycerol into 12 mL glass tubes.
- Sterilized by autoclaving at 121 °C for 20 min.
- Stored at +5 °C for no longer than a month.

1.7.3 Preparation of Cultures

- Grow sporulating fungal cultures on slant agar under optimal growth conditions and on suitable mediums (www.vkm.ru).
- Wash off spores from agar surface with 5 mL of cool sterile 10% glycerol.
- Titer of spores' suspension should be not less than 10^6 spores/mL.

1.7.4 Silica Gel Inoculation

- Add 75–100 silica gel granules (40 grade, 9–16 mesh) in a sterile Petri dish.
- Add 1 mL spore suspension to sterile and dry silica gel.
- Shake the Petri dish with the granules.
- Put the Petri dish in desiccator and store in the refrigerator 12 h at 4–7 °C.

1.7.5 Filling of Vials

- Add silica gel granules with fungal spores (20–25 pieces) to 3 plastic ampoules with a sterile spoon.
- Place cryovials in the boxes and transfer them to the refrigerators (5 and −12 °C) and the ultralow temperature freezer (−70 °C).

1.7.6 Control of Viability

- Place ampoule in a special metal container, thermostatic inside by expanded polystyrene, to prevent defrosting.
- Transfer one granule of silica gel from ampoule on fresh suitable agar medium and incubate under optimal conditions.
- The remaining granules were resealed and stored as described. Thus, each ampoule with fungal spores adsorbed on silica gel may be used repeatedly.

Result

The real storage time estimates obtained in VKM are given in Table 1 and Annex 2. They are not final data: the cultures are still being stored, and we expect to get longer storage times later on. Some cells of the table are empty; this is the case if the culture is not stored by this method.

There is at present clear that more than 98% of fungal cultures preserved by cryoconservation method remain viable after 20 years of storage. For lyophilization and storage in sterile soil methods, these figures after 30 years of storage are 95 and 85%, respectively. For long-term storage of fungal cultures on silica gel, the temperature −70 °C should be chosen. At this temperature, over 90% of spore-forming fungi retain their viability after 30 years of the experiment.

Conclusion

The conservation techniques used in VKM presents effective preservation of the stock of filamentous fungi from different taxonomic groups. The possibility and practical time estimates of secure long-term storage of fungal cultures belonging to 1600 species and 590 genera were shown. The represented information could be used as a reference for researchers intending to maintain pure cultures of microorganisms for a long time. The data produced are also accessible online on the VKM Web site.

Annexies

Annex 1: Fields Attributes in the Table «Database Preservation Methods»

Code	Counter
EntryDate	Date/Time
Method	Text
Col	Text
Strain	Numerical
dep	Text
pat	Text
Curator	Text
Dubl-cart	Text
Dubl fond	Text
Dubl fond new	Text
Ampules	Numerical
Data	Date/Time
Result	Text
Data2	Date/Time
Result2	Text
Days	Numerical
Year	Numerical
Comments	Text
Data3	Date/Time
Result3	Text
Data4	Date/Time
Result4	Text
Data5	Date/Time
Result5	Text
EditDate	Date/Time
Protector	Text
Programm	Text
Location	Text
Type	Text

Annex 2: Maximal Preservation Times for VKM Fungal Species

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
1	*Absidia caerulea* Bainier 1889	5	19.70	5	45.95	5	12.42
2	*Absidia cuneospora* G.F. Orr et Plunkett 1959	1	25.41	1	27.38		
3	*Absidia cylindrospora* Hagem 1908			2	31.17	2	37.64
4	*Absidia glauca* Hagem 1908	8	24.13	10	40.90	8	49.99
5	*Absidia repens* van Tieghem 1878	1	23.67	1	45.19		
6	*Absidia spinosa* Lendner 1907	2	19.75	2	39.43	2	23.32
7	*Achlya bisexualis* Coker et Couch 1927	4	0.51				
8	*Achlya bonariensis* Beroqui 1969	1	0.16				
9	*Achlya colorata* Pringsheim 1882	2	6.32				
10	*Achlya intricata* Beneke 1948	1	0.15				
11	*Achlya radiosa* Maurizio 1899	1	28.12				
12	*Achlya sparrowii* Reischer 1949	1	23.01				
13	*Acladium curvatum* Bonorden 1851			1	35.41	1	0.73
14	*Acremonium alternatum* Link 1809	3	17.73	4	30.79	3	2.72
15	*Acremonium arxii* W. Gams 1971	2	19.46	2	27.32	2	3.20
16	*Acremonium atrogriseum* (Panasenko 1964) W. Gams 1971	3	17.47	3	32.65		
17	*Acremonium bacillisporum* (Onions et G.L. Barron 1967) W. Gams 1971	2	22.13	3	22.08		
18	*Acremonium bactrocephalum* W. Gams 1971	1	22.13	5	25.25	1	0.53
19	*Acremonium biseptum* W. Gams 1971			1	25.30		
20	*Acremonium breve* (Sukapure et Thirumalachar 1966) W. Gams 1971	3	19.31	12	40.33	1	3.56
21	*Acremonium cavaraeanum* (Jasevoli 1924) W. Gams 1971	1	19.93	1	6.05		
22	*Acremonium cereale* (P. Karsten 1887) W. Gams 1971	1	17.75	1	19.92	1	3.27
23	*Acremonium charticola* (J. Lindau 1907) W. Gams 1971	4	21.04	8	25.98	1	0.09
24	*Acremonium chrysogenum* (Schol-Schwarz 1965) W. Gams 1971			2	9.96		
25	*Acremonium crotocinigenum* (Schol-Schwarz 1965) W. Gams 1971			4	32.50	3	2.72
26	*Acremonium cymosum* W. Gams 1971	2	6.54	2	28.44		
27	*Acremonium domschii* W. Gams 1971			2	28.36	2	2.84
28	*Acremonium egyptiacum* (J.F.H. Beyma 1933) W. Gams 1971	1	16.93	1	29.72	1	0.09
29	*Acremonium fuci* Summerbell et al. 2004			3	2.15		
30	*Acremonium hyalinulum* (Saccardo 1878) W. Gams 1971			2	31.29		
31	*Acremonium implicatum* (J.C. Gilman et E.V. Abbott 1927) W. Gams 1975	2	19.57	5	27.42	3	0.68
32	*Acremonium incrustatum* W. Gams 1971	2	17.73	2	25.80	1	3.49

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
33	*Acremonium kiliense* Gruetz 1925	4	22.13	4	26.11	2	0.09
34	*Acremonium lichenicola* W. Gams 1971			1	24.26		
35	*Acremonium murorum* (Corda 1839) W. Gams 1971			11	5.74		
36	*Acremonium persicinum* (Nicot 1958) W. Gams 1971	2	19.37	2	43.88	1	26.53
37	*Acremonium polychromum* (J.F.H. Beyma 1928) W. Gams 1971	5	29.82	5	35.47	3	0.09
38	*Acremonium rutilum* W. Gams 1971	1	8.02	1	19.10		
39	*Acremonium salmoneum* W. Gams et Lodha 1975			4	2.70		
40	*Acremonium sclerotigenum* (Moreau et R. Moreau 1941 ex Valenta 1948) *W.* Gams 1971	3	20.23	3	36.56	2	3.73
41	*Acremonium strictum* W. Gams 1971	19	19.81	24	39.59	17	9.71
42	*Acremonium tubakii* W. Gams 1971			2	31.23		
43	*Acrophialophora fusispora* (S.B. Saksena 1953) Samson 1970	1	17.49	1	30.36		
44	*Acrostalagmus albus* Preuss 1851	1	19.59	1	32.45	1	3.51
45	*Acrostalagmus luteoalbus* (Link 1809) Zare et al. 2004	12	19.35	12	37.43	5	13.56
46	*Acrothecium robustum* J.C. Gilman et E.V. Abbott 1927	1	19.30	1	31.30	1	25.73
47	*Actinomucor elegans* (Eidam 1884) C.R. Benjamin et Hesseltine 1957	5	13.17	7	42.07	7	50.21
48	*Agaricus arvensis* Schaeffer 1774	1	20.04				
49	*Agaricus bisporus* (J. Lange 1926) Imbach 1946	35	26.07				
50	*Agaricus squarrosus* Oeder 1770	1	23.59				
51	*Albifimbria verrucaria* (Albertini et Schweinitz 1805) L. Lombard et Crous *2016*	3	19.83	3	48.27	1	2.57
52	*Albonectria rigidiuscula* (Berkeley et Broome 1875) Rossman et Samuels 1999	1	19.85	1	34.34	1	7.45
53	*Alternaria alternariae* (Cooke 1871) Woudenberg et Crous 2013	2	20.53	2	32.26		
54	*Alternaria alternata* (Fries 1832) Keissler 1912	13	19.30	43	48.71		
55	*Alternaria atra* (Preuss 1852) Woudenberg et Crous 2013	9	19.60	13	48.35	1	8.34
56	*Alternaria botrytis* (Preuss 1851) Woudenberg et Crous 2013	12	19.56	19	48.41	1	35.79
57	*Alternaria brassicae* (Berkeley 1836) Saccardo 1880	4	19.78	3	0.50		
58	*Alternaria brassicicola* (Schweinitz 1832) Wiltshire 1947	6	19.28	7	43.57		
59	*Alternaria chartarum* Preuss 1848	8	19.46	22	45.05		
60	*Alternaria cheiranthi* (Libert 1827) P.C. Bolle 1924	1	19.79	1	19.67		
61	*Alternaria consortialis* (Thuemen 1876) Groves et Hughes 1953	4	19.49	4	45.05		
62		1	19.79				

	Alternaria cucumerina (Ellis et Everhart 1895) J.A. Elliott 1917 var. *cucumerina*						
63	*Alternaria dauci* (J.G. Kuehn 1855) J.W. Groves et Skolko 1944	5	19.79				
64	*Alternaria dianthicola* Neergaard 1945	1	19.78				
65	*Alternaria geophila* Daszewska 1912	1	12.56				
66	*Alternaria godetiae* (Neergaard 1933) Neergaard 1945	1	12.56				
67	*Alternaria grandis* E.G. Simmons 2000			1	1.74		
68	*Alternaria japonica* Yoshii 1941	3	19.32	3	27.64		
69	*Alternaria leucanthemi* Nelen 1962	1	19.78	1	24.95		
70	*Alternaria macrospora* Zimmermann 1904	2	12.56	2	30.03		
71	*Alternaria multirostrata* E.G. Simmons et C.R. Jackson 1968	1	17.80	1	11.28		
72	*Alternaria nobilis* (Vize 1877) E.G. Simmons 2002	1	19.78				
73	*Alternaria oudemansii* (E.G. Simmons 1967) Woudenberg et Crous 2013	2	19.32	1	42.78		
74	*Alternaria radicina* Meier et al. 1922	2	17.60	2	38.20		
75	*Alternaria silybi* Gannibal 2011			3	1.97		
76	*Alternaria simmonsii* Gannibal 2011			2	1.97		
77	*Alternaria solani* Sorauer 1896	6	19.78	4	30.40		
78	*Alternaria tenuissima* (Kunze 1818) Wiltshire 1933			6	26.81		
79	*Alternariaster helianthi* (Hansford 1943) E.G. Simmons 2007			2	7.12		
80	*Amanita citrina* (Schaeffer 1762) Persoon 1797	1	17.92				
81	*Amauroascus aureus* (Eidam 1887) Arx 1971	1	6.18	1	33.15		
82	*Amblyosporium botrytis* Fresenius 1863	2	15.34	2	31.28	2	12.18
83	*Amerosporium concinnum* Petrak 1953	1	19.54	1	47.12		
84	*Ampelomyces artemisiae* (Voglino 1905) Rudakov 1979	1	12.58	1	11.42		
85	*Ampelomyces heraclei* (Dejeva 1967) Rudakov 1979	1	12.18	1	13.21		
86	*Ampelomyces humuli* (Fautrey 1890) Rudakov 1979			1	16.55		
87	*Ampelomyces polygoni* (Potebnia 1907) Rudakov 1979	2	12.28	2	34.50		
88	*Ampelomyces quercinus* (Sydow 1915) Rudakov 1979			2	0.02		
89	*Ampelomyces quisqualis* Cesati 1852	1	12.58	1	29.31		
90	*Ampelomyces ulicis* (Adams 1907) Rudakov 1979	1	12.38	2	29.15		
91	*Ampelomyces uncinulae* (Fautrey 1893) Rudakov 1979	1	12.58	1	40.64		
92	*Antrodia sinuosa* (Fries 1821) P. Karsten 1881	1	8.16				
93	*Apenidiella antarctica* Ivanushkina et al. 2019			1	7.34		
94	*Apenidiella strumelloidea* (Milko et Dunaev 1986) W. Quaedvlieg et P.W. *Crous* 2014			1	29.09		

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
95	*Aphanoascus fulvescens* (Cooke 1879) Apinis 1968	1	20.42	1	43.15	1	21.16
96	*Aphanocladium album* (Preuss 1848) W. Gams 1971	4	17.73	4	27.66		
97	*Aphanomyces helicoides* Minden 1915	1	33.22				
98	*Apiospora montagnei* Saccardo 1875			1	9.90		
99	*Aplanes treleaseanus* (Humphrey 1893) Coker 1927	1	15.93				
100	*Aposphaeria caespitosa* (Fuckel 1869) Jaczewski 1917	1	19.21	1	36.11		
101	*Arachniotus aurantiacus* (Kamyschko 1967) Arx 1971			1	43.59	1	21.16
102	*Arcticomyces warmingii* (Rostrup 1888) Savile 1959	1	24.47	1	1.64		
103	*Armillaria cepistipes* Velenovsky 1920	6	13.55				
104	*Armillaria lutea* Gillet 1874	5	23.45				
105	*Armillaria mellea* (Vahl 1790) P. Kummer 1871	5	33.22				
106	*Arthrinium arundinis* (Corda 1838) Dyko et Sutton 1981	3	12.58	12	24.70		
107	*Arthrinium phaeospermum* (Corda 1837) M.B. Ellis 1965			2	13.34		
108	*Arthrinium saccharicola* F. Stevens 1917			1	16.91		
109	*Arthrinium sphaerospermum* Fuckel 1874	1	19.21	3	47.40	1	3.85
110	*Arthrobotrys arthrobotryoides* (Berlese 1888) J. Lindau 1907	1	19.32			1	3.91
111	*Arthrobotrys cladodes* Drechsler 1937	2	15.74	2	23.73	2	7.77
112	*Arthrobotrys conoides* Drechsler 1937	4	19.32	4	9.44	3	13.48
113	*Arthrobotrys longa* Mekhtieva 1973	1	10.65	1	9.96	1	0.53
114	*Arthrobotrys longispora* Preuss 1853	1	25.39			1	3.91
115	*Arthrobotrys oligospora* Fresenius 1850	6	27.60	7	24.76	7	7.81
116	*Arthrobotrys oviformis* Soprunov 1958			1	2.40	1	0.19
117	*Arthrobotrys robusta* Duddington 1951	1	19.41	1	31.56	1	7.99
118	*Arthrobotrys superba* Corda 1839	7	26.36	7	26.32	7	3.91
119	*Ascochyta cucumeris* Fautrey et Roumeguere 1891	2	20.34	2	27.91		
120	*Ascochyta malvicola* Saccardo 1878	1	19.22	1	11.74		
121	*Ascochyta pisi* Libert 1830	3	19.45	1	44.50	1	8.67
122	*Ascochyta viciae* Libert 1837	1	20.31	1	26.76		
123	*Ascotricha chartarum* Berkeley 1838	1	18.97	1	10.75		
124	*Aspergillus aculeatus* Iizuka 1953			7	18.19	2	0.35
125	*Aspergillus alliaceus* Thom et Church 1926			6	41.09	5	30.10
126	*Aspergillus amylovorus* Panasenko 1964 ex Samson 1979	1	12.47	1	27.12	1	27.64

127	*Aspergillus asperescens* Stolk 1954			2	9.95	2	0.50
128	*Aspergillus aureolatus* Muntanola-Cvetkovic et Bata 1964			1	37.60	1	9.58
129	*Aspergillus aureoterreus* Samson et al. 2011			1	33.83	1	33.41
130	*Aspergillus awamori* Nakazawa 1915			13	45.82	13	37.03
131	*Aspergillus awamori* Nakazawa 1915 var. *fumeus* Nakazawa et al. 1936			1	17.96	1	30.17
132	*Aspergillus brasiliensis* Varga et al. 2007			2	36.25	2	22.13
133	*Aspergillus brunneouniseriatus* Suj. Singh et B.K. Bakshi 1961			1	21.90		
134	*Aspergillus caespitosus* Raper et Thom 1944			2	30.61	2	19.26
135	*Aspergillus calidoustus* Varga et al. 2008			7	20.53		
136	*Aspergillus candidus* Link 1809			11	39.94	6	46.75
137	*Aspergillus carbonarius* (Bainier 1880) Thom 1916			2	40.31	2	25.81
138	*Aspergillus carneus* (van Tieghem 1877) Blochwitz in Thom and Raper 1945			5	39.44	2	30.62
139	*Aspergillus clavatus* Desmazieres 1834			10	44.38	9	46.84
140	*Aspergillus crustosus* Raper et Fennell 1965					1	0.15
141	*Aspergillus duricaulis* Raper et Fennell 1965			1	21.55	1	21.92
142	*Aspergillus echinulatus* (Delacroix 1893) Thom et Church 1926			1	9.60	1	38.16
143	*Aspergillus ficuum* (Reichardt 1867) Thom et Currie 1916			2	15.58	1	20.92
144	*Aspergillus fischeri* Wehmer 1907			7	38.87	6	47.71
145	*Aspergillus flavipes* (Bainier et R. Sartory 1911) Thom et Church 1926			8	41.10	7	20.90
146	*Aspergillus flavus* Link 1809			16	41.63	12	46.87
147	*Aspergillus flavus* Link 1809 var. *columnaris* Raper et Fennell 1965			1	32.17	1	37.25
148	*Aspergillus foetidus* Thom et Raper 1945			2	30.88	2	27.21
149	*Aspergillus fumigatus* Fresenius 1863			15	44.89	10	46.83
150	*Aspergillus giganteus* Wehmer 1901			3	42.69	3	35.55
151	*Aspergillus gorakhpurensis* Kamal et Bhargava 1969					1	0.12
152	*Aspergillus hennebergii* Blochwitz 1935			1	6.46	1	0.14
153	*Aspergillus heteromorphus* Batista et H. Maia 1957			1	27.48	1	3.35
154	*Aspergillus insuetus* (Bainier 1908) Thom et Church 1929			1	15.90	1	37.09
155	*Aspergillus janus* Raper et Thom 1944			2	36.04	2	46.78
156	*Aspergillus japonicus* Saito 1906			10	34.48	8	32.73
157	*Aspergillus kanagawaensis* Nehira 1951			2	39.62	2	42.25
158	*Aspergillus melleus* Yukawa 1911			4	39.08	4	20.75
159	*Aspergillus neoafricanus* Samson et al. 2011			1	36.34	1	33.42
160	*Aspergillus neoniveus* Samson et al. 2011					1	0.37
161	*Aspergillus nidulans* (Eidam 1883) G. Winter 1884			13	48.25	9	47.04

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
162	*Aspergillus niger* van Tieghem 1867			109	44.57	30	46.83
163	*Aspergillus niveus* Blochwitz 1929			6	37.67	4	37.78
164	*Aspergillus nomius* Kurtzman et al. 1987					2	0.07
165	*Aspergillus nutans* McLennan et Ducker 1954			2	46.76	2	37.38
166	*Aspergillus ochraceus* G. Wilhelm 1877			21	43.70	16	36.29
167	*Aspergillus oryzae* (Ahlburg 1878) E. Cohn 1884			25	43.68	19	42.05
168	*Aspergillus oryzae* (Ahlburg 1878) E. Cohn 1884 var. *effusus* (Tiraboschi 1908) Y. Ohara 1951			1	34.42	1	9.58
169	*Aspergillus pallidus* Kamyschko 1963	1	12.47	1	18.59	1	48.04
170	*Aspergillus parasiticus* Speare 1912			2	3.30		
171	*Aspergillus parvulus* G. Smith 1961			1	46.73	1	30.34
172	*Aspergillus penicilliformis* Kamyschko 1963			1	38.97	1	28.21
173	*Aspergillus penicillioides* Spegazzini 1896	1	6.90	3	5.91		
174	*Aspergillus phoenicis* (Corda 1840) Thom et Currie 1916			5	32.96	1	7.66
175	*Aspergillus proliferans* G. Smith 1943			1	10.75	1	11.13
176	*Aspergillus pseudodeflectus* Samson et Mouchacca 1975			1	36.19	1	38.11
177	*Aspergillus puniceus* Kwon-Chung et Fennell 1965			3	31.40	1	4.32
178	*Aspergillus quadrilineatus* Thom et Raper 1939			3	39.84	3	30.07
179	*Aspergillus raperi* Stolk et J.A. Meyer 1957					2	0.37
180	*Aspergillus repens* (Corda 1842) Saccardo 1882			11	47.00	11	46.92
181	*Aspergillus restrictus* G. Smith 1931			1	24.65	1	24.65
182	*Aspergillus rugulosus* Thom et Raper 1939			5	37.90	5	37.05
183	*Aspergillus sclerotiorum* G.A. Huber 1933			5	31.55	3	30.64
184	*Aspergillus silvaticus* Fennell et Raper 1955			1	36.62	1	33.75
185	*Aspergillus sojae* Sakaguchi et K. Yamada ex Murakami 1971			1	37.49	1	36.16
186	*Aspergillus stellatus* Curzi 1934			2	36.15	2	37.24
187	*Aspergillus subsessilis* Raper et Fennell 1965	1	12.44	2	46.65	1	26.30
188	*Aspergillus sulphureus* (Fresenius 1863) Wehmer 1901			3	40.12	1	42.08
189	*Aspergillus sydowii* (Bainier et R. Sartory 1913) Thom et Church 1926			30	38.84	7	46.83
190	*Aspergillus tamarii* Kita 1913			3	38.72	1	42.06
191	*Aspergillus terreus* Thom 1918	8	6.68	28	47.42	25	47.04
192	*Aspergillus terricola* Marchal et É.J. Marchal 1893			3	36.09	3	46.84

193	*Aspergillus terricola* Marchal et É.J. Marchal 1893 var. *americanus* Marchal et É.J. Marchal 1921			1	33.83	1	7.34
194	*Aspergillus tubingensis* Mosseray 1934			1	12.64	1	0.19
195	*Aspergillus umbrosus* Bainier et R. Sartory 1912			1	13.10	1	0.23
196	*Aspergillus unguis* (Weill et L. Gaudin 1919) Dodge 1935	3	12.47	5	41.10	5	42.25
197	*Aspergillus ustus* (Bainier 1881) Thom et Church 1926			17	42.48	13	46.82
198	*Aspergillus varians* Wehmer 1897			1	18.57	1	25.81
199	*Aspergillus versicolor* (Vuillemin 1903) Tiraboschi 1908			46	42.57	12	46.87
200	*Aspergillus viridinutans* Ducker et Thrower 1954			2	28.31	1	47.20
201	*Aspergillus wentii* Wehmer 1896			12	40.01	6	27.64
202	*Asterosporium orientale* Melnik 1988			1	0.07		
203	*Athelia rolfsii* (Curzi 1932) C.C. Tu et Kimbrough 1978	1	19.30				
204	*Aureobasidium melanogenum* (Hermanides-Nijhof 1977) Zalar et al. 2014			9	46.31	2	12.60
205	*Aureobasidium microstictum* (Bubak 1907) W.B. Cooke 1962	1	19.85	3	35.55		
206	*Aureobasidium pullulans* (de Bary 1866) G. Arnaud 1918	14	20.22	29	47.98	3	5.00
207	*Backusella circina* J.J. Ellis et Hesseltine 1969	2	23.57	2	44.37		
208	*Backusella indica* (Baijal et B.S. Mehrotra 1965) G. Walther et *de* Hoog 2013	1	20.66	1	25.49	1	20.12
209	*Backusella lamprospora* (Lendner 1908) Benny et R.K. Benjamin 1975	2	25.41	4	39.34	3	8.78
210	*Backusella oblongiellipticа* (H. Naganishi et al. ex Pidoplichko et Milko *1971)* G. Walther et de Hoog 2013	1	20.62	1	34.63		
211	*Backusella recurva* (E.E. Butler 1952) G. Walther et de *Hoog* 2013	1	25.31	1	19.65		
212	*Backusella tuberculispora* (Schipper 1978) G. Walther et de Hoog 2013	1	25.31	1	36.53		
213	*Backusella variabilis* (A.K. Sarbhoy 1965) G. Walther et de Hoog 2013	1	19.59	1	46.64	1	19.25
214	*Basidiobolus magnus* Drechsler 1964	1	20.06				
215	*Basidiobolus meristosporus* Drechsler 1955	1	20.21				
216	*Beauveria bassiana* (Balsamo-Crivelli 1835) Vuillemin 1912	12	29.66	14	46.45	12	22.79
217	*Beauveria brongniartii* (Saccardo 1892) Petch 1926	6	19.50	6	33.01	5	10.92
218	*Beauveria caledonica* Bissett et Widden 1988			1	3.00	1	0.70
219	*Beauveria felina* (De Candolle 1815) J.W. Carmichael 1980			1	13.51	1	0.60
220	*Benjaminiella poitrasii* (R.K. Benjamin 1960) Arx 1981	2	19.56	2	38.65	2	46.30
221	*Berkeleyomyces basicola* (Berkeley et Broome 1850) W.J. Nel et al. 2017	2	19.51	2	39.60		
222	*Bionectria ochroleuca* (Schweinitz 1832) Schroers et Samuels 1997			1	26.61		
223	*Bipolaris australiensis* (M.B. Ellis 1971) Tsuda et Ueyama 1981	6	19.30	14	49.80	2	8.53
224	*Bipolaris bicolor* (Mitra 1931) Shoemaker 1959			1	24.97		

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
225	*Bipolaris cynodontis* (Marignoni 1909) Shoemaker 1959	4	19.12	4	49.79	1	8.43
226	*Bipolaris sorokiniana* (Saccardo 1890) Shoemaker 1959	4	19.35	8	49.80		
227	*Bipolaris spicifera* (Bainier 1908) Subramanian 1971			1	23.92		
228	*Bipolaris victoriae* (F. Meehan et H.C. Murphy 1946) Shoemaker 1959	1	17.47	1	26.99		
229	*Biscogniauxia nummularia* (Bulliard 1790) Kuntze 1891	1	19.32	1	7.17	1	0.10
230	*Bispora antennata* (Persoon 1801) E.W. Mason 1953	2	19.16	3	27.07		
231	*Bispora betulina* (Corda 1838) S. Hughes 1958	2	17.45	2	34.55		
232	*Bispora effusa* Peck 1891	1	20.22	1	15.09		
233	*Bjerkandera adusta* (Willdenow 1787) P. Karsten 1879	7	8.31				
234	*Blakeslea trispora* Thaxter 1914	14	26.15	16	48.52	7	11.74
235	*Blumeriella jaapii* (Rehm 1907) Arx 1961	1	8.61	1	9.62		
236	*Boeremia hedericola* (Durieu et Montagne 1855) Aveskamp et al. 2010			1	4.61		
237	*Boeremia lycopersici* (Cooke 1885) Aveskamp et al. 2010			1	14.02		
238	*Botryodiplodia malorum* (Berkeley 1836) Petrak et Sydow 1926	1	20.00				
239	*Botryosphaeria rhodina* (Berkeley et M.A. Curtis 1889) Arx 1970	1	19.98	1	26.19	1	0.12
240	*Botryosporium longibrachiatum* (Oudemans 1890) Maire 1903	2	21.41				
241	*Botryotinia narcissicola* (P.H. Gregory 1941) N.F. Buchwald 1949	1	18.96	1	28.02	1	0.11
242	*Botryotinia polyblastis* (P.H. Gregory 1938) N.F. Buchwald 1949			1	27.67		
243	*Botryotrichum piluliferum* Saccardo et Marchal 1885	6	19.23	7	48.07		
244	*Botryotrichum verrucosum* (Pugh et al. 1964) X. Wei Wang et Houbraken 2018			1	17.82		
245	*Botryoxylon geniculatum* (Corda 1839) Ciferri 1962	1	17.71	1	28.41		
246	*Botrytis aclada* Fresenius 1850	2	19.30	2	37.80		
247	*Botrytis anthophila* Bondartsev 1913	2	19.20	2	40.66		
248	*Botrytis bifurcata* J.H. Miller et al. 1958			1	6.88		
249	*Botrytis cinerea* Persoon 1794	14	19.23	24	37.74		
250	*Botrytis convallariae* (Klebahn 1930) Ondrej 1972 ex Boerema et Hamers *1988*	3	15.32	3	15.42		
251	*Botrytis convoluta* Whetzel et Drayton 1932	2	19.23	2	47.64		
252	*Botrytis fabae* Sardina 1929	1	19.12				
253	*Botrytis galanthina* (Berkeley et Broome 1873) Saccardo 1886	1	19.23	1	27.67		
254	*Botrytis gladiolorum* Timmermans 1941	2	19.30	2	38.79		
255	*Botrytis hyacinthi* Westerdijk et J.F.H. Beyma 1928	1	19.20				

256	*Botrytis lutescens* Saccardo et Roumeguere 1882	1	19.20				
257	*Botrytis squamosa* J.C. Walker 1925	1	19.33				
258	*Botrytis tulipae* (Libert 1830) Lind 1913	1	19.50	1	13.16		
259	*Bovista pusilla* (Batsch 1789) Persoon 1801	1	16.61				
260	*Brachysporium nigrum* (Link 1824) S. Hughes 1958	1	20.58	1	26.08		
261	*Burgoa anomala* (Hotson 1912) Goidanich 1937			1	8.98		
262	*Byssochlamys nivea* Westling 1909	2	19.44	2	36.44	2	44.03
263	*Cadophora fastigiata* Lagerberg et Melin 1928	6	19.41	7	28.68		
264	*Cadophora luteo-olivacea* (J.F.H. Beyma 1940) T.C. Harrington et McNew 2003			3	0.54		
265	*Cadophora malorum* (Kidd et Beaumont 1924) W. Gams 2000	7	19.76	9	45.39		
266	*Cadophora melinii* Nannfeldt 1934	1	19.49	5	45.37		
267	*Calcarisporium arbuscula* Preuss 1851	4	1.99	5	28.45		
268	*Calcarisporium griseum* Spegazzini 1902	3	17.70	3	27.88		
269	*Celosporium* sp.			1	6.56		
270	*Cephalotrichum gorgonifer* (Bainier 1907) Sandoval-Denis et al. 2016	1	17.70	1	27.05		
271	*Cephalotrichum microsporum* (Saccardo 1878) P.M. Kirk 1984			3	11.54		
272	*Cephalotrichum purpureofuscum* (Schweinitz 1832) S. Hughes	1	19.39	2	31.05		
273	*Cephalotrichum stemonitis* (Persoon 1801) Nees 1809	4	19.41	10	36.99	1	5.68
274	*Ceratellopsis equiseticola* (Boudier 1917) Corner 1950	1	23.34				
275	*Ceratocystis adiposa* (E.J. Butler 1906) C. Moreau 1952					1	0.10
276	*Ceratocystis paradoxa* (Dade 1928) C. Moreau 1952	2	19.85	2	40.51	2	26.12
277	*Ceratocystis pilifera* (Fries 1822) C. Moreau 1952	2	18.86	2	39.58		
278	*Cercospora armoraciae* Saccardo 1876	1	20.34	1	17.87	1	0.53
279	*Cercospora beticola* Saccardo 1876			2	25.47		
280	*Cercospora carotae* (Passerini 1887) Kaznowski et Siemaszko 1929	1	20.34	1	23.40		
281	*Cercospora rosicola* Passerini 1875	1	16.67			1	22.04
282	*Cercospora violae* Saccardo 1876			1	23.85		
283	*Ceriporiopsis gilvescens* (Bresadola 1908) Domanski 1963	1	12.58				
284	*Cerrena unicolor* (Bulliard 1788) Murrill 1903	1	12.58				
285	*Chaetocladium brefeldii* van Tieghem et G. Le Monnier 1873	2	17.59	2	38.59	2	16.98
286	*Chaetocladium jonesii* (Berkeley et Broome 1854) Fresenius 1863	1	23.67	1	27.42		
287	*Chaetocytostroma* sp.			1	20.53		
288	*Chaetomidium pilosum* (C. Booth et Shipton 1966) Arx 1975	1	14.46	1	43.99	1	24.33
289	*Chaetomium amesii* Sergeeva 1965	1	19.28	1	31.01	1	24.33

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
290	*Chaetomium angustispirale* Sergeeva 1956	1	19.28	1	43.64	1	23.33
291	*Chaetomium aureum* Chivers 1912	2	20.51	1	42.33	2	23.86
292	*Chaetomium brasiliense* Bat. et Pontual 1948			1	38.87		
293	*Chaetomium crispatum* (Fuckel) Fuckel 1870	1	20.04				
294	*Chaetomium elatum* Kunze 1817	3	20.51	3	43.64	3	24.33
295	*Chaetomium fieberi* Corda 1837			1	32.08	1	11.25
296	*Chaetomium funicola* Cooke 1873	1	19.28	1	26.15	1	0.72
297	*Chaetomium globosum* Kunze 1817	11	19.44	21	48.45	14	28.00
298	*Chaetomium homopilatum* Omvik 1953	1	19.33	2	35.37	2	0.32
299	*Chaetomium indicum* Corda 1840	1	19.28	2	42.28	1	23.47
300	*Chaetomium megalocarpum* Bainier 1910	2	19.28	2	43.64	2	23.47
301	*Chaetomium nozdrenkoae* Sergeeva 1961	1	18.87	1	35.25	1	0.11
302	*Chaetomium perlucidum* Sergeeva 1956	1	19.28	1	35.29	1	0.27
303	*Chaetomium seminis-citrulli* Sergeeva 1956	1	25.68	1	43.67	1	0.11
304	*Chaetomium spirale* Zopf 1881	1	19.28	1	37.32		
305	*Chaetomium subaffine* Sergeeva 1961	1	19.28	1	35.29	1	23.47
306	*Chaetomium subspirilliferum* Sergeeva 1960	1	20.42	1	25.61	1	22.62
307	*Chaetomium trilaterale* Chivers 1912			1	11.80		
308	*Chaunopycnis alba* W. Gams 1979			1	2.01		
309	*Chloridium caesium* (Nees et T. Nees 1818) Réblová et Seifert 2016			1	27.64		
310	*Chloridium virescens* (Persoon 1797) W. Gams et Holubova-Jechova 1976 var. *caudigerum* (Hoehnel 1903) W. Gams et Holubova-Jechova 1976	1	19.67	1	30.31		
311	*Chlorophyllum rhacodes* (Vittadini 1835) Vellinga 2002	1	12.02				
312	*Choanephora conjuncta* Couch 1925	1	3.78				
313	*Choanephora cucurbitarum* (Berkeley et Ravenel 1875) Thaxter 1903	1	20.21	1	20.93		
314	*Choanephora infundibulifera* (Currey 1873) Saccardo 1891	1	25.41	1	33.99		
315	*Chondrostereum purpureum* (Persoon 1794) Pouzar 1959	1	20.10				
316	*Chordomyces antarcticus* Bilanenko et al. 2015			6	8.00		
317	*Chromelosporium fulvum* (Link 1824) McGinty et al. 1975	2	1.88	2	18.07		
318	*Chrysonilia sitophila* (Montagne 1843) Arx 1981			1	43.95	1	22.14
319	*Chrysosporium keratinophilum* D. Frey 1959 ex J.W. Carmichael 1962	2	9.57	2	32.84	1	5.95
320	*Chrysosporium lobatum* Scharapov 1978			1	38.25	1	29.86

321	*Chrysosporium lucknowense* Garg 1966	6	5.62	6	22.03		
322	*Chrysosporium merdarium* (Link 1818 ex Greville 1823) J.W. Carmichael 1962	3	11.87	4	25.63	1	35.73
323	*Chrysosporium queenslandicum* Apinis et R.G. Rees 1976	2	31.45	2	38.32	2	31.26
324	*Chrysosporium tropicum* J.W. Carmichael 1962	3	9.10	3	37.15	2	25.87
325	*Chrysosporium undulatum* P. Vidal et al. 1999	3	20.41	4	42.69	3	35.66
326	*Circinella muscae* (Sorokin 1870) Berlese et de Toni 1888	4	22.71	5	43.04	5	47.39
327	*Circinella umbellata* van Tieghem et G. Le Monnier 1873	1	19.68	1	40.08	1	37.94
328	*Cistella* sp.			2	1.87		
329	*Cladobotryum dendroides* (Bulliard 1791) W. Gams et Hoozemans 1970	3	26.47	3	38.61	1	3.20
330	*Cladobotryum multiseptatum* de Hoog 1978			1	16.25		
331	*Cladobotryum varium* Nees 1817	5	21.79	7	43.99	5	7.73
332	*Cladobotryum verticillatum* (Link 1809) S. Hughes 1958			2	43.99		
333	*Cladophialophora chaetospira* (Grove 1886) Crous et Arzanlou 2007			1	33.14		
334	*Cladosporium aecidiicola* Thuemen 1876	1	19.20	1	26.20		
335	*Cladosporium allicinum* (Fries 1817: Fries 1832) Bensch et al. 2012			1	33.11		
336	*Cladosporium antarcticum* K. Schubert et al. 2007			1	7.32		
337	*Cladosporium brevicompactum* Pidoplichko et Deniak 1941			2	34.24		
338	*Cladosporium cladosporioides* (Fresenius 1850) G.A. de Vries 1952	4	19.53	56	47.50	4	6.72
339	*Cladosporium colocasiae* Sawada 1916	1	19.35	1	19.16		
340	*Cladosporium cucumerinum* Ellis et Arthur 1889			1	48.92		
341	*Cladosporium elegantulum* Pidoplichko et Deniak 1938			2	26.47	1	7.58
342	*Cladosporium gossypiicola* Pidoplichko et Deniak 1941			2	34.83		
343	*Cladosporium halotolerans* Zalar et al. 2007			2	28.46		
344	*Cladosporium herbarum* (Persoon 1794) Link 1816	19	19.35	101	47.52	10	5.76
345	*Cladosporium lycoperdinum* Cooke 1883			1	41.76		
346	*Cladosporium macrocarpum* Preuss 1848	4	19.01	5	47.72		
347	*Cladosporium pseudocladosporioides* Bensch et al. 2010			1	23.56		
348	*Cladosporium sphaerospermum* Penzig 1882	7	19.30	22	47.50	2	14.36
349	*Cladosporium straminicola* Pidoplichko et Deniak 1938			1	26.05		
350	*Cladosporium transchelii* Pidoplichko et Deniak 1938			1	13.10		
351	*Clathrus archeri* (Berkeley 1859) Dring 1980	1	1.65				
352	*Clavariadelphus pistillaris* (Linnaeus 1753) Donk 1933	1	19.70				
353	*Claviceps paspali* F. Stevens et J.G. Hall 1910	3	18.95				
354	*Claviceps purpurea* (Fries 1823) Tulasne 1853	3	26.36	7	25.22		

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
355	*Clitocybe odora* (Bulliard 1784) P. Kummer 1871	1	0.01				
356	*Clonostachys byssicola* Schroers 2001			2	3.67		
357	*Clonostachys rosea* (Link 1816) Schroers et al. 1999 f. *catenulata* (J.C. Gilman et E.V. Abbott 1927) Schroers 2001	8	20.68	8	34.61	3	19.96
358	*Clonostachys rosea* (Link 1816) Schroers et al. 1999 f. *rosea*	17	19.47	18	42.09	16	15.09
359	*Clonostachys solani* (Harting 1846) Schroers et W. Gams 2001			2	1.65		
360	*Clonostachys solani* (Harting 1846) Schroers et W. Gams 2001 f. *nigrovirens* (J.F.H.Beyma 1931) Schroers 2001			2	32.42	1	4.92
361	*Coemansia aciculifera* Linder 1943	1	21.05				
362	*Cokeromyces recurvatus* Poitras 1950	3	24.14	3	42.75	3	22.74
363	*Colletoconis aecidiophila* (Spegazzini 1886) de Hoog et al. 1978	1	16.87	1	30.04		
364	*Colletotrichum coccodes* (Wallroth 1833) S. Hughes 1958			4	20.10		
365	*Colletotrichum dematium* (Persoon 1801) Grove 1918			1	6.08		
366	*Colletotrichum gloeosporioides* (Penzig 1882) Penzig et Saccardo 1884	2	19.33	4	37.54		
367	*Colletotrichum musae* (Berkeley et M.A. Curtis 1874) Arx 1957	1	19.32	1	24.90		
368	*Colpoma quercinum* (Persoon 1796) Wallroth 1823			1	37.71		
369	*Conidiobolus coronatus* (Costantin 1897) Batko 1964	4	15.93				
370	*Conidiobolus thromboides* Drechsler 1953	2	6.78				
371	*Coniochaeta verticillata* (van Emden 1973) Dania García et al. 2006	1	18.96	1	39.44	1	0.10
372	*Coniophora puteana* (Schumacher 1803) P. Karsten 1868	4	12.02				
373	*Coniothyrium concentricum* (Desmazieres 1840) Saccardo 1878	1	19.01	1	12.63		
374	*Coniothyrium hellebori* Cooke et Massee 1886	1	17.70	1	27.85		
375	*Coniothyrium rosarum* Cooke et Harkness 1882	2	19.40	2	16.28		
376	*Coniothyrium wernsdorffiae* Laubert 1905	1	19.01	1	20.34		
377	*Coprinellus disseminatus* (Persoon 1801) J.E. Lange 1938	1	32.89				
378	*Coprinellus ephemerus* (Bulliard 1786) Redhead et al. 2001	1	32.87				
379	*Coprinellus micaceus* (Bulliard 1785) Vilgalys et al. 2001	3	32.83				
380	*Coprinellus radians* (Desmazieres 1828) Vilgalys et al. 2001	1	32.83				
381	*Coprinopsis atramentaria* (Bulliard 1783) Redhead et al. 2001	2	32.83				
382	*Coprinopsis gonophylla* (Quélet 1884) Redhead et al. 2001	2	22.97				
383	*Coprinopsis kimurae* (Hongo et Aoki 1966) Redhead et al. 2001	1	7.92				
384	*Coprinus comatus* (O.F. Mueller 1780) Persoon 1797	2	32.83				
385	*Coprinus domesticus* (Bolton 1788) Gray 1821	1	27.36				

386	*Coprinus sterquilinus* (Fries 1821) Fries 1838	2	32.87				
387	*Coriolopsis trogii* (Berkeley 1850) Domanski 1974	1	34.10				
388	*Coriolus* sp.	1	19.42				
389	*Cortinarius bulbosus* Gray 1821	1	22.97				
390	*Cortinarius caperatus* (Persoon 1796) Fries 1838	1	23.53				
391	*Corynascella inaequalis* (Pidoplichko et al. 1973) Arx 1975	1	18.95	1	42.84	1	27.97
392	*Corynascus sepedonium* (C.W. Emmons 1932) Arx 1973	1	20.42	1	39.91	1	21.16
393	*Cosmospora arxii* (W. Gams 1971) Gräfenhan et Schroers 2011	1	16.01	1	35.44	1	0.53
394	*Cosmospora berkeleyana* (P. Karsten 1891) Gräfenhan et al. 2011			8	24.26	1	21.27
395	*Cosmospora lavitskiae* (Zhdanova 1966) Gräfenhan et Seifert 2011	1	4.33	1	30.12	1	0.09
396	*Crassicarpon hotsonii* Koukol 2016			1	13.43		
397	*Cryphonectria parasitica* (Murrill 1906) M.E. Barr 1978	5	19.35	5	41.47	1	0.19
398	*Cryptococcus depauperatus* (Petch 1932) Boekhout et al. 2015	1	23.96				
399	*Cunninghamella blakesleeana* Lendner 1927	3	14.1			2	13.81
400	*Cunninghamella echinulata* (Thaxter 1891) Thaxter ex Blakeslee1905	13	25.18	15	42.96	12	49.77
401	*Cunninghamella homothallica* Kominami et Tubaki 1952	1	20.08				
402	*Cunninghamella japonica* (Saito 1905) Pidoplichko et Milko 1971	6	25.18	7	37.02	6	35.84
403	*Cunninghamella vesiculosa* P.C. Misra 1966	1	21.95				
404	*Curvularia clavata* B.L. Jain 1962			1	13.68		
405	*Curvularia comoriensis* Bouriquet et Jauffret 1955 ex M.B. Ellis 1966	1	17.80	1	26.87		
406	*Curvularia fallax* Boedijn 1933			1	13.60		
407	*Curvularia geniculata* (Tracy et Earle 1896) Boedijn 1933	4	19.30	5	45.17		
408	*Curvularia inaequalis* (Shear 1907) Boedijn 1933	5	19.76	6	28.20	1	4.33
409	*Curvularia kusanoi* (Y. Nisikado 1928) Manamgoda et al. 2014			1	18.74		
410	*Curvularia lunata* (Wakker 1898) Boedijn 1933	6	19.35	9	44.30		
411	*Curvularia nodulosa* (Saccardo 1886) Manamgoda et al. 2014			1	23.88		
412	*Curvularia protuberata* Nelson et Hodges 1965			1	13.50		
413	*Cyathus olla* (Batsch 1783) Persoon 1801	1	14.05				
414	*Cylindrium cordae* Grove 1886			1	27.86	1	3.49
415	*Cylindrocarpon album* (Saccardo 1877) Wollenweber 1917	1	17.67	1	34.66	1	3.51
416	*Cylindrocarpon chlamydospora* Schischkina et Tzanava 1973	1	19.97	1	18.49		
417	*Cylindrocarpon congoense* J.A. Meyer 1958	1	19.56	1	28.46	1	2.49
418	*Cylindrocarpon destructans* (Zinssmeister 1918) Scholten 1964	6	15.66	12	21.12	2	2.43
419	*Cylindrocarpon didymum* (Hartig 1846) Wollenweber 1926	2	19.56	3	21.12		
420	*Cylindrocarpon gracile* Bugnicourt 1939	3	29.09	3	1.25	1	3.07

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
421	*Cylindrocarpon heteronema* (Berkeley et Broome 1865) Wollenweber 1916	3	19.53	4	28.67	1	2.49
422	*Cylindrocarpon lucidum* C. Booth 1966			1	20.70		
423	*Cylindrocarpon obtusisporum* (Cooke et Harkness 1884) Wollenweber 1926	1	25.95	1	18.94	1	2.45
424	*Cylindrocarpon peronosporae* (Fautrey et Lambotte 1896) Rudakov 1981			1	37.38	1	0.08
425	*Cylindrocarpon tenue* Bugnicourt 1939	1	3.81				
426	*Cylindrocephalum stellatum* (Harz 1871) Saccardo 1886	1	12.09	1	38.72	1	3.20
427	*Cylindrophora alba* Bonorden 1851			1	19.78		
428	*Cylindrophora hoffmannii* Daszewska 1912	1	17.73	1	28.06	1	5.65
429	*Cytospora* sp.			1	1.21		
430	*Dacrymyces stillatus* Nees 1816	1	6.62				
431	*Dactylaria acerosa* Matsushima 1975			1	0.77		
432	*Dactylaria dimorphospora* Veenbaas-Rijks 1973	1	16.10	1	23.58		
433	*Dactylellina asthenopaga* (Drechsler 1937) M. Scholler et al.1999	1	19.30	1	0.08		
434	*Daedalea quercina* (Linnaeus 1753) Persoon 1801	2	34.05				
435	*Daedaleopsis confragosa* (Bolton 1791) J. Schröter 1888 var. *confragosa*	1	10.36				
436	*Dematioscypha delicata* (Berkeley et Broome 1859) Hosoya 2014	1	19.38	1	26.79		
437	*Dendrodochium toxicum* Pidoplichko et Bilai 1947	1	19.33	1	28.29	1	4.79
438	*Dendrostilbella macrospora* W. Bally 1917	1	7.72	1	14.23	1	4.92
439	*Dendrostilbella mycophila* (Persoon 1822) Seifert 1985			1	37.05	1	2.89
440	*Dendryphion nanum* (Nees 1816) S. Hughes 1958			1	24.14	1	0.53
441	*Dendryphion penicillatum* (Corda 1838) Fries 1846			1	24.88	2	3.44
442	*Dichobotrys* sp.	1	19.28	1	18.88		
443	*Dichotomomyces cejpii* (Milko 1964) D.B. Scott 1970	1	18.96	1	19.72	1	23.17
444	*Dictyostelium discoideum* (Bosc 1811) E. Fischer 1888	1	5.75	1	24.44		
445	*Dictyuchus monosporus* Leitgeb 1870	2	12.57				
446	*Dicyma ampullifera* Boulanger 1897	1	19.21	1	12.36		
447	*Dicyma olivacea* (Emoto et Tubaki 1970) Arx 1982	1	19.40	1	48.10		
448	*Dicyma ovalispora* (S. Hughes 1951) Arx 1982	1	6.69	1	12.32		
449	*Didymella glomerata* (Corda 1840) Q. Chen et L. Cai 2015	11	19.47	8	46.45		
450	*Didymella musae* (P. Joly 1961) Q. Chen et L. Cai *2015*			4	19.59		
451	*Didymella pinodella* (L.K. Jones 1927) Q. Chen et L. Cai *2015*	1	20.54				
452	*Didymella pinodes* (Berkeley et A. Bloxam 1861) Petrak 1924	1	19.22	1	22.41		

453	*Didymella pomorum* (Thümen 1879) Q. Chen et L. Cai 2015	4	19.56	4	46.45		
454	*Didymopsis helvellae* (Corda 1854) Saccardo et Marchall 1885			1	28.44	1	2.73
455	*Dimargaris bacillispora* R.K. Benjamin 1959	1	19.37	1	8.30		
456	*Dinemasporium strigosum* (Persoon 1801) Saccardo 1881	1	17.80	1	20.15		
457	*Diplocladium majus* Bonorden 1851	2	21.84	2	38.61	2	2.84
458	*Diplocladium penicilloides* Saccardo 1886			2	27.21	2	3.73
459	*Diplodina acerina* (Passerini 1875) B. Sutton 1980			1	6.56		
460	*Dipodascopsis tothii* (Zsolt 1963) L.R. Batra et Millner 1978			1	42.60	1	0.10
461	*Dipodascopsis uninucleata* (Biggs 1937) L.R. Batra et Millner 1978 var. *uninucleata*	2	4.03	2	42.60		
462	*Dipodascus aggregatus* Francke-Grosmann 1952	2	18.88	2	42.63	2	0.10
463	*Dipodascus armillariae* W. Gams 1983	1	19.62	1	15.67		
464	*Discula brunneotingens* E.I. Meyer 1953	1	20.32	1	29.45		
465	*Discula pinicola* (Naumov 1926) Petrak 1927 var. *mammosa* Lagerberg et al. 1927	1	19.33	1	39.74		
466	*Dispira cornuta* van Tieghem 1875	1	21.05				
467	*Dissoacremoniella silvatica* Kirilenko 1970	1	28.88	1	22.90		
468	*Dothiora prunorum* (Dennis et Buhagiar 1973) Crous 2016	1	16.93	1	27.42		
469	*Drechmeria coniospora* (Drechsler 1941) W. Gams et H.-B. Jansson 1985			1	13.88	1	22.04
470	*Drechslera avenacea* (M.A. Curtis ex Cooke 1889) Shoemaker 1959			2	37.32		
471	*Drechslera campanulata* (Leveille 1841) B. Sutton 1976	3	19.38	2	23.17		
472	*Drechslera poae* (Baudys 1916) Shoemaker 1962			1	33.87		
473	*Duddingtonia flagrans* (Duddington 1949) R.C. Cooke 1969	1	19.32	1	18.30	1	2.49
474	*Echinobotryum rubrum* Sorokin ex Jaczewski 1917			1	25.93	1	0.08
475	*Eladia saccula* (E. Dale 1926) G. Smith 1961	1	20.56	1	44.21		
476	*Emericellopsis alkalina* Bilanenko et Georgieva 2013			3	7.77		
477	*Emericellopsis donezkii* Beliakova 1974	7	20.48	7	46.22	6	0.09
478	*Emericellopsis glabra* (J.F.H. Beyma 1940) Backus et Orpurt 1961	1	19.33	3	46.54		
479	*Emericellopsis humicola* (Cain 1956) Gilman 1956	1	19.33	1	48.87	1	23.47
480	*Emericellopsis maritima* Beliakova 1970	1	14.88	1	17.90		
481	*Emericellopsis minima* Stolk 1955	10	20.42	10	46.48	9	22.75
482	*Emericellopsis pallida* Beliakova 1974	1	20.44	1	49.00	1	23.10
483	*Emericellopsis robusta* van Emden et W. Gams 1971			2	44.91		
484	*Emericellopsis terricola* J.F.H. Beyma 1940	1	19.33	1	49.00	1	0.10
485	*Engyodontium album* (Limber 1940) de Hoog 1978	2	23.78	3	18.23		

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
486	*Entomophthora dipterigena* (Thaxter 1888) Saccardo et Traverso 1891	1	26.20				
487	*Entomophthora thaxteriana* I.M. Hall et J. Bell 1963	5	23.20				
488	*Entyloma gaillardianum* Vanky 1982	1	12.35	1	18.84		
489	*Epicoccum nigrum* Link 1815	5	19.81	11	35.51		
490	*Epicoccum sorghinum* (Saccardo 1878) Aveskamp et al. 2010			4	3.29		
491	*Epithyrium obscurum* (Passerini 1885) Saccardo 1931			1	11.05		
492	*Eremascus fertilis* Stoppel 1907	1	18.86	1	5.73		
493	*Eremothecium ashbyi* Guilliermond 1935	5	17.32				
494	*Eremothecium gossypii* (S.F. Ashby et W. Nowell 1926) Kurtzman 1995	2	16.33				
495	*Eupenicillium pinetorum* Stolk 1968					1	0.10
496	*Eurotium amstelodami* L. Mangin 1909			16	43.08	9	46.64
497	*Eurotium chevalieri* L. Mangin 1909			8	42.86	5	46.87
498	*Eurotium halophilicum* C.M. Christensen et al. 1959			1	28.92	1	0.10
499	*Eurotium herbariorum* (F.H. Wiggers 1780) Link 1809	1	18.88	18	37.95	1	21.57
500	*Eurotium rubrum* Jos. König et al. 1901			7	43.26	6	37.41
501	*Eurotium tonophilum* Ohtsuki 1962	1	20.01	1	39.91	1	19.77
502	*Eutypa* sp.			1	0.04	1	2.69
503	*Evlachovaea kintrischica* B. Borisov et Tarasov 1999			1	15.54		
504	*Exobasidium bisporum* Sawada ex Ezuka 1991	1	23.62	1	1.64		
505	*Exobasidium karstenii* Saccardo et Trotter 1912	1	23.62				
506	*Exobasidium myrtilli* Siegmund 1879	1	19.46	1	1.64		
507	*Exobasidium pachysporum* Nannfeldt 1981	1	2.04				
508	*Exobasidium vaccinii* (Fuckel 1861) Woronin 1867	2	23.62	2	1.81		
509	*Exophiala castellanii* Iwatsu et al. 1984	2	20.40	2	23.19		
510	*Exophiala heteromorpha* (Nannfeldt 1934) de Hoog et Haase 2003	1	19.54	1	21.63		
511	*Exophiala lecanii-corni* (Benedek et Specht 1933) Haase et de Hoog 1999			1	18.56		
512	*Exophiala moniliae* de Hoog 1977			1	18.56		
513	*Exophiala salmonis* J.W. Carmichael 1966	1	17.78	1	10.32		
514	*Exophiala xenobiotica* de Hoog et al. 2006			3	2.11		
515	*Exserohilum pedicellatum* (A.W. Henry 1924) K.J. Leonard et Suggs 1974	1	19.48	1	40.52		
516	*Exserohilum rostratum* (Drechsler 1923) K.J. Leonard et Suggs 1974			1	18.33		
517	*Farlowiella carmichaeliana* (Berkeley 1836) Saccardo 1891			1	26.00	1	3.49

518	*Farrowia seminuda* (L.M. Ames 1949) D. Hawksworth 1975			1	38.09		
519	*Fennellomyces linderi* (Hesseltine et Fennell 1955) Benny et R.K. Benjamin *1975*	1	19.64	1	15.02	1	29.69
520	*Fibroporia vaillantii* (de Candolle 1815) Parmasto 1968	1	20.10				
521	*Flammulina velutipes* (Curtis 1782) Singer 1951	6	34.05				
522	*Fomes fomentarius* (Linnaeus 1753) J.J. Kickx 1867	4	34.14				
523	*Fomitopsis pinicola* (Swartz 1810) P. Karsten 1881	8	20.04	1	0.99		
524	*Fomitopsis rosea* (Albertini et Schweinitz 1805) P. Karsten 1881	1	20.10				
525	*Fonsecaea pedrosoi* (Brumpt 1922) Negroni 1936	1	19.72	1	39.24		
526	*Fulvia* fulva (Cooke 1883) Ciferri 1954	3	19.01	3	22.51		
527	*Fusarium agaricorum* Sarrazin 1887	1	17.67	1	27.21	1	2.62
528	*Fusarium aquaeductuum* (Rabenhorst et Radlkofer 1861) Lagerheim et Rabenhorst 1891	3	0.19	2	33.19	2	2.52
529	*Fusarium aquaeductuum* (Rabenhorst et Radlkofer 1861) Lagerheim et Rabenhorst 1891 var. *medium* Wollenweber 1931			1	23.04	1	5.64
530	*Fusarium arthrosporioides* Sherbakoff 1915	1	13.96	1	21.18		
531	*Fusarium avenaceum* (Fries 1832) Saccardo 1886	4	19.99	3	44.73	3	14.34
532	*Fusarium avenaceum* (Fries 1832) Saccardo 1886 var. *herbarum* (Corda 1839) Saccardo 1886			1	26.39	1	2.90
533	*Fusarium cerealis* (Cooke 1878) Saccardo 1886	1	2.21	1	26.52		
534	*Fusarium chlamydosporum* Wollenweber et Reinking 1925			2	29.57		
535	*Fusarium concolor* Reinking 1935	1	1.88	2	16.36		
536	*Fusarium culmorum* (W.G. Smith 1884) Saccardo 1895	3	17.67	3	32.72	3	11.43
537	*Fusarium decemcellulare* Brick 1908	2	19.50	2	44.40	2	11.93
538	*Fusarium epistroma* (Hoehnel 1909) C. Booth 1971			2	32.43	2	3.04
539	*Fusarium equiseti* (Corda 1838) Saccardo 1886	5	19.28	7	44.85	3	26.61
540	*Fusarium expansum* Schlechtendal 1824			1	29.47	1	2.68
541	*Fusarium fujikuroi* Nirenberg 1976	1	7.65	1	39.07	1	24.70
542	*Fusarium graminearum* Schwabe 1839	4	15.66	4	35.19	3	3.98
543	*Fusarium graminearum* Schwabe 1839 f. oxalis			1	38.91	1	7.09
544	*Fusarium heterosporum* Nees et T. Nees 1818			3	31.04	1	7.09
545	*Fusarium heterosporum* Nees et T. Nees 1818 var. *pucciniophilum Saccardo* et Sydow 1899	1	17.73	1	23.19	1	2.62
546	*Fusarium incarnatum* (Roberge 1849) Saccardo 1886	4	16.56	4	28.98	4	2.97
547	*Fusarium javanicum* Koorders 1907	2	13.36	2	35.60	2	7.90
548	*Fusarium lateritium* Nees 1816	7	20.47	8	39.14	4	21.85

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
549	*Fusarium merismoides* Corda 1838	3	17.90	3	33.20	1	3.64
550	*Fusarium oxysporum* Schlechtendal 1824	14	31.06	28	38.36	7	24.51
551	*Fusarium oxysporum* Schlechtendal 1824 f. sp. *batatas* (G.F. Atkinson *1892)* W.C. Snyder et H.N. Hansen 1940			1	18.10	1	25.21
552	*Fusarium oxysporum* Schlechtendal 1824 f. sp. *conglutinans* W.C. Snyder et *H.N.* Hansen 1940			2	23.35	1	25.52
553	*Fusarium oxysporum* Schlechtendal 1824 f. sp. *lycopersici* W.C. Snyder et *H.N.* Hansen 1940			2	29.35	2	23.85
554	*Fusarium oxysporum* Schlechtendal 1824 f. sp. *vasinfectum* W.C. Snyder et *H.N.* Hansen 1940			1	25.04	1	26.69
555	*Fusarium poae* (Peck 1903) Wollenweber 1913	3	17.81	4	45.15	3	12.68
556	*Fusarium redolens* Wollenweber 1913	3	21.67	3	21.54		
557	*Fusarium sambucinum* Fuckel 1863	11	7.69	9	37.19	7	13.96
558	*Fusarium sambucinum* Fuckel 1863 var. *ossicola* (Berkeley et M.A.Curtis 1875) Bilai 1955			1	11.59	1	0.73
559	*Fusarium sarcochroum* (Desmazieres 1850) Saccardo 1879	1	4.99	1	21.31	1	2.90
560	*Fusarium solani* (Martius 1842) Saccardo 1881	12	16.16	23	34.24	10	16.90
561	*Fusarium sporotrichioides* Sherbakoff 1915	6	17.73	6	47.20	6	18.61
562	*Fusarium tricinctum* (Corda 1838) Saccardo 1886	7	17.47	7	37.16	4	4.75
563	*Fusarium ventricosum* Appel et Wollenweber 1913			3	29.13	2	12.68
564	*Fusarium verticillioides* (Saccardo 1881) Nirenberg 1976	25	30.64	26	42.97	23	28.25
565	*Fusarium viride* (Lechmere 1912) Wollenweber 1917	1	17.67	1	23.96	1	2.62
566	*Fusarium wolgense* Rodigin 1942			1	37.26	1	3.04
567	*Fusicladium peltigericola* Crous et Diederich 2010			1	2.12		
568	*Fusicladium pomi* (Fries 1825) Lind 1913	1	19.82				
569	*Fusicoccum castaneum* Saccardo 1882			1	11.21		
570	*Fusicolla epistroma* (Höhn. 1909) Gräfenhan and Seifert 2011			1	3.70		
571	*Gabarnaudia betae* (Delacroix 1897) Samson et W. Gams 1974	2	11.05	3	33.00		
572	*Gaeumannomyces caricis* J. Walker 1980	1	5.62				
573	*Gaeumannomyces graminis* (Saccardo 1875) Arx et D.L. Olivier 1952 var. *graminis*	1	25.68				
574	*Galactomyces geotrichum* (E.E. Butler et L.J. Petersen 1972) Redhead et *Malloch* 1977			3	45.89	3	3.45
575	*Galactomyces reessii* (van der Walt 1959) Redhead et Malloch 1977	1	19.27	1	46.16	1	3.45

576	*Ganoderma lipsiense* (Batsch 1786) G.F. Atkinson 1908	3	17.39				
577	*Ganoderma lucidum* (Curtis 1781) P. Karsten 1881	1	9.55				
578	*Geastrum fimbriatum* Fries 1829	1	16.63				
579	*Geomyces asperulatus* Sigler et J.W. Carmichael 1976			1	8.54		
580	*Geomyces pannorum* (Link 1824) Sigler et J.W. Carmichael 1976	38	20.25	166	39.85	8	3.20
581	*Geosmithia lavendula* (Raper et Fennell 1948) Pitt 1980			1	44.17	1	5.31
582	*Geosmithia namyslowskii* (K.M. Zalessky 1927) Pitt 1980			1	43.86	1	22.92
583	*Geotrichum amycelicum* Redaelli et Ciferri 1935	1	18.86	1	11.15		
584	*Geotrichum bipunctatum* Rolland et Fautrey 1894			1	38.59	1	2.90
585	*Geotrichum candidum* Link 1809	24	31.24	42	45.14	15	2.90
586	*Geotrichum fragrans* (Berkhout 1923) Morenz 1960 ex Morenz 1964	4	16.13	4	31.67	1	10.53
587	*Geotrichum klebahnii* (Stautz 1931) Morenz 1964	3	19.79	3	29.62		
588	*Gibberella fujikuroi* (Sawada 1917) Wollenweber 1931	3	19.32	3	26.87	3	30.36
589	*Gibberella zeae* (Schweinitz 1821) Petch 1936	2	19.77	3	16.40	1	6.20
590	*Gibellula pulchra* Cavara 1894			1	6.13		
591	*Gibellulopsis nigrescens* (Pethybridge 1919) Zare et al. 2007			6	41.27	6	13.62
592	*Gilbertella persicaria* (E.D. Eddy 1925) Hesseltine 1960	1	11.41	1	27.13	1	37.52
593	*Gilmaniella humicola* G.L. Barron 1964			2	13.68		
594	*Gliocephalotrichum bulbilium* J.J. Ellis et Hesseltine 1962			1	15.86		
595	*Gliocladiopsis tenuis* (Bugnicourt 1939) Crous et M.J. Wingfeld 1993			1	14.46		
596	*Gliocladium album* (Preuss 1851) Petch 1926			2	26.90	2	2.49
597	*Gliocladium ammoniphilum* Pidoplichko et Bilai 1953	1	19.26	1	28.55	1	9.57
598	*Gliocladium aurifilum* (W. Gerard 1874) Seifert et al. 1985	1	0.54	1	14.41		
599	*Gliocladium cholodnyi* Pidoplichko 1931	2	16.15	2	26.27	2	9.13
600	*Gliocladium comtus* Rudakov 1981	1	7.81	1	30.99	1	3.73
601	*Gliocladium viride* Matruchot 1893			4	32.23	1	3.32
602	*Gliomastix cerealis* (P. Karsten 1887) C.H. Dickinson 1968	2	19.22	2	36.53	1	10.70
603	*Gliomastix inflata* C.H. Dickinson 1968			2	23.17	2	3.22
604	*Gliomastix luzulae* (Fuckel 1870) E.W. Mason 1953 ex S. Hughes *1958*	2	6.22	3	29.44	3	2.74
605	*Gliomastix murorum* (Corda 1838) S. Hughes 1958 var. *felina* (Marchal 1895) S. Hughes 1958	5	30.42	6	44.79	4	9.25
606	*Gliomastix murorum* (Corda 1838) S. Hughes 1958 var. *murorum*	10	30.71	9	33.64	6	14.07
607	*Gloeophyllum odoratum* (Wulfen 1788) Imazeki 1943	1	23.78				
608	*Gloeophyllum sepiarium* (Wulfen 1786) P. Karsten 1882	5	34.14				
609	*Gongronella butleri* (Lendner 1926) Peyronel et Dal Vesko 1955	6	24.55	6	32.12	3	5.42

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
610	*Gongronella lacrispora* Hesseltine et J.J. Ellis 1961	1	15.38	1	27.66		
611	*Gonytrichum macrocladum* (Saccardo 1880) S. Hughes 1951	3	17.68	4	30.36		
612	*Graphium penicillioides* Corda 1837			1	18.14		
613	*Graphium putredinis* (Corda 1839) S. Hughes 1958			1	23.88		
614	*Grifola frondosa* (Dickson 1785) Gray 1821	2	12.58				
615	*Guepiniopsis buccina* (Persoon 1801) L.L. Kennedy 1959	1	25.88				
616	*Gymnoascus reessii* Baranetzky 1872			1	36.56	1	3.35
617	*Gymnopilus sapineus* (Fries 1821) Murrill 1912	1	22.97				
618	*Gymnostellatospora japonica* Udagawa 1993			1	2.30		
619	*Hansfordia pulvinata* (Berkeley et M.A. Curtis 1875) S. Hughes 1958	2	19.44	2	26.42		
620	*Hansfordia triumfettae* (Hahsford 1943) S. Hughes 1952			1	27.00		
621	*Haplotrichum capitatum* (Link 1809) Link 1824	2	30.60	2	30.56	2	25.26
622	*Hapsidospora milkoi* Beliakova 1975	1	2.27	1	34.82	1	23.86
623	*Harposporium lilliputanum* Dixon 1952	1	19.30	1	21.69		
624	*Harposporium sinense* C.Y. Wang et K.Q. Zhang 2007	1	20.85				
625	*Harzia acremonioides* (Harz 1871) Costantin 1888	4	19.49	4	45.20		
626	*Hebeloma versipelle* (Fries 1838) Gillet 1876	1	22.96	1	6.77		
627	*Helicodendron tubulosum* (Riess 1853) Linder 1929	1	19.31	1	11.44		
628	*Helicostylum elegans* Corda 1842	1	17.59	1	44.19	1	15.39
629	*Helicostylum pulchrum* (Preuss 1851) Pidoplichko et Milko 1971	2	25.41	2	27.11	2	38.98
630	*Helminthosporium solani* Durieu et Montagne 1849	1	19.61	1	9.56		
631	*Hemicarpenteles ornatum* (Subramanian 1972) Arx 1974			1	39.58	1	5.09
632	*Hericium coralloides* (Scopoli 1772) Persoon 1794	4	32.55				
633	*Hericium erinaceus* (Bulliard 1781) Persoon 1797	4	25.07				
634	*Hesseltinella vesiculosa* H.P. Upadhyay 1970	1	21.26	1	40.75		
635	*Heterobasidion annosum* (Fries 1821) Brefeld 1888	1	20.10				
636	*Hirsutella thompsonii* F.E. Fischer 1950			1	15.26		
637	*Holwaya mucida* (Schulzer 1860) Korf et Abawi 1971 var. *mucida*			1	2.30		
638	*Hormiactis alba* Preuss 1851			1	23.26	1	2.73
639	*Hormoconis resinae* (Lindau 1906) Arx et G.A. de Vries 1973	12	19.49	12	47.50	2	17.28
640	*Hormonema macrosporum* L. Voronin 1986	1	19.81	1	26.36		
641	*Humicola fuscoatra* Traaen 1914	3	28.79	4	32.96	1	33.60

642	*Humicola grisea* Traaen 1914 var. *thermoidea* Cooney et Emerson 1964			1	11.62		
643	*Humicola insolens* Cooney et R. Emerson 1964			1	16.82		
644	*Hydnoporia tabacina* (Sowerby 1797) V. Spirin et al. 2019	1	19.56				
645	*Hyphopichia burtonii* (Boidin et al. 1964) Arx et Van der Walt 1976			1	38.10		
646	*Hyphozyma sanguinea* (C. Ramirez 1952) de Hoog et M.T. Smith 1981	1	28.74	1	20.45		
647	*Hyphozyma variabilis* de Hoog et M.T. Smith 1981	2	19.30				
648	*Hyphozyma variabilis* de Hoog et M.T. Smith 1981 var. *odora* de Hoog et M. T. Smith 1981			1	21.64		
649	*Hyphozyma variabilis* de Hoog et M.T. Smith 1981 var. *variabilis*			1	20.45		
650	*Hypomyces ochraceus* (Persoon 1801) Tulasne et C. Tulasne 1865			3	22.53		
651	*Inocutis dryophila* (Berkeley 1904) Fiasson et Niemelä 1984	1	20.73				
652	*Inonotus obliquus* (Acharius ex Persoon 1801) Pilat 1942	2	34.08				
653	*Inonotus rheades* (Persoon 1825) Bondartsev et Singer 1941	2	15.29				
654	*Irpex lacteus* (Fries 1818) Fries 1828	1	12.40				
655	*Isaria farinosa* (Holmskjold 1781) Fries 1832	7	20.34	8	35.81	3	18.38
656	*Isaria fumosorosea* Wize 1904	6	21.44	6	43.70	1	2.32
657	*Isaria javanica* (Friedrichs et Bally 1923) Samson et Hywel-Jones 2005			1	6.21		
658	*Isaria tenuipes* Peck 1879			1	6.84		
659	*Juxtiphoma eupyrena* (Saccardo 1879) Valenzuela-Lopez et al. 2017			3	17.62		
660	*Kickxella alabastrina* Coemans 1862	1	6.69	1	6.46		
661	*Kuehneromyces lignicola* (Peck 1872) Redhead 1984	1	23.50				
662	*Kuehneromyces mutabilis* (Schaeffer 1774) Singer et A.H. Smith 1946	6	24.26				
663	*Laccaria bicolor* (Maire 1937) P.D. Orton 1960	1	14.95				
664	*Laccaria laccata* (Scopoli 1772) Cooke 1884	2	22.96				
665	*Lactarius helvus* (Fries 1821) Fries 1838	1	24.01				
666	*Laetiporus sulphureus* (Bulliard 1789) Murrill 1920	6	19.99	1	8.56		
667	*Lasiodiplodia theobromae* Patouillard 1892) Griffon et Maublanc 1909	1	7.36	1	17.95		
668	*Lecanicillium dimorphum* (J.D. Chen 1985) Zare et W. Gams 2001			2	38.36		
669	*Lecanicillium fungicola* (Preuss 1851) Zare et W. Gams 2008	3	20.40	3	39.64		
670	*Lecanicillium fusisporum* (W. Gams 1971) Zare et W. Gams 2001			1	25.03		
671	*Lecanicillium lecanii* (Zimmermann 1898) Zare et W. Gams 2001	4	19.84	4	39.18	1	4.47
672	*Lecanicillium longisporum* (Petch 1925) Zare et W. Gams 2001	1	19.26	1	37.00	1	2.54
673	*Lecanicillium muscarium* (Petch 1931) Zare et W. Gams 2001	14	22.33	15	36.34	3	4.97
674	*Lecanicillium psalliotae* (Treschew 1941) Zare et W. Gams 2001	6	20.44	7	36.72		
675	*Leccinum scabrum* (Bulliard 1783) Gray 1821	1	12.08				

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
676	*Lecythophora decumbens* (J.F.H. Beyma 1942) E. Weber et al. 2002	1	20.40	1	38.10		
677	*Lecythophora fasciculata* (J.F.H. Beyma 1939) E. Weber et al. 2002	1	20.40	1	42.03		
678	*Lecythophora hoffmannii* (J.F.H. Beyma 1939) W. Gams et McGinnis 1983	3	20.52	5	42.40		
679	*Lecythophora mutabilis* (J.F.H. Beyma 1944) W. Gams et McGinnis 1983	1	20.40	4	45.70		
680	*Lentinula edodes* (Berkeley 1878) Pegler 1976	5	26.21				
681	*Lentinus sulcatus* Berkeley 1845	1	12.13				
682	*Lentinus tigrinus* (Bulliard 1782) Fries 1825	3	20.98				
683	*Lenzites betulina* (Linnaeus 1753) Fries 1838	3	13.47				
684	*Lepista luscina* (Fries 1818) Singer 1951	1	0.21				
685	*Lepista nuda* (Bulliard 1790) Cooke 1871	1	1.82				
686	*Leptobacillium leptobactrum* (W. Gams 1971) Zare et W. Gams 2016	2	3.35	2	21.46		
687	*Leptographium lundbergii* Lagerberg et Melin 1927			1	11.42		
688	*Leptosphaeria coniothyrium* (Fuckel 1870) Saccardo 1875	1	18.96	1	6.03		
689	*Leucoagaricus leucothites* (Vittadini 1835) Wasser 1977	1	3.04				
690	*Leucoagaricus nympharum* (Kalchbrenner 1873) Bon 1977	1	12.07				
691	*Leuconeurospora pulcherrima* (G. Winter 1876) Malloch et Cain 1970	1	0.54				
692	*Lichtheimia blakesleeana* (Lendner 1924) Kerst. Hoffmann et al. 2009	4	28.53	4	44.36	4	48.92
693	*Lichtheimia corymbifera* (Cohn 1884) Vuillemin 1903	12	23.06	18	40.93	18	50.21
694	*Lichtheimia hyalospora* (Saito 1906) Kerst. Hoffmann et al. 2009	1	19.38	1	19.04	1	29.82
695	*Linderina pennispora* Raper et Fennell 1952	1	11.78	1	46.29		
696	*Lobosporangium transversale* (Malloch) M. Blackwell et Benny 2004	1	7.17				
697	*Lycoperdon perlatum* Persoon 1796	1	21.10				
698	*Lycoperdon pyriforme* Schaeffer 1774	2	20.04				
699	*Macrolepiota mastoidea* (Fries 1821) Singer 1951	1	12.20				
700	*Macrolepiota procera* (Scopoli 1772) Singer 1948	1	12.32				
701	*Macrophoma mantegazziana* (Penzig 1882) Berlese et Voglino 1886	1	0.97	1	26.65		
702	*Magnusiomyces magnusii* (F. Ludwig 1886) Redhead et Malloch 1977			1	31.43	1	0.11
703	*Malbranchea flavorosea* Sigler et J.W. Carmichael 1976			1	7.19		
704	*Mammaria* echinobotryoides Cesati 1854			1	1.99		
705	*Marasmius oreades* (Bolton 1792) Fries 1836	1	34.16				
706	*Mariannaea elegans* (Corda 1838) Samson 1974	6	31.50	6	45.32	4	9.56
707	*Melanconium apiocarpum* Link 1825	2	18.22	1	24.70		

708	*Melanconium bicolor* Nees 1817			1	24.70		
709	*Melanocarpus albomyces* (Cooney et R. Emerson 1964) Arx 1975	2	18.61	2	34.85		
710	*Melanospora betae* Panasenko 1938	1	4.07	1	44.99	1	22.75
711	*Melanospora damnosa* (Saccardo 1895) Lindau 1897	2	29.97	2	22.29		
712	*Melanospora phaseoli* Roll-Hansen 1948	1	0.12	1	45.85		
713	*Memnoniella echinata* (Rivolta 1884) Galloway 1933	2	20.54	3	32.39		
714	*Menispora ciliata* Corda 1837	1	19.25	1	45.78		
715	*Menispora tortuosa* Corda 1839			1	9.82		
716	*Merimbla ingelheimense* (J.F.H. Beyma 1942) Pitt 1980			2	36.14	1	22.89
717	*Metarhizium anisopliae* (Metschnikoff 1879) Sorokin 1883	6	19.56	6	32.32	4	15.82
718	*Microascus cirrosus* Curzi 1930	1	19.32				
719	*Microascus trigonosporus* C.W. Emmons et B.O. Dodge var. *terreus* Kamyschko *1966*	1	20.42	1	36.69	1	21.16
720	*Microbotryum silenes-inflatae* (de Candolle 1815 ex Liro 1924) G. Deml et Oberwinkler 1982	2	26.41	2	19.05		
721	*Microbotryum vinosum* (Tulasne et C. Tulasne 1847) Denchev 1994	1	23.70	1	19.05		
722	*Microbotryum violaceum* (Persoon 1797) G. Deml et Oberwinkler 1982	2	21.38	2	19.05		
723	*Microdiplodia pruni* Diedicke 1914	1	19.90	1	9.94		
724	*Microdochium nivale* (Fries 1825) Samuels et I.C. Hallett 1983	1	6.04	1	31.05	1	23.41
725	*Microsphaeropsis olivacea* (Bonorden 1869) Höhnell 1917	1	19.40	1	12.05		
726	*Mirandina corticola* G. Arnaud 1952 ex Matsushima 1975	1	19.27	1	31.46		
727	*Monascus floridanus* P.F. Cannon et E.L. Barnard 1987			1	6.75		
728	*Monilia brunnea* J.C. Gilman et E.V. Abbott 1927	1	15.22	1	27.24		
729	*Monilia diversispora* J.F.H. Beyma 1933	1	15.22	1	35.69		
730	*Monilia medoacensis* (Saccardo 1913) J.F.H. Beyma 1933			1	28.21		
731	*Monilia megalospora* (Berkeley et M.A. Curtis 1869) Saccardo 1886			1	26.72	1	22.73
732	*Moniliella suaveolens* (Lindner 1895 ex Lindner 1906) Arx 1972 var. *nigra* (Burri et Staub 1909) de Hoog 1979	4	15.42	4	23.99		
733	*Moniliella suaveolens* (Lindner 1895 ex Lindner 1906) Arx 1972 var. *suaveolens*	1	18.97	1	35.38		
734	*Monilinia fructigena* (Aderhold et Ruhland 1905) Honey 1936	2	20.03				
735	*Monochaetia concentrica* (Berkeley et Broome 1874) Saccardo et D. Saccardo 1906			1	9.21		
736	*Monochaetia dimorphospora* T. Yokoyama 1975			1	24.55		
737	*Monochaetia karstenii* (Corda 1839) Nag Raj 1985			3	13.39		
738	*Monocillium dimorphosporum* W. Gams 1971			2	27.09		

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
739	*Monocillium indicum* S.B. Saksena 1955	1	16.32	2	15.10		
740	*Monocillium nordinii* (Bourchier 1961) W. Gams 1971	1	19.84	2	22.59		
741	*Monocillium tenue* W. Gams 1971			2	24.20	1	0.53
742	*Monodictys paradoxa* (Corda 1938) S. Hughes 1958	1	17.68	1	11.72		
743	*Monosporium meliolicola* Spegazzini 1910			1	23.10		
744	*Mortierella alliacea* Linnemann 1953	1	13.86				
745	*Mortierella alpina* Peyronel 1913	5	21.33	3	43.35	2	27.46
746	*Mortierella ambigua* B.S. Mehrotra 1963	1	11.73	1	35.99		
747	*Mortierella angusta* Linnemann 1969	1	23.44				
748	*Mortierella beljakovae* Milko 1973	1	3.79				
749	*Mortierella bisporalis* (Thaxter 1914) Bjoerling 1936	2	13.09	2	2.74		
750	*Mortierella capitata* Marchal 1891	1	21.26	1	35.16	1	13.30
751	*Mortierella dichotoma* Linnemann 1936 ex W. Gams 1977	1	22.23	1	25.53	1	1.08
752	*Mortierella elasson* Sideris et G.E. Paxton 1929	2	10.95			2	5.88
753	*Mortierella elongata* Linnemann 1941	2	28.89	5	4.40		
754	*Mortierella exigua* Linnemann 1941	3	34.07	3	36.68		
755	*Mortierella gamsii* Milko 1974	7	18.90				
756	*Mortierella gemmifera* M. Ellis 1940	3	23.25	5	25.48	2	21.69
757	*Mortierella globalpina* W. Gams et Veenbaas-Rijks 1976	1	13.86				
758	*Mortierella globulifera* O. Rostrup 1916	3	33.28	1	2.76	2	41.39
759	*Mortierella horticola* Linnemann 1941	2	32.84				
760	*Mortierella humilis* Linnemann 1936 ex W. Gams 1977	5	14.00				
761	*Mortierella hyalina* (Harz 1871) W. Gams 1970 var. *hyalina*	3	23.57	2	31.26	3	1.08
762	*Mortierella jenkinii* (A.L. Smith 1898) Naumov 1935	3	13.28	2	36.62		
763	*Mortierella lignicola* (G.W. Martin 1937) W. Gams et R. Moreau 1959	1	13.97	1	45.23	1	23.94
764	*Mortierella minutissima* van Tieghem 1878	4	34.13	3	14.31	1	1.02
765	*Mortierella mutabilis* Linnemann 1941	2	22.07	2	14.53		
766	*Mortierella nigrescens* van Tieghem 1878	1	18.26				
767	*Mortierella oligospora* Bjoerling 1936	1	27.05	1	2.52	1	1.08
768	*Mortierella parvispora* Linnemann 1941	6	33.99	5	27.66	4	22.30
769	*Mortierella polycephala* Coemans 1863			1	24.88		
770	*Mortierella pulchella* Linnemann 1941	1	23.24	1	25.48		

771	*Mortierella pusilla* Oudemans 1902	1	33.99	1	25.11		
772	*Mortierella reticulata* van Tieghem et G. Le Monnier 1873	1	6.98	1	45.16	1	45.75
773	*Mortierella sarnyensis* Milko 1973	1	13.86				
774	*Mortierella sclerotiella* Milko 1967	1	22.97				
775	*Mortierella strangulata* van Tieghem 1875	1	3.79	1	25.26		
776	*Mortierella stylospora* Dixon-Stewart 1932	1	19.96	1	27.89	1	34.43
777	*Mortierella turficola* Y. Ling 1930	1	11.03				
778	*Mortierella verticillata* Linnemann 1941	8	23.25	8	39.71	4	24.60
779	*Mortierella zonata* Linnemann 1936 ex W. Gams 1977	1	13.96	1	5.09		
780	*Mortierella zychae* Linnemann 1941	5	26.76	5	31.68	3	20.46
781	*Mucobasispora tarikii* Moustafa et Abdul-Wahid 1990			1	23.92		
782	*Mucor abundans* Povah 1917	1	24.09	1	29.37	1	5.99
783	*Mucor aligarensis* B.S. Mehrotra et B.R. Mehrotra 1969			1	23.28		
784	*Mucor amphibiorum* Shipper 1978	1	15.38	1	27.73	1	0.58
785	*Mucor bacilliformis* Hesseltine 1954	1	20.55	1	28.79		
786	*Mucor bainieri* B.S. Mehrotra et Baijal 1963	1	20.70	1	32.41		
787	*Mucor circinelloides* van Tieghem 1875 var. *circinelloides*	15	25.31	17	44.90	14	45.75
788	*Mucor circinelloides* van Tieghem 1875 var. *janssenii* (Lendner 1907) Schipper 1976	6	22.62	7	44.93	6	49.10
789	*Mucor circinelloides* van Tieghem 1875 var. *lusitanicus* (Bruderlein 1916) Schipper 1976	6	25.18	8	44.18	7	28.44
790	*Mucor durus* G. Walther et de Hoog 2013	1	19.68	1	42.97	1	37.90
791	*Mucor exponens* (Burgeff 1924) G. Walther et de Hoog 2013	4	25.41	4	30.87		
792	*Mucor flavus* Bainier 1903	19	28.49	19	47.04	16	26.71
793	*Mucor fragilis* Bainier 1884	1	24.09	1	37.17	1	30.73
794	*Mucor fuscus* Bainier 1903	3	20.68	3	45.03	3	32.05
795	*Mucor genevensis* Lendner 1908	4	25.31	4	37.18		
796	*Mucor griseocyanus* Hagem 1908	3	25.41	3	38.26	3	24.30
797	*Mucor guilliermondii* Nadson et Philippow 1925	1	24.48	1	37.53	1	30.03
798	*Mucor heterogamus* Vuillemin 1903			1	27.33	1	1.66
799	*Mucor hiemalis* Wehmer 1903 var. *corticolus* (Hagem 1910) Schipper 1973	3	20.71	3	43.93	2	9.59
800	*Mucor hiemalis* Wehmer 1903 var. *hiemalis*	18	33.53	19	46.92	14	45.95
801	*Mucor hiemalis* Wehmer 1903 var. *silvaticus* (Hagem 1908) Schipper 1973	3	20.56	3	38.71	3	2.61
802	*Mucor inaequisporus* Dade 1937	1	19.64	1	39.72	1	0.10
803	*Mucor indicus* Lendner 1930	3	33.53	3	40.30	3	47.89

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
804	*Mucor laxorrhizus* Y. Ling 1930	5	19.59	5	31.36	5	20.52
805	*Mucor luteus* Linnemann 1936	2	19.66	2	41.88	2	16.22
806	*Mucor megaolocarpus* G. Walther et de Hoog 2013	1	19.75	1	9.93		
807	*Mucor microsporus* Namyslowski 1910	1	19.57	1	28.08		
808	*Mucor moelleri* (Vuillemin 1903) Lendner 1908	5	23.57	5	47.23	5	14.60
809	*Mucor mousanensis* Baijal et B.S. Mehrotra 1966	1	19.70	1	45.34	1	24.54
810	*Mucor mucedo* Linnaeus 1753	9	25.41	9	46.64	7	29.90
811	*Mucor odoratus* Treschew 1940	2	25.31	2	16.18		
812	*Mucor piriformis* A. Fischer 1892	5	25.41	5	35.21		
813	*Mucor plasmaticus* van Tieghem 1875	1	19.68	1	44.21	1	5.23
814	*Mucor plumbeus* Bonorden 1864	11	24.15	18	40.64	16	49.99
815	*Mucor psychrophilus* Milko 1971	1	25.41	1	14.88		
816	*Mucor racemosus* Fresenius 1850 var. *chibinensis* (Neophytova 1955) Schipper 1976	2	11.86	4	40.59	3	34.29
817	*Mucor racemosus* Fresenius 1850 var. *racemosus*	23	24.25	34	43.94	29	50.21
818	*Mucor racemosus* Fresenius 1850 var. *sphaerosporus* (Hagem 1908) Schipper 1970	3	25.41	3	44.91	3	47.36
819	*Mucor ramosissimus* Samoutsevitch 1927	1	19.64	1	17.67		
820	*Mucor saturninus* Hagem 1910	1	20.72	1	35.92	1	8.11
821	*Mucor sinensis* Milko et Beliakova 1971	2	21.24	2	36.82	2	33.55
822	*Mucor strictus* Hagem 1908	2	24.09	2	32.51	2	8.38
823	*Mucor ucrainicus* Milko 1971	1	23.18	1	25.68		
824	*Mucor zonatus* Milko 1967	2	19.75	2	27.40	1	5.69
825	*Mucor zychae* Baijal et B.S. Mehrotra 1965 var. *zychae*	2	25.31	2	44.13		
826	*Mutinus caninus* (Hudson 1778) Fries 1849	1	13.37				
827	*Myceliophthora fergusii* (Klopotek 1974) Oorschot 1977			2	7.38		
828	*Myceliophthora lutea* Costantin 1892			1	34.75	1	3.73
829	*Myceliophthora thermophila* (Apinis 1962) van Oorschot 1977	3	20.51	3	20.37	2	19.25
830	*Mycena epipterygia* (Scopoli 1772) Gray 1821	1	12.20				
831	*Mycena pura* (Persoon 1794) P. Kummer 1871	1	34.16				
832	*Mycogone cervina* Ditmar 1817	1	19.86				
833	*Mycogone nigra* (Morgan 1895) C.N. Jensen 1912	4	19.54	4	47.62		
834	*Mycogone rosea* Link 1809	4	19.38	4	38.41		

835	*Mycosticta cytosporicola* Frolov 1968	2	17.68	2	37.24		
836	*Mycotypha africana* R.O. Novak et Backus 1963			1	21.41		
837	*Mycotypha indica* P.M. Kirk et Benny 1985			1	22.42		
838	*Myrothecium* sp.	2	19.19	2	43.47		
839	*Myxotrichum setosum* (Eidam 1882) G.F. Orr et Plunkett 1963			2	38.43		
840	*Myxotrichum stipitatum* (Eidam 1882) G.F. Orr et Kuehn 1963	1	4.07	1	45.26		
841	*Nadsoniella nigra* Issatschenko 1914 var. *hesuelica* Lyakh et Ruban 1970	1	18.86	1	12.62		
842	*Nakataea sigmoidea* (Cavara 1889) Hara 1939	1	19.31				
843	*Nectria cosmariospora* Cesati et de Notaris 1863	2	18.95	2	35.78	2	3.45
844	*Nectria inventa* Pethybridge 1919			1	27.90	1	3.49
845	*Nematogonum mycophilum* (Saccardo 1886) Rogerson et W. Gams 1981	1	21.73	1	20.12		
846	*Neoantrodia serialis* (Fries 1821) Audet 2017	1	17.47				
847	*Neocamarosporium betae* (Berlese 1888) Ariyawansa et K.D. Hyde 2015	2	19.33	2	34.74		
848	*Neocosmospora vasinfecta* E.F. Smith 1899 var. *africana* (von Arx 1955) Cannon et D. Hawksworth 1984	2	20.10	2	48.22		
849	*Neonectria galligena* (Bresadola 1901) Rossman et Samuels 1999	1	18.96				
850	*Neoscytalidium dimidiatum* (Penzig 1887) Crous et Slippers 2006	1	19.40				
851	*Neottiospora caricina* (Desmazieres 1836) Hoehnel 1924			1	31.07		
852	*Neovossia setariae* (Ling 1945) Yu et Lou 1962	1	19.41				
853	*Neurospora crassa* Shear et B.O. Dodge 1927	71	18.95	77	44.92	9	23.18
854	*Neurospora sitophila* Shear et B.O. Dodge 1927	4	19.44	4	42.28	4	22.14
855	*Neurospora toroi* F.L. Tai 1935	1	18.97	1	41.71	1	21.16
856	*Newbya pascuicola* M.C. Vick et M.W. Dick 2002	1	20.71				
857	*Niesslia exilis* (Albertini et Schweinitz 1805) G. Winter 1885	1	18.96	1	7.35	1	4.37
858	*Nigrospora gorlenkoana* Novobranova 1972	2	19.47	2	39.55		
859	*Nigrospora gossypii* Jaczewski 1929	1	17.47	1	33.25		
860	*Nigrospora oryzae* (Berkeley et Broome 1873) Petch 1924			4	42.86	1	35.79
861	*Nodulisporium verrucosum* (J.F.H. Beyma 1929) G. Smith 1954	1	19.72				
862	*Nomuraea rileyi* (Farlow 1883) Samson 1974	1	9.85	1	12.70		
863	*Ochrocladosporium elatum* (Harz 1871) Crous et U. Braun 2007	1	12.58	1	27.80	1	7.91
864	*Ochroconis constricta* (E.V. Abbott 1927) de Hoog et Arx 1973			1	18.32		
865	*Oedocephalum* sp.	1	15.56	1	28.99	1	15.98
866	*Oidiodendron cereale* (Thuemen 1880) G.L. Barron 1962	5	0.18	4	28.60		
867	*Oidiodendron echinulatum* G.L. Barron 1962	2	19.58	2	31.49		
868	*Oidiodendron griseum* Robak 1934			2	13.34		

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
869	*Oidiodendron periconioides* Morrall 1968			1	1.79		
870	*Oidiodendron sulphureoochraceum*			1	1.95		
871	*Oidiodendron truncatum* G.L. Barron 1962			3	8.70		
872	*Olpitrichum* sp.	1	20.47	1	19.95	1	2.49
873	*Oospora nicotianae* Pezzolato 1899	1	19.77	1	29.63	1	2.89
874	*Oospora oryzae* Ferraris 1902	1	19.95	1	1.05	1	2.90
875	*Oospora sajanica* Ogarkov 1979	1	16.12	1	25.73		
876	*Oospora sulphurea* (Preuss 1852) Saccardo et Voglino 1886	1	28.81	1	19.34		
877	*Oospora sulphurella* (Saccardo et Roumeguere 1881) Saccardo 1886	1	19.56	1	26.34		
878	*Oospora tenuis* (P. Maze 1910) Berkhout 1923	1	31.12	1	30.92	1	0.09
879	*Oospora variabilis* (Lindner 1898) J. Lindau 1907	1	19.86	1	14.69		
880	*Ophiostoma piceae* (Münch 1907) Sydow et P. Sydow 1919			2	22.04		
881	*Ovadendron sulphureo-ochraceum* (J.F.H. Beyma 1933) Sigler et J.W. Carmichael 1976	1	19.79	1	43.59		
882	*Paecilomyces borysthenicus* B.A. Borisov et Tarasov 1997	2	5.07	2	12.90		
883	*Paecilomyces carneus* (Duche et R. Heim 1931) A.H.S. Brown *et* G. Smith 1957	2	4.41	2	8.12	2	0.21
884	*Paecilomyces fulvus* Stolk et Samson 1971			1	5.53		
885	*Paecilomyces inflatus* (Burnside 1927) J.W. Carmichael 1962			3	8.19		
886	*Paecilomyces marquandii* (Massee 1898) S. Hughes 1951	4	19.56	5	44.40	3	21.91
887	*Paecilomyces penicillatus* (Höhnel 1904) Samson 1974			1	5.44		
888	*Paecilomyces suffultus* (Petch 1944) Samson 1974	1	6.10				
889	*Paecilomyces variotii* Bainier 1907	20	19.41	30	45.44	16	47.90
890	*Paecilomyces zollerniae* Stolk et Samson 1971	1	12.37	1	12.56		
891	*Panus conchatus* (Bulliard 1787) Fries 1838	1	23.84				
892	*Papulaspora biformospora* Kirilenko 1971	1	19.64	1	20.69		
893	*Paraconiothyrium fuckelii* (Saccardo 1878) Verkley et Gruyter 2012			1	9.65		
894	*Paraconiothyrium sporulosum* (W. Gams et Domsch 1969) Verkley 2004	2	19.40	2	16.28		
895	*Paradendryphiella salina* (G.K. Sutherland 1916) Woudenberg et Crous 2013			4	10.24		
896	*Paramyrothecium roridum* (Tode 1790) L. Lombard et Crous 2016	3	19.49	4	45.17		
897	*Paraphoma fimeti* (Brunaud 1889) Gruyter et al. 2010			6	2.11		
898	*Parasitella parasitica* (Bainier 1884) Sydow 1903	2	33.43	2	46.09		

899	*Penicillium adametzii* K.M. Zalessky 1927			4	40.39	4	45.81
900	*Penicillium albicans* Bainier 1907			2	22.03	2	37.14
901	*Penicillium albidum* Sopp 1912			1	4.99	1	0.95
902	*Penicillium alicantinum* C. Ramirez et A.T. Martinez 1980			1	38.03	1	12.20
903	*Penicillium anatolicum* Stolk 1968			1	33.19	1	15.32
904	*Penicillium aragonense* C. Ramirez et A.T. Martinez 1981			1	38.03	1	12.20
905	*Penicillium arenicola* Chalabuda 1950			1	22.92	1	26.18
906	*Penicillium atramentosum* Thom 1910			1	21.02	1	2.56
907	*Penicillium aurantioflammiferum* C. Ramirez et al. 1980			1	27.31	1	12.20
908	*Penicillium aurantiogriseum* Dierckx 1901	9	20.52	62	44.47	36	42.96
909	*Penicillium bilaiae* Chalabuda 1950			1	43.15	1	18.52
910	*Penicillium brevicompactum* Dierckx 1901	6	20.50	23	45.39	15	18.10
911	*Penicillium brunneum* Udagawa 1959			1	37.98	1	12.07
912	*Penicillium camemberti* Thom 1906			11	42.00	10	24.37
913	*Penicillium canescens* Sopp 1912	31	20.50	60	47.85	44	49.17
914	*Penicillium capsulatum* Raper et Fennell 1948	2	19.51	3	44.92	3	28.71
915	*Penicillium castellonense* C. Ramirez et A.T. Martinez 1981			1	38.03	1	12.20
916	*Penicillium chermesinum* Biourge 1923			4	42.92	4	45.97
917	*Penicillium chrysogenum* Thom 1910	17	18.95	96	45.01	53	55.63
918	*Penicillium cinerascens* Biourge 1923			1	41.75	1	28.22
919	*Penicillium citreonigrum* Dierckx 1901	3	18.95	13	43.65	10	46.51
920	*Penicillium citrinum* Thom 1910	9	18.31	27	45.08	17	33.21
921	*Penicillium commune* Thom 1910	10	2.52	23	44.45	22	38.12
922	*Penicillium coprophilum* (Berkeley et M.A. Curtis 1868) Seifert et Samson 1986					2	0.98
923	*Penicillium cordubense* C. Ramirez et A.T. Martinez 1981			1	38.01	1	12.20
924	*Penicillium corylophilum* Dierckx 1901			3	28.97	2	15.77
925	*Penicillium crustosum* Thom 1930			31	45.20	10	47.85
926	*Penicillium cyaneum* (Bainier et R. Sartory 1913) Biourge 1923 ex Thom 1930			1	44.58	1	9.56
927	*Penicillium daleae* K.M. Zalessky 1927	1	2.27	2	29.98	1	22.64
928	*Penicillium decumbens* Thom 1910	4	18.08	17	43.07	8	37.99
929	*Penicillium dierckxii* Biourge 1923	2	18.31	7	44.77	7	28.88
930	*Penicillium digitatum* (Persoon 1801) Saccardo 1881			3	44.23	3	21.28
931	*Penicillium diversum* Raper et Fennell 1948			3	40.50	1	38.07

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
932	*Penicillium dodgei* Pitt 1980			1	27.75	1	12.58
933	*Penicillium duclauxii* Delacroix 1892			7	44.65	7	32.90
934	*Penicillium expansum* Link 1809			27	42.88	7	41.58
935	*Penicillium fagi* A.T. Martinez et C. Ramirez 1978			1	38.05	1	12.07
936	*Penicillium fellutanum* Biourge 1923			3	1.96	1	1.15
937	*Penicillium funiculosum* Thom 1910	5	20.50	14	44.53	9	38.16
938	*Penicillium glabrum* (Wehmer 1893) Westling 1911			23	40.77	9	31.15
939	*Penicillium gladioli* Machacek 1928	2	20.46	2	36.75	2	7.91
940	*Penicillium glaucum* Link 1805	1	20.52	1	44.50	1	48.00
941	*Penicillium grancanariae* C. Ramirez et al. 1978			1	38.05	1	5.70
942	*Penicillium granulatum* Bainier 1905			17	45.20	5	21.61
943	*Penicillium griseofulvum* Dierckx 1901			11	43.90	8	43.32
944	*Penicillium herqueri* Bainier et R. Sartory 1912			4	45.41	4	28.33
945	*Penicillium hirayamae* Udagawa 1959			1	20.37	1	20.18
946	*Penicillium hirsutum* Dierckx 1901 var. *hirsutum*			1	2.77	1	5.41
947	*Penicillium hispanicum* C. Ramirez et al. 1978			1	38.05	1	12.07
948	*Penicillium humuli* J.F.H. Beyma 1937			1	44.47	1	47.94
949	*Penicillium ilerdanum* C. Ramirez et al. 1980			1	26.66	1	12.20
950	*Penicillium indonesiae* Pitt 1980			2	38.31	2	37.22
951	*Penicillium inflatum* Stolk et Malla 1971			2	2.95	1	0.15
952	*Penicillium insectivorum* (Sopp 1912) Biourge 1923			1	44.21	1	38.19
953	*Penicillium islandicum* Sopp 1912	1	2.23	3	33.12	3	23.12
954	*Penicillium italicum* Wehmer 1894			3	39.11	3	22.63
955	*Penicillium janczewskii* K.M. Zalessky 1927			13	44.47	8	47.96
956	*Penicillium jensenii* K.M. Zalessky 1927			9	47.35	8	43.44
957	*Penicillium lagena* (Delitsch 1943) Stolk et Samson 1983	3	19.46	4	39.10	4	25.92
958	*Penicillium lanosum* Westling 1911			3	44.45	3	38.02
959	*Penicillium lapidosum* Raper et Fennell 1948			5	44.45	4	42.21
960	*Penicillium lehmanii* Pitt 1980			2	40.41	2	47.99
961	*Penicillium lineatum* Pitt 1980			1	10.51	1	11.55
962	*Penicillium lividum* Westling 1911			15	38.20	1	15.33
963	*Penicillium malacaense* C. Ramirez et A.T. Martinez 1980			1	38.01	1	12.20

964	*Penicillium martensii* Biourge 1923 var. *moldavicum* Solovei 1975	1	2.27	1	20.16	1	10.71
965	*Penicillium megasporum* Orpurt et Fennell 1955			3	20.18	2	20.34
966	*Penicillium melanoconidium* Dierckx 1901			2	12.89	2	0.10
967	*Penicillium melinii* Thom 1930	1	3.99	6	41.94	4	37.14
968	*Penicillium miczynskii* K.M. Zalessky 1927	1	4.02	7	43.38	6	47.84
969	*Penicillium minioluteum* Dierckx 1901			37	38.03	2	12.20
970	*Penicillium mirabile* Beliakova et Milko 1972			1	36.90	1	9.56
971	*Penicillium multicolor* Grigorieva-Manoilova et Poradielova 1915			1	42.24	1	20.77
972	*Penicillium multicolor* Novobranova 1972			1	12.94	1	15.09
973	*Penicillium murcianum* C. Ramirez et A.T. Martinez 1981			1	31.98	1	32.01
974	*Penicillium nalgiovense* Laxa 1932			7	9.24	7	0.08
975	*Penicillium novae-zeelandiae* J.F.H. Beyma 1940			5	44.78	5	9.70
976	*Penicillium ochrochloron* Biourge 1923			7	44.30	5	26.52
977	*Penicillium olivicolor* Pitt 1980					1	0.32
978	*Penicillium olsonii* Bainier et R. Sartory 1912					2	0.95
979	*Penicillium onobense* C. Ramirez et A.T. Martinez 1981			1	38.05	1	12.20
980	*Penicillium ovetense* C. Ramirez et A.T. Martinez 1981			1	38.03	1	12.20
981	*Penicillium oxalicum* Currie et Thom 1915			6	38.05	6	38.11
982	*Penicillium palitans* Westling 1911			9	9.63	4	0.25
983	*Penicillium palmense* C. Ramirez et al. 1978			1	38.05	1	12.20
984	*Penicillium paxilli* Bainier 1907	3	19.51	8	44.47	7	18.52
985	*Penicillium phoeniceum* J.F.H. Beyma 1933			4	44.92	4	15.02
986	*Penicillium piceum* Raper et Fennell 1948	1	20.55	3	41.96	3	47.78
987	*Penicillium pinophilum* Thom 1910			3	37.80	2	32.70
988	*Penicillium polonicum* K.M. Zalessky 1927			6	6.49	6	0.08
989	*Penicillium purpurogenum* Stoll 1904			19	46.91	9	50.92
990	*Penicillium quercetorum* Baghdadi 1968	1	4.06	1	44.72	1	38.26
991	*Penicillium raistrickii* G. Smith 1933			5	36.68	3	20.13
992	*Penicillium resticulosum* Birkinshaw et al. 1942			1	26.72	1	44.28
993	*Penicillium restrictum* J.C. Gilman et E.V. Abbott 1927	2	18.31	14	44.23	8	38.51
994	*Penicillium roqueforti* Thom 1906	7	3.99	16	45.00	15	43.04
995	*Penicillium roseopurpureum* Dierckx 1901			8	44.69	5	28.62
996	*Penicillium rubrum* Stoll 1904	5	20.50	13	45.08	13	43.02
997	*Penicillium rugulosum* Thom 1910	3	28.56	23	44.08	17	43.08
998	*Penicillium sclerotiorum* J.F.H. Beyma 1937			7	42.22	7	34.33

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
999	*Penicillium severskii* Schechovtsov 1981			1	11.10	1	5.72
1000	*Penicillium simplicissimum* (Oudemans 1903) Thom 1930	3	18.31	26	45.25	19	47.55
1001	*Penicillium solitum* Westling 1911	13	18.00	15	44.01	15	38.32
1002	*Penicillium spinulosum* Thom 1910	6	4.02	25	43.47	17	46.00
1003	*Penicillium terraconense* C. Ramirez et A.T. Martinez 1980			1	38.04	1	2.55
1004	*Penicillium thomii* Maire 1917	4	2.27	9	42.32	9	36.25
1005	*Penicillium thymicola* Frisvad et Samson 2004			1	6.41	1	0.31
1006	*Penicillium turbatum* Westling 1911			1	28.44	1	43.16
1007	*Penicillium turolense* C. Ramirez et A.T. Martinez 1981			1	38.04	1	12.20
1008	*Penicillium umbonatum* Sopp 1912			1	32.95		
1009	*Penicillium valentinum* C. Ramirez et A.T. Martinez 1980			1	38.03	1	12.20
1010	*Penicillium vanbeymae* Pitt 1980			1	42.32	1	7.00
1011	*Penicillium variabile* Sopp 1912			37	42.19	17	42.68
1012	*Penicillium vasconiae* C. Ramirez et A.T. Martinez 1980			1	37.93	1	12.20
1013	*Penicillium velutinum* J.F.H. Beyma 1935			11	44.65	10	38.52
1014	*Penicillium verrucosum* Dierckx 1901	6	0.58	18	42.32	9	32.75
1015	*Penicillium verruculosum* Peyronel 1913	14	18.08	23	42.33	13	28.85
1016	*Penicillium vinaceum* J.C. Gilman et E.V. Abbott 1927			4	44.59	4	43.16
1017	*Penicillium viridicatum* Westling 1911			5	6.06	1	0.09
1018	*Penicillium vulpinum* (Cooke et Massee 1888) Seifert et Samson 1985			10	44.53	10	42.56
1019	*Penicillium waksmanii* K.M. Zalessky 1927	1	2.27	10	44.57	4	47.96
1020	*Penicillium westlingii* K.M. Zalessky 1927			1	29.52	1	21.65
1021	*Penicillium zacinthae* C. Ramírez et A.T. Martínez 1981			1	26.64	1	12.20
1022	*Penidiella* sp.			5	2.10		
1023	*Perenniporia medulla-panis* (Jacquin 1778) Donk 1967	1	18.55				
1024	*Periconia igniaria* E.W. Mason et M.B. Ellis 1953			1	9.90		
1025	*Periconia macrospinosa* Lefebvre et Aar.G. Johnson 1949	2	19.49	2	28.22		
1026	*Periconiella cocoes* M.B. Ellis 1967			1	18.12		
1027	*Pestalotia pezizoides* de Notaris 1841	2	19.86	2	39.30		
1028	*Pestalotiopsis guepinii* (Desmazieres 1840) Steyaert 1949			6	10.76		
1029	*Pestalotiopsis sydowiana* (Bresadola 1895) B. Sutton 1961	1	19.29				
1030	*Petriella sordida* (Zukal 1890) G.L. Barron et J.C. Gilman 1961			1	10.23		

1031	*Phacidium lacerum* Fries 1818			1	0.33		
1032	*Phaeococcomyces nigricans* (Rich et Stern 1958) de Hoog 1979			1	23.58	1	0.53
1033	*Phaeoisaria triseptata* Holubova-Jechova 1988			1	18.69		
1034	*Phaeosphaeria* sp.			1	1.56		
1035	*Phallus hadriani* Ventenat 1798	1	29.07				
1036	*Phallus impudicus* Linnaeus 1753 var. *togatus* (Kalchbrenner 1883) Costantin et L.M. Dufour 1895	3	21.77				
1037	*Phanerochaete sanguinea* (Fries 1828) Pouzar 1973	1	3.25				
1038	*Phellinus igniarius* (Linnaeus 1753) Quelet 1886	6	21.17				
1039	*Phellinus lundellii* Niemelae 1972	3	20.02				
1040	*Phellinus populicola* Niemelae 1975	3	27.22				
1041	*Phialophora atrovirens* (J.F.H. Beyma 1935) Schol-Schwarz 1970	1	20.40	1	28.65		
1042	*Phialophora bubakii* (Laxa 1930) Schol-Schwarz 1970	2	20.42	10	36.70		
1043	*Phialophora cyclaminis* J.F.H. Beyma 1942			2	6.36		
1044	*Phialophora lagerbergii* (Melin et Nannfeldt 1934) Conant 1937	1	19.38	1	13.60		
1045	*Phialophora melinii* (Nannfeldt 1934) Conant 1937			13	5.86		
1046	*Phialophora verrucosa* Medlar 1915	1	19.76	1	19.52		
1047	*Phlebia ochraceofulva* (Bourdot et Galzin 1911) Donk 1957	1	16.82				
1048	*Phlebia rufa* (Persoon 1801) M.P. Christiansen 1960	1	14.95				
1049	*Phlebia tremellosa* (Schrader 1794) Nakasone et Burdsall 1984	2	19.68				
1050	*Phlebiopsis gigantea* (Fries 1815) Juelich 1978	3	20.04				
1051	*Pholiota adiposa* (Batsch 1786) P. Kummer 1871	1	20.10				
1052	*Pholiota aurivella* (Batsch 1786) P. Kummer 1871	1	19.72				
1053	*Pholiota lenta* (Persoon 1801) Singer 1951	2	14.04				
1054	*Pholiota microspora* (Berkeley 1850) Saccardo 1887	1	8.08				
1055	*Phoma herbarum* Westendorp 1852			5	4.99		
1056	*Phoma leveillei* Boerema et G.J. Bollen 1975			4	13.49		
1057	*Phoma lingam* (Tode 1791) Desmazieres 1849			4	19.59		
1058	*Phomatospora* sp.	1	18.86	1	26.61		
1059	*Phomopsis castanea* (Saccardo 1879) Petrak 1921			1	11.21		
1060	*Phomopsis castaneae* Moriondo 1963			1	11.10		
1061	*Phomopsis helianthi* Muntanola-Cvetcovic et al. 1981	1	8.26	1	5.68		
1062	*Phycomyces blakesleeanus* Burgeff 1925	8	24.13	8	31.79	2	1.51
1063	*Phycomyces nitens* (C. Agardh 1823) Kunze 1823	2	23.06	2	43.00	1	0.34
1064	*Phyllosticta castaneae* Ellis et Everhart 1894			1	11.05		

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
1065	*Phyllosticta pucciniospila* C. Massalongo 1900	1	11.45	1	23.98		
1066	*Phytophthora cactorum* (Lebert et Cohn 1870) J. Schroeter 1886	1	17.05				
1067	*Phytophthora capsici* Leonian 1922	2	28.12				
1068	*Phytophthora cinnamomi* Rands 1922	4	18.78				
1069	*Phytophthora cryptogea* Pethybridge et Lafferty 1919	1	18.03				
1070	*Phytophthora drechsleri* Tucker 1931	3	26.22				
1071	*Phytophthora megasperma* Drechsler 1931	1	0.19				
1072	*Pidoplitchkoviella terricola* Kirilenko 1975	1	20.22	1	42.69		
1073	*Piedraia hortae* Fonseca et Leao 1928	2	19.35	1	34.74	1	23.85
1074	*Piedraia hortae* Fonseca et Leao 1928 var. *paraguayensis* Fonseca et Leao 1928			1	32.46	1	0.11
1075	*Piedraia sarmentoi* M.J. Pereira 1930	1	19.35	1	25.02	1	21.46
1076	*Pilaira anomala* (Cesati 1851) J. Schroeter 1886			1	26.00	1	1.11
1077	*Pilaira caucasica* Milko 1970	1	24.12	1	16.20		
1078	*Pilaira moreaui* Y. Ling 1926	1	23.57	1	27.53		
1079	*Pilobolus crystallinus* (F.H. Wiggers 1780) Tode 1784			1	15.64		
1080	*Pilobolus longipes* van Tieghem 1878	1	3.68	1	15.64		
1081	*Pilobolus umbonatus* Buller 1934			1	15.65		
1082	*Piptoporus betulinus* (Bulliard 1788) P. Karsten 1881	3	26.12				
1083	*Pirella circinans* Bainier 1882	2	24.09	1	46.10	1	14.40
1084	*Pirella circinans* Bainier 1882 var. *volgogradensis* (Milko 1974) Benny et Schipper 1988			1	37.71		
1085	*Pirella naumovii* (Milko 1970) Benny et Schipper 1992	1	19.32	1	15.35	1	46.87
1086	*Pithoascus schumacheri* (E.C. Hansen 1877) Arx 1973					1	0.32
1087	*Plectosphaerella cucumerina* (Lindfors 1919) W. Gams 1968	1	20.79	1	39.44		
1088	*Plenodomus tracheiphila* (Petri 1929) Gruyter et al. 2013	1	12.56				
1089	*Pleotrichocladium opacum* (Corda 1837) Hernández-Restrepo et al. 2017	3	19.43	4	32.79		
1090	*Pleurocytospora* sp.			1	7.59		
1091	*Pleurodesmospora coccorum* (Petch 1924) Samson et al. 1980			1	28.25		
1092	*Pleurophoma cava* (Schulzer 1871) Boerema 1996	3	19.81	3	44.72		
1093	*Pleurotus cornucopiae* (Paulet 1793) Rolland 1910	1	20.02				
1094	*Pleurotus eryngii* (De Candolle 1815) Quelet 1872	1	18.92				
1095	*Pleurotus ostreatus* (Jacquin 1774) P. Kummer 1871	70	34.08				

1096	*Pleurotus pulmonarius* (Fries 1821) Quelet 1872	4	8.16				
1097	*Pochonia bulbillosa* (W. Gams et Malla 1988) Zare et W. *Gams* 2001	5	19.99	7	28.36	2	2.72
1098	*Pochonia chlamydosporia* (Goddard 1913) Zare et W. Gams 2001	7	19.47	7	31.72		
1099	*Poitrasia circinans* (H. Naganishi et N. Kawakami 1955) P.M. Kirk 1984	1	20.08	1	44.36		
1100	*Polycephalomyces tomentosus* (Schrad. 1799) Seifert 1985	1	19.54	1	40.41	1	1.55
1101	*Polyporus ciliatus* Fries 1815	1	10.36				
1102	*Polyporus tomentosus* Fries 1821	1	9.07				
1103	*Polyscytalum pustulans* (M.N. Owen et Wakefield 1919) M.B. Ellis 1976	1	15.57	1	38.53		
1104	*Porodaedalea pini* (Brotero 1804) Murrill 1905	1	22.97				
1105	*Preussia fleischhakii* (Auerswald 1866) Cain 1961			1	43.78	1	23.47
1106	*Protomyces macrosporus* Unger 1834	1	19.01				
1107	*Pseudallescheria boydii* (Shear 1922) McGinnis et al. 1982	3	20.28	3	44.50	2	22.75
1108	*Pseudallescheria ellipsoidea* (Arx et Fassatiova 1973) McGinnis et al. 1982			1	42.46	1	23.42
1109	*Pseudeurotium bakeri* C. Booth 1961	1	20.48	1	43.04		
1110	*Pseudeurotium desertorum* Mouchacca 1971	1	18.88	1	41.28		
1111	*Pseudeurotium hygrophilum* (Sogonov et al. 2005) Minnis et D.L. Lindner 2013			9	1.67		
1112	*Pseudeurotium ovale* Stolk 1955 var. *milkoi* Beliakova 1969			2	44.73		
1113	*Pseudeurotium ovale* Stolk 1955 var. *ovale*	3	34.44	1	45.50	1	0.11
1114	*Pseudeurotium zonatum* J.F.H. Beyma 1937	9	20.42	20	44.28	10	24.97
1115	*Pseudogymnoascus caucasicus* Cejp et Milko 1966	1	29.35	1	39.38	1	25.13
1116	*Pseudogymnoascus roseus* Raillo 1929	2	22.61	5	47.77		
1117	*Puccinia adoxae* R. Hedwig 1805	1	19.60	1	19.05		
1118	*Puccinia bupleuri* (Opiz 1852) F. Rudolphi 1829	1	19.60	1	1.84		
1119	*Puccinia punctiformis* (F. Strauss 1811) Roehling 1813	1	23.64	1	19.05		
1120	*Purpureocillium lilacinum* (Thom 1910) Luangsa-ard et al. 2011	16	22.80	28	44.55	17	19.37
1121	*Pycnidiella resinae* (Ehrenberg 1818) Hoehnel 1915			1	25.43		
1122	*Pycnoporus cinnabarinus* (Jacquin 1776) P. Karsten 1881	2	23.74				
1123	*Pyrenochaeta* sp.			1	6.54		
1124	*Pyrenophora biseptata* (Saccardo et Roumeguere 1881) Crous 2013	1	19.30	1	33.34	1	4.33
1125	*Pyrenophora triseptata* (Drechsler 1923) Rossman et K.D. Hyde 2015			1	4.24		
1126	*Pyricularia grisea* Saccardo 1880	3	19.85	1	13.76		
1127	*Pyronema omphalodes* (Bulliard 1791) Fuckel 1870	1	18.96	1	17.28		
1128	*Pythium heterothallicum* W.A. Campbell et F.F. Hendrix 1968	2	33.45				
1129	*Pythium intermedium* de Bary 1881	1	6.50				

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
1130	*Pythium irregulare* Buisman 1927	2	33.47				
1131	*Pythium mamillatum* Meurs 1928	1	17.05				
1132	*Pythium oedichilum* Drechsler 1930	1	33.28				
1133	*Pythium paroecandrum* Drechsler 1930	1	33.58				
1134	*Pythium spinosum* Sawada 1926	1	0.15				
1135	*Pythium sylvaticum* W.A. Campbell et F.F. Hendrix 1967	2	20.59				
1136	*Quambalaria cyanescens* (de Hoog et G.A. de Vries 1973) Z.W. *de* Beer et al. 2006	3	6.48				
1137	*Radiomyces embreei* R.K. Benjamin 1960			2	44.93	2	46.27
1138	*Radiomyces spectabilis* Embree 1959	1	25.31	1	39.17	1	24.89
1139	*Remotididymella destructiva* (Plowright 1881) Valenzuela-Lopez et al. 2017			3	16.86		
1140	*Rhinocladiella atrovirens* Nannfeldt 1934	3	17.70	5	13.39		
1141	*Rhinotrichum aureum* Cooke et Massee 1889			1	28.38		
1142	*Rhinotrichum lanosum* Cooke 1871	1	19.47	1	21.52		
1143	*Rhizoctonia solani* J.G. Kuehn 1858	22	20.04	1	5.53	4	21.24
1144	*Rhizoctonia tuliparum* (Klebahn 1905) Whetzel et J.M. Arthur 1924			1	14.94		
1145	*Rhizomucor miehei* (Cooney et R. Emerson 1964) Schipper 1978	1	19.68	1	26.51	1	46.10
1146	*Rhizomucor pusillus* (Lindt 1886) Schipper 1978	4	19.70	4	43.33	4	43.99
1147	*Rhizomucor tauricus* (Milko et Schkurenko 1970) Schipper 1978	2	19.64	2	45.07	2	24.69
1148	*Rhizopus arrhizus* A. Fischer 1892	23	20.72	26	45.19	25	50.01
1149	*Rhizopus microsporus* van Tieghem 1875 var. *chinensis* (Saito 1904) Schipper et Stalpers 1984	5	20.77	5	45.28	5	48.43
1150	*Rhizopus microsporus* van Tieghem 1875 var. *microsporus*	8	20.75	11	39.38	10	49.99
1151	*Rhizopus microsporus* van Tieghem 1875 var. *oligosporus* (Saito 1905) Schipper et Stalpers 1984	2	12.43	2	44.83	2	49.50
1152	*Rhizopus microsporus* van Tieghem 1875 var. *rhizopodiformis* (Cohn 1884) Schipper et Stalpers 1984			4	15.00	3	12.48
1153	*Rhizopus stolonifer* (Ehrenberg 1818) Vuillemin 1902 var. *stolonifer*	20	24.15	24	45.19	19	50.21
1154	*Rhodocollybia butyracea* (Bulliard 1792) Lennox 1979	1	2.82				
1155	*Robillarda sessilis* (Saccardo 1878) Saccardo 1880			1	18.99		
1156	*Rosellinia mammiformis* (Persoon 1801) Cesati et de Notaris 1863	1	19.28	1	31.68		
1157	*Russula aurora* (Krombholz 1836) Bresadola 1892	1	19.40				
1158	*Russula decolorans* (Fries 1821) Fries 1838	1	34.23				

1159	*Russula grisea* (Batsch 1786) Fries 1838	1	34.03				
1160	*Saksenaea vasiformis* S.B. Saksena 1953	1	27.79				
1161	*Saprochaete gigas* (Smit et L. Meyer 1928) de Hoog et *M.T.* Smith 2004	1	19.87	1	43.67	1	0.12
1162	*Saprolegnia asterophora* de Bary 1860	1	15.20				
1163	*Saprolegnia blelhamensis* (M.W. Dick 1969) Milko 1979	3	13.34				
1164	*Saprolegnia ferax* (Gruithuisen 1821) Nees 1843	2	13.81				
1165	*Saprolegnia litoralis* Coker 1923	1	13.10				
1166	*Saprolegnia mixta* de Bary 1883	1	0.17				
1167	*Saprolegnia terrestris* Cookson 1937 ex R.L. Seymour 1970	1	0.17				
1168	*Saprolegnia unispora* (Coker et Couch 1923) R.L. Seymour 1970	2	0.17				
1169	*Sarocladium strictum* (W. Gams 1971) Summerbell 2011	4	19.91	4	26.96	2	23.67
1170	*Schizophyllum commune* Fries 1815	4	34.08				
1171	*Sclerotinia borealis* Bubák et Vleugel 1917	19	3.85				
1172	*Sclerotinia nivalis* I. Saito 1997	25	3.85				
1173	*Sclerotinia ricini* G.H. Godfrey 1919	1	18.97	1	27.35	1	0.11
1174	*Sclerotinia sclerotiorum* (Libert 1837) de Bary 1884	2	31.23				
1175	*Scopulariopsis acremonium* (Saccardo 1882) Bainier 1907 (Thom 1910) Thom 1930	1	19.33	1	23.60	1	4.86
1176	*Scopulariopsis asperula* (Saccardo 1882) S. Hughes 1958	1	19.26	1	39.91	1	18.38
1177	*Scopulariopsis brevicaulis* (Saccardo 1882) Bainier 1907	15	20.49	17	48.08	12	46.09
1178	*Scopulariopsis brumptii* Salvanet-Duval 1935	3	15.25	4	21.54	1	3.33
1179	*Scopulariopsis coprophila* (Cooke et Massee 1887) W. Gams 1971			1	31.33		
1180	*Scopulariopsis croci* J.F.H. Beyma 1944			1	35.42	1	23.92
1181	*Scopulariopsis flava* (Sopp 1912) F.J. Morton et G. Smith 1963 (Thom 1910) Thom 1930	1	19.30	1	23.56	1	20.68
1182	*Scopulariopsis halophilica* Tubaki 1973	1	28.84				
1183	*Scopulariopsis koningii* (Oudemans 1902) Vuuillemin 1911			1	30.89		
1184	*Scytalidium terminale* G.V. Rao et de Hoog 1975	1	19.50	1	29.45		
1185	*Seimatosporium pestalozzioides* (Saccardo 1884) B. Sutton 1975			1	9.19		
1186	*Sepedonium macrosporum* Saccardo et Cavara 1900			1	39.83	1	25.33
1187	*Septoria lycopersici* Spegazzini 1881	1	17.25				
1188	*Septoria rosarum* Westendorp 1851				19.42		
1189	*Serpula lacrymans* (Wulfen 1781) J. Schroeter 1885	2	20.11				
1190	*Simplicillium lamellicola* (F.E.W. Smith 1924) Zare et W. Gams 2001	4	19.57	4	24.26		
1191	*Simplicillium obclavatum* (W. Gams 1984) Zare et W. Gams 2001			1	3.83		

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
1192	*Sistotrema brinkmannii* (Bresadola 1903) J. Eriksson 1948	1	0.16				
1193	*Sordaria fimicola* (Roberge ex Desmazières 1849) Cesati et de Notaris *1863*	2	19.37	2	43.76	2	23.47
1194	*Sorosporium saponariae* F. Rudolphi 1830	1	17.70				
1195	*Spadicesporium acrosporum* V.N. Borisova et Dvoinos 1982	1	19.43	1	43.13		
1196	*Spadicesporium acrosporum-majus* V.N. Borisova et Dvoinos 1982	1	19.43	1	11.45		
1197	*Spadicesporium bifurcatum* V.N. Borisova et Dvoinos 1982	1	19.43	1	19.24		
1198	*Spadicesporium bifurcatum-majus* V.N. Borisova et Dvoinos 1982	1	19.43	1	13.52		
1199	*Spadicesporium copiosum* V.N. Borisova et Dvoinos 1982	1	19.43	1	32.34		
1200	*Spadicesporium persistens* V.N. Borisova et Dvoinos 1982	1	19.43	1	40.85		
1201	*Spadicesporium ramosum* V.N. Borisova et Dvoinos 1982	1	19.78	1	43.13		
1202	*Sparassis crispa* (Wulfen 1781) Fries 1821	1	7.49				
1203	*Sphaceloma* sp.			1	23.55		
1204	*Sphaeropsis sapinea* (Fries 1823) Dyko et B. Sutton 1980	2	19.90				
1205	*Sphaerostilbella aureonitens* (Tulasne et C. Tulasne 1865) Seifert et al. 1985			1	22.34		
1206	*Sphaerostilbella penicillioides* (Corda 1840) Rossman et al. 2015	6	19.19	6	34.71	2	3.20
1207	*Sporocadus lichenicola* Corda 1839			1	10.24		
1208	*Sporodiniopsis dichotoma* van Hoehnel 1903	1	15.90	1	15.95	1	10.80
1209	*Sporormiella australis* (Spegazzini 1887) S.I. Ahmed et Cain 1972			1	2.41	1	5.07
1210	*Sporormiella intermedia* (Auerswald 1868) S.I. Ahmed et Cain ex Kobayasi 1969			1	38.09		
1211	*Sporothrix fungorum* de Hoog et G.A. de Vries 1973			1	1.94		
1212	*Sporotrichum aeruginosum* Schweinitz 1886 var. *microsporum* Karsten 1905			1	32.35		
1213	*Sporotrichum bombycinum* (Corda 1839) Rabenhorst 1844	3	19.31	3	29.59	2	35.25
1214	*Sporotrichum gorlenkoanum* Kuritzina et Sizova 1967			1	23.64	1	20.27
1215	*Sporotrichum laxum* Nees 1816	1	19.31	1	26.27		
1216	*Sporotrichum mycophilum* Link 1818			1	27.68		
1217	*Sporotrichum pruinosum* J.C. Gilman et E.V. Abbott 1927	7	19.82	13	42.71	5	35.05
1218	*Sporotrichum roseolum* Oudemans et Beijerinck 1903	1	15.20	1	26.16	1	2.28
1219	*Stachybotrys chartarum* (Ehrenberg 1818) S. Hughes 1958	10	19.32	14	48.10	2	9.56
1220	*Stachybotrys cylindrospora* C.N. Jensen 1912			1	29.41		
1221	*Stachylidium variabile* Schulzer et Saccardo 1884			1	1.59		
1222	*Stagonospora paludosa* (Saccardo et Spegazzini 1879) Saccardo 1884	1	19.90				

1223	*Stagonosporopsis hortensis* (Saccardo et Malbranche 1882) Petrak 1921	1	20.34	1	27.54		
1224	*Stagonosporopsis trachelii* (Allescher 1895) Aveskamp et al. 2010	1	20.34				
1225	*Stemphyliomma* sp.			1	26.05	1	35.79
1226	*Stemphylium botryosum* Wallroth 1833	1	17.06	1	23.43		
1227	*Stemphylium sarciniforme* (Cavara 1890) Wiltshire 1938	3	17.80	3	25.45		
1228	*Stenocarpella maydis* (Berkeley 1847) B. Sutton 1980	1	21.31	1	11.57		
1229	*Stephanoma* sp.	1	16.24	1	16.03		
1230	*Stereum hirsutum* (Willdenow 1787) Persoon 1800	3	20.04				
1231	*Stereum sanguinolentum* (Albertini et Schweinitz 1805) Fries 1838	1	23.21				
1232	*Stilbella bulbicola* Hennings 1905	1	19.46	1	44.40		
1233	*Stilbotulasnella conidiophora* Bandoni et Oberwinkler 1982	1	23.91				
1234	*Striaticonidium brachysporum* (Nicot 1961) L. Lombard et Crous 2016	1	12.92	1	18.96		
1235	*Striaticonidium cinctum* (Corda 1842) L. Lombard et Crous 2016	1	19.30	1	28.18		
1236	*Strobilomyces strobilaceus* (Scopoli 1770) Berkeley 1851	1	26.53				
1237	*Stropharia rugosoannulata* Farlow ex Murrill 1922	1	12.21				
1238	*Syncephalastrum racemosum* Cohn ex J. Schroeter 1886	12	19.75	12	42.96	12	50.78
1239	*Syncephalis cornu* van Tieghem et G. Le Monnier 1873			1	46.84	1	45.50
1240	*Syncephalis nodosa* van Tieghem 1875	1	24.13	1	23.87	1	32.34
1241	*Taeniolella aquatilis* (Woronichin 1925) Milko 1985	1	19.33	1	31.75		
1242	*Talaromyces emersonii* Stolk 1965			1	30.14	1	37.44
1243	*Talaromyces flavus* (Kloecker 1902) Stolk et Samson 1972	2	18.08	4	43.58	4	46.02
1244	*Talaromyces luteus* (Zukal 1889) C.R. Benjamin 1955			9	43.25	9	36.49
1245	*Talaromyces stipitatus* (Thom 1935) C.R. Benjamin 1955			2	33.85	2	33.96
1246	*Talaromyces thermophilus* Stolk 1965			1	31.21	1	1.25
1247	*Talaromyces ucrainicus* Udagawa 1966	2	18.08	3	44.17	3	36.26
1248	*Talaromyces wortmannii* (Kloecker 1903) C.R. Benjamin 1955			3	22.58	3	12.07
1249	*Taphrina bergeniae* Döbbeler 1979	1	19.01	1	10.22		
1250	*Taphrina betulina* Rostrup 1883	1	13.27				
1251	*Taphrina carnea* Johanson 1886	1	8.12				
1252	*Taphrina coerulescens* Lindquist et Wright	1	13.25				
1253	*Taphrina flavorubra* W.W. Ray 1939	1	13.27				
1254	*Taphrina pruni* (Fuckel 1861) Tulasne 1866	1	19.01				
1255	*Taphrina purpurascens* B.L. Robinson 1887	1	13.27	1	8.72		
1256	*Taphrina robinsoniana* Giesenhagen 1892	1	13.25				
1257	*Taphrina sadebeckii* Johanson 1885	1	13.25				

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
1258	*Taphrina tosquinetii* (Westendorp 1861) Tulasne 1866	1	18.91	1	9.59		
1259	*Tapinella panuoides* (Batsch 1783) E.-J. Gilbert 1931	2	7.02				
1260	*Teberdinia hygrophila* Sogonov et al. 2005			1	4.67		
1261	*Tetraploa aristata* Berkeley et Broome 1850	1	30.33				
1262	*Thamnidium elegans* Link 1809	3	17.72	3	40.96	3	33.55
1263	*Thamnostylum piriforme* (Bainier 1880) Arx et H.P. Upadhyay 1970	4	24.14	4	37.27	4	48.92
1264	*Thelebolus microsporus* (Berkeley et Broome 1865) Kimbrough 1967			3	6.54		
1265	*Thelebolus polysporus* (P. Karsten 1871) Otani et Kanzawa 1970	1	20.60	2	33.25		
1266	*Thermomyces ibadanensis* Apinis et Eggins 1966			2	28.76		
1267	*Thielavia appendiculata* Srivastava et al. 1966			1	46.06		
1268	*Thielavia hyrcaniae* Nicot 1961	1	4.06	1	42.75		
1269	*Thielavia inaequalis* Pidoplichko et al. 1973	2	4.07	2	45.58	2	0.10
1270	*Thielavia ovispora* Pidoplichko et al. 1973	3	19.33	3	43.57	3	24.15
1271	*Thielavia terrestris* (Apinis 1963) Malloch et Cain 1972	1	20.52	1	12.62		
1272	*Thielavia terricola* (J.C. Gilman et E.V. Abbott 1927) Emmons 1930	5	19.24	5	43.99	5	24.81
1273	*Thyrostroma carpophilum* (Leveille 1843) B. Sutton 1997	1	19.92				
1274	*Thysanophora canadensis* Stolk et Hennebert 1968	1	17.70	1	7.31		
1275	*Thysanophora penicillioides* (Roumeguere 1890) W.B. Kendrick 1961	6	19.21	6	32.25		
1276	*Tilachlidium pinnatum* Preuss 1851			1	29.01		
1277	*Tilletia laevis* J.G. Kühn 1873	1	23.91	1	19.05		
1278	*Tilletiopsis albescens* Gokhale 1972	1	16.41	1	20.08		
1279	*Tilletiopsis washingtonensis* Nyland 1950	3	28.64	3	29.65		
1280	*Tolypocladium cylindrosporum* W. Gams 1971	5	20.73	5	36.11	1	5.13
1281	*Tolypocladium geodes* W. Gams 1971	6	13.72	6	13.67		
1282	*Tolypocladium inflatum* W. Gams 1971	9	19.28	13	35.21	2	0.09
1283	*Tolypocladium microsporum* (Jaap 1916) Bissett 1983			1	3.92		
1284	*Torula ligniperda* (Willkomm 1866) Saccardo 1906	1	12.49	1	30.12		
1285	*Trametes gibbosa* (Persoon 1795) Fries 1838	1	23.84				
1286	*Trametes hirsuta* (Wulfen 1788) Lloyd 1924	5	26.58				
1287	*Trametes ochracea* (Persoon 1794) Gilbertson et Ryvarden 1987	1	8.25				
1288	*Trametes pubescens* (Schumacher 1803) Pilat 1939	3	34.10				
1289	*Trametes versicolor* (Linnaeus 1753) Lloyd 1920	10	34.10				

1290	*Trametes zonatella* Ryvarden 1978	1	0.41				
1291	*Tricellula aquatica* J. Webster 1959	1	16.22	1	29.30		
1292	*Tricellula aurantiaca* (Haskins 1958) Arx 1970	1	31.01	1	34.22		
1293	*Trichaptum abietinum* (Dickson 1793) Ryvarden 1972	2	25.23				
1294	*Trichocladium asperum* Harz 1871	2	19.56	2	47.73		
1295	*Trichocladium griseum* (Traaen 1914) X. Wei Wang et Houbraken 2018	2	19.30	7	48.38		
1296	*Trichocladium nigrospermum* (Schweinitz 1832) X. Wei Wang et Houbraken 2018	3	17.78	3	32.27	1	35.79
1297	*Trichoderma asperellum* Samuels et al. 1999			2	7.71	1	8.48
1298	*Trichoderma atroviride* P. Karsten 1892	5	20.24	9	46.30	4	30.59
1299	*Trichoderma aureoviride* Rifai 1969	4	19.82	4	41.99	4	29.94
1300	*Trichoderma citrinoviride* Bissett 1984			3	1.92		
1301	*Trichoderma deliquescens* (Sopp 1912) Jaklitsch 2011			1	21.12	1	12.36
1302	*Trichoderma flavofuscum* (J.H. Miller et al. 1957) Bissett 1991			1	25.05	1	0.09
1303	*Trichoderma ghanense* Yoshim. Doi et al. 1987			1	25.05	1	0.09
1304	*Trichoderma hamatum* (Bonorden 1851) Bainier 1906	1	20.36	1	25.62	1	10.01
1305	*Trichoderma harzianum* Rifai 1969	24	19.96	32	45.53	12	12.42
1306	*Trichoderma koningii* Oudemans 1902	4	19.82	4	42.75	4	35.83
1307	*Trichoderma lignorum* (Tode 1790) Harz 1872			1	2.99		
1308	*Trichoderma longibrachiatum* Rifai 1969	16	21.37	22	46.32	9	37.65
1309	*Trichoderma polysporum* (Link 1816) Rifai 1969	4	19.99	4	46.07	3	3.56
1310	*Trichoderma pseudokoningii* Rifai 1969	8	19.81	9	45.02	8	38.35
1311	*Trichoderma reesei* E.G. Simmons 1968	6	19.82	6	36.31	5	35.98
1312	*Trichoderma saturnisporum* Hammill 1970	2	0.54	2	25.05	1	4.92
1313	*Trichoderma virens* (J.H. Miller et al. 1957) Arx 1987	2	18.70	3	44.87	2	18.38
1314	*Trichoderma viride* Persoon 1794	13	20.47	12	40.53	11	29.81
1315	*Trichoderma viride* Persoon 1794 var. *kizhanicum* Krapivina 1975			1	32.63	1	8.12
1316	*Trichoderma viridescens* (A.S. Horne et H.S. Williamson 1923) Jaklitsch et Samuels 2006			5	26.99	4	26.87
1317	*Trichosporiella cerebriformis* (G.A. de Vries et Kleine-Natrop) W. Gams 1971	2	19.59	3	19.22		
1318	*Trichosporon dulcitum* (Berkhout 1923) Weijman 1979			1	27.05		
1319	*Trichosporum herbarum* Jaap 1916	1	0.54	1	27.82	1	3.73
1320	*Trichothecium roseum* (Persoon 1794) Link 1809	12	20.43	13	47.13	9	10.41
1321	*Tritirachium oryzae* (Vincens 1910) de Hoog 1972	5	17.67	5	45.93	4	3.38

(continued)

No.	Name of species	Cryopreservation		Freeze-drying		Soil	
		Number of strains	Max storage time (years)	Number of strains	Max storage time (years)	Number of strains	Max storage time (years)
1322	*Tropicoporus linteus* (Berkeley et M.A. Curtis 1858) L.W. Zhou et Y.C. Dai 2015	1	22.97				
1323	*Truncatella angustata* (Persoon 1801) S. Hughes 1958	3	19.47	4	46.76		
1324	*Tympanosporium parasiticum* W. Gams 1974	1	18.61	1	35.89	1	0.53
1325	*Ugola praticola* (Pidoplichko 1950) Stalpers 1984			1	30.96	1	8.47
1326	*Umbelopsis isabellina* (Oudemans 1902) W. Gams 2003	7	23.57	8	42.26	7	47.18
1327	*Umbelopsis longicollis* (Dixon-Stewart 1932) Y.N. Wang et al. 2015	4	19.43	4	38.84	4	50.23
1328	*Umbelopsis nana* (Linnemann 1941) Arx 1984	3	24.12	2	35.21	2	27.28
1329	*Umbelopsis ramanniana* (Moeller 1903) W. Gams 2003	11	19.43	11	40.99	11	50.21
1330	*Umbelopsis vinacea* (Dixon-Stewart 1932) Arx 1984	3	24.73	3	39.93	3	34.51
1331	*Ustilago avenae* (Persoon 1801) Rostrup 1890	1	18.51	1	1.64		
1332	*Ustilago cordae* Liro 1924	1	19.56	1	1.84		
1333	*Ustilago cynodontis* (Passerini 1870) Hennings 1893	1	21.45	1	18.84		
1334	*Ustilago filiformis* (Schrank 1793) Rostrup 1890	1	9.34	1	18.84		
1335	*Ustilago hordei* (Persoon 1801) Lagerheim 1889	1	12.32	1	18.84		
1336	*Ustilago maydis* (de Candolle 1815) Corda 1842	2	13.39				
1337	*Valsa sordida* Nitschke 1870			1	17.51		
1338	*Venturia tremulae* Aderhold 1897			1	2.11		
1339	*Verticillium albo-atrum* Reinke et Berthold 1879	3	19.33	3	29.00	3	5.02
1340	*Verticillium bulbillosum* W. Gams et Malla 1971			4	29.29		
1341	*Verticillium candidulum* W. Gams et Malla 1971			1	29.62		
1342	*Verticillium cellulose* W. Gams et Malla 1971			1	9.19		
1343	*Verticillium dahliae* Klebahn 1913	4	19.37	4	43.73	4	13.62
1344	*Verticillium epiphytum* Hansford 1943			1	38.28		
1345	*Verticillium fumosum* Seman 1968	1	19.27	1	31.86	1	4.74
1346	*Verticillium lecanii* (Zimmermann 1898) Viegas 1939	6	19.37	13	39.89	4	3.33
1347	*Verticillium longisporum* (C. Stark 1961) Karapapa et al. 1997			1	2.97		
1348	*Verticillium psalliotae* Treschew 1941			1	24.57		
1349	*Verticillium tenerum* Nees 1816			2	24.68		
1350	*Verticillium tricorpus* I. Isaac 1953	2	19.33	2	37.17	2	7.30
1351	*Verticillium villosum* Rudakov 1981			1	41.45		
1352	*Verticillium zaregamsianum* Inderbitzin et al. 2011	7	20.76	7	35.67	5	6.53

1353	*Viennotidia humicola* (Samson et W. Gams 1974) P.F. Cannon et *D.* Hawksworth 1982			1	27.31	1	27.65
1354	*Volutella ciliata* (Albertini et Schweinitz 1805) Fries 1832	2	19.84	2	21.53		
1355	*Volutella roseola* Cooke 1872	1	21.38	1	15.43		
1356	*Wallemia sebi* (Fries 1832) Arx 1970	3	19.60	2	15.96		
1357	*Wallrothiella subiculosa* Hoehnel 1912			1	21.53		
1358	*Wardomyces anomalus* Brooks et Hansford 1923			1	13.50		
1359	*Westerdykella dispersa* (Clum 1955) Cejp et Milko 1964	1	19.35	1	34.61	1	25.11
1360	*Westerdykella multispora* (Saito et Minoura ex Cain 1961) Cejp et Milko 1964	1	3.99	1	43.57	1	23.47
1361	*Xeromyces bisporus* L.R. Fraser 1953	1	18.86	1	15.48	1	0.21
1362	*Xylobolus frustulatus* (Persoon 1801) Boidin 1958	1	20.01				
1363	*Zasmidium biverticillatum* (Arzanlou et P.W. Crous 2007) S.I.R. Videira et *P.W.* Crous 2017			1	25.54		
1364	*Zygosporium echinosporum* Bunting et E.W. Mason 1941	1	19.56	1	36.47	1	1.00
1365	*Zymoseptoria passerinii* (Saccardo 1884) Quaedvlieg et Crous 2011			1	1.74		
1366	*Zymoseptoria pseudotritici* B. McDonald et al. 2012			1	6.45		

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Chapter 2
Sabouraud Agar and Other Fungal Growth Media

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2.1 Sabouraud Dextrose Agar

2.1.1 History

Sabouraud agar medium was developed by the French dermatologist Raymond J. A. Sabouraud (pronounced sah-bū-rō′) in the late 1800s to support the growth of fungi, particularly dermatophytes [1, 2]. Sabouraud's medical investigations focused on bacteria and fungi that cause skin lesions, and he developed many agars and techniques to culture pathogens such as dermatophytes and *Malassezia* species. The long incubation period (multiple weeks) of dermatophytes and the need to avoid bacterial contamination while culturing them, was one driving force behind the development of this medium. Additionally, Sabouraud sought to provide a medium that would yield reliable results for fungal identification *across* laboratories. He recommended that all mycologists detail their exact media formulations and sources of ingredients as well as the temperatures and times of specimen incubation, in order to standardize observations and reduce media-derived sources of differences in appearance [3].

Ironically, given Sabouraud's original desire to standardize the construction of fungal media, there are currently many sources of confusion and variation in both the names and ingredients associated with Sabouraud agar, also called Sabouraud's agar (abbreviated either SDA or SAB). Due to the old-fashioned use of the term "dextrose" to refer to D-glucose, the medium has been referred to as Sabouraud dextrose agar as well as Sabouraud glucose agar, the name most appropriate and consistent with standard chemical nomenclature [3]. Finally, a more recent modification of Sabouraud agar by Emmons is either called Sabouraud agar (Modified), or Sabouraud agar, Emmons [4]. Many of the historical details behind these names and ingredient variations are described in Odds' excellent review article [3].

2.1.2 Theory

Sabouraud agar is a selective medium that is formulated to allow the growth of fungi and inhibit the growth of bacteria. The available means of inhibiting bacterial growth in Sabouraud's pre-antibiotic era was via an acidic medium (pH 5.6). Currently, the addition of antibiotics or antimicrobials to the acidic medium is used to inhibit

bacterial growth (and sometimes saprophytic fungi, depending on the particular antimicrobial used).

Sabouraud agar medium is complex and undefined but contains few ingredients. Peptones, as soluble protein digests, are sources of nitrogenous growth factors that can vary significantly according to the particular protein source. The most variation is present in the source and method of these protein digests. Both Difco and BBL brand Sabouraud agars use pancreatic digests of casein as their peptone source, but they and other vendors also use a combination of pancreatic digest of casein and peptic digest of animal tissues. Sabouraud's original formulation contained a peptone termed "Granulée de Chassaing," which is no longer available. Mold morphology can vary slightly based on the peptones used, but pigmentation and sporulation can be consistent if one uses a consistent method of medium preparation, with the ingredients from the same source each time. Researchers should also explicitly describe the commercial or laboratory-prepared components used in their medium.

Sabouraud originally used sugar maltose as an energy source, and although this medium is still commercially available, glucose (formerly referred to as dextrose) is currently used most frequently. Glucose is present at a high level of 4% in Sabouraud's formulation to assist in vigorous fermentation and acid production by any bacteria present, inhibiting later bacterial growth [5].

In 1977, Emmons formulated an alternative version of Sabouraud's agar, which contains half the amount of glucose (2%) and a neutral pH of 6.8–7.0. The neutral pH seems to enhance the growth of some pathogenic fungi, such as dermatophytes. Agar concentrations ranging from 1.5–2.0% are found in commercial preparations of Sabouraud agar in both the original formula and Emmons modification and serve to solidify the medium in tube and plate medium.

2.1.3 Materials

Sabouraud agar can be either made from individual ingredients (see Tables 2.1 and 2.2), purchased either as a dehydrated powder that must be dissolved in water, autoclaved, and dispensed, or as a prepared medium that can be purchased in a tube, plate, or broth form from a variety of commercial sources. Various antimicrobials can be added to either the original recipe (Sabouraud agar) or Sabouraud agar, Modified/Emmons (see Table 2.2).

Table 2.1 Ingredients for Sabouraud agar and Sabouraud agar (Emmons)

Ingredient	Sabouraud agar (per liter)	Sabouraud agar (Emmons) (per liter)
Pancreatic digest of casein	10 g	10 g
Glucose	40 g	20 g
Agar	15–20 g	15–20 g

Table 2.2 Antimicrobial and other additives to Sabouraud agar

Ingredient	Amount (per liter)	Notes on preparation for use
Chloramphenicol*	50 mg	Dissolve in 10 mL 95% ethanol
Cycloheximide*	0.5 g	Dissolve in 2 mL acetone
Gentamicin sulfate	50 mg	Dissolve in 5 mL water; add before autoclaving
Lecithin	0.7 g	Add directly with other powdered medium ingredients before autoclaving
Tween 80	5 g	
Olive oil	N/A	Spread 0.1 mL sterile olive oil on surface of each agar plate

*Add these to molten, autoclaved media once it has been tempered in water bath to 45–50 °C

1. Deionized, distilled water.
2. Autoclave.
3. Graduated cylinder, 1000 mL.
4. Erlenmeyer flask (2 L if making 1 L of medium).
5. Analytical balance (if using antimicrobial agents).
6. Balance for weighing media ingredients.
7. Stir bar.
8. Stirring hotplate.
9. Slant tube rack for holding media tubes after autoclaving to solidify with a slanted surface.
10. Pancreatic digest of casein.
11. Glucose.
12. Chloramphenicol.
13. Gentamicin sulfate.
14. Cycloheximide.
15. Tween 80 (polysorbate 80).
16. Lecithin.
17. Olive oil, sterilized by autoclaving.
18. Sterile glass test tubes with caps.
19. Sterile Petri dishes, 100 mm diameter.

2.1.4 Method of Sabouraud Agar Preparation

2.1.4.1 Standard Preparation

1. Combine all ingredients, except any antimicrobials to be used, in ~900 mL of deionized water in a graduated cylinder while stirring with a magnetic stir bar.
2. Adjust to pH 5.6 with hydrochloric acid and adjust final volume to 1 L.
3. Transfer contents to a 2 L flask and boil on a heating/stirring plate while stirring, for 1 min.
4. Cover the opening of the flask loosely with aluminum foil and autoclave for 15 minutes at 121 °C under pressure of 15 lb. in^{-2}.

5. Cool to ~45–50 °C (roughly until one can support the flask underneath with an ungloved hand). If the antimicrobials chloramphenicol or cycloheximide are to be added, aseptically add them at this point and swirl medium gently (see Sect. 2.1.4.3).
6. Pour into Petri dishes or tubes and leave them at room temperature (15–30 °C) overnight to solidify and dry. When pouring plates, fill each Petri dish with at least 25 mL of medium to allow for medium dehydration during the longer incubation period required for fungi. If preparing tubes, slant the rack of covered tubes immediately after pouring in a slant tube rack, either at a 5° or 20° slant.
7. Store all media at 4 °C, regardless of whether they contain antimicrobials.

2.1.4.2 Method of Sabouraud Agar, Emmons Modification Preparation

1. Combine all ingredients, except any antimicrobials to be used, in ~900 mL of deionized water in a graduated cylinder while stirring with a magnetic stir bar.
2. Adjust to pH 6.8–7.0 with hydrochloric acid and adjust final volume to 1 L.
3. Transfer contents to a 2 L flask and boil on a heating/stirring plate while stirring, for one minute.
4. Cover the opening of the flask loosely with aluminum foil and autoclave for 15 minutes at 121 °C under pressure of 15 lb. in^{-2}.
5. Cool to ~45–50 °C (roughly until one can support the flask underneath with an ungloved hand). If the antimicrobials chloramphenicol or cycloheximide are to be added, aseptically add them at this point and swirl medium gently (see Sect. 2.1.4.3).
6. Pour into Petri dishes or tubes and leave at room temperature overnight to solidify and dry. Fill each Petri dish with at least 25 mL of medium to allow for medium dehydration during the longer incubation period required for fungi. If preparing tubes, slant the rack of covered tubes immediately after pouring in a slant tube rack, either at a 5° or 20° slant.
7. Store all media at 4 °C, regardless of whether they contain antimicrobials.

2.1.4.3 Variations on Standard Sabouraud Agar

Either Sabouraud agar or its Emmons version can be made more selective by adding antimicrobials (see Table 2.2). Antimicrobials commonly used are the aminoglycoside gentamicin, which inhibits gram-negative bacteria, chloramphenicol, which inhibits a wide range of gram-positive and gram-negative bacteria, and cycloheximide, which inhibits primarily saprophytic fungi, but not dermatophytes or yeasts [6, 7]. Chloramphenicol and gentamicin are used at 50 mg L^{-1} and cycloheximide at 0.5 g L^{-1} (See Table 2.2) [8]. Chloramphenicol and cycloheximide should only be added after the media has been autoclaved and then cooled to ~45–50 °C (See step 5 in Sect. 2.1.4.1). Gentamicin may be added to the medium ingredients before autoclaving.

Lecithin and Tween 80 are added to Sabouraud agar (Table 2.2) that is used in monitoring environmental surfaces that may have been treated with antiseptics and quaternary ammonium compounds, as these additives neutralize the cleaning compounds [9]. Sterile olive oil can be spread on the surface of Sabouraud agar plates to grow lipophilic *Malassezia* species [10].

2.1.5 Methods of Inoculation and Incubation

Sabouraud agar plates can be inoculated by streaking for isolation, as with standard bacteriological media, by exposing the medium to ambient air, or by tamping clinical sample material (e.g., hair, skin scrapings) onto the surface of the agar medium. When growing cultures in tubes, the caps should be screwed on loosely to admit air, as dermatophytes and most molds are obligate aerobes. Isolation of fungi is performed on plates, while slants are primarily used for maintaining pure, or stock, cultures once isolated. If using selective Sabouraud media, a control plate/tube without antimicrobials should also be inoculated for comparison. Typically, molds are incubated at room temperature or slightly warmer (25–30 °C), yeasts are incubated at 28–30 °C or both 30 °C and 37 °C if suspected to be dimorphic fungi.

Incubation times will vary, from approximately 2 days for the growth of yeast colonies such as *Malasezzia* to 2–4 weeks for growth of dermatophytes or dimorphic fungi such as *Histoplasma capsulatum*. Indeed, the incubation time required to acquire fungal growth is one diagnostic indicator used to identify or confirm fungal species. Dermatophytes, in particular, show characteristic incubation times ranging from 5–7 days (some *Epidermophyton* or *Microsporum* species) to 3–4 weeks for some *Trichophyton* species [11]. Cultures should be examined twice weekly and be held for 4–6 weeks before being reported as negative if infection by systemic agents such as *Histoplasma*, *Blastomyces*, or *Coccidioides* species is suspected.

2.1.6 Results

Depending on the antimicrobials used, different types of microorganisms and groups of fungi may grow on Sabouraud agar (See Table 2.3). Typically, saprophytic fungi are inhibited by cycloheximide and/or chloramphenicol, but yeasts and dermatophytes grow well in their presence. Conversely, even Sabouraud agar is unable to support the growth of a few dermatophytes in the absence of additives. For example, some *Trichophyton* species require additional growth factors, such as thiamine and inositol (*T. verrucosum*) or nicotinic acid (*T. equinum*), and may not grow well, if at all, on Sabouraud agar [12]. *T. mentagrophytes* and *T. rubrum,* however, grow well on Sabouraud agar. Similarly, the growth of *Malassezia* species is significantly impaired without the addition of olive oil overlaid on the surface of a Sabouraud agar plate [10].

Table 2.3 Expected growth of various microbes on Sabouraud agar containing antimicrobials

Microbe	Growth on SAB + CAM[a]	Growth on SAB + CHX[b]
Candida albicans	Yes	Yes
Cryptococcus neoformans	Yes	No
Aspergillus Niger	Yes	No
Trichophyton mentagrophytes	Yes	Yes
Microsporum audouinii	Yes	Yes
Blastomyces dermatitidis	Yes (mold phase at 25 °C)	Yes (mold phase at 25 °C)
	No (yeast phase at 37 °C)	No (yeast phase at 37 °C)
Histoplasma capsulatum	Yes (mold phase at 25 °C)	Yes (mold phase at 25 °C)
	No (yeast phase at 37 °C)	No (yeast phase at 37 °C)
Rhizopus spp.	Yes	No
Sporothrix schenckii	Yes	Yes
Penicillium roquefortii	Yes	No
Escherichia coli	No	No

[a]SAB + CAM = Sabouraud agar plus chloramphenicol
[b]SAB + CHX = Sabouraud agar plus cycloheximide

Mold morphology should be observed on both the top (obverse) and bottom (reverse) surfaces, as differences can be seen on each surface.

Variation from lot to lot as well as between commercial vendors of Sabouraud agar can significantly impact the qualitative and quantitative growth of fungi. One study comparing five different commercial preparations of Sabouraud glucose agar observed significant differences in the quantitation of yeasts as well as the color of *Aspergillus* colonies; however, the dermatophytes yielded reliably similar appearances on the five media sources tested [13].

2.2 Potato Dextrose Agar

2.2.1 History

The use of potato extract as a growth source in fungal media was published by New Zealand mycologist Ross Beever in 1970 [14], as part of a detailed study into the most efficacious component of potatoes as a growth medium. After analysis of the carbon, nitrogen, mineral salts, and other growth factor components of the potato extract medium, it was concluded that no one component was responsible for the stimulation of the growth of fungi.

2.2.2 Theory

Potato Dextrose Agar (PDA) contains dextrose as a carbohydrate source which serves as a growth stimulant and potato infusion that provides a nutrient base for luxuriant growth of most fungi. Agar is added as the solidifying agent. Potato Dextrose Agar (PDA) is a general-purpose medium for the identification, cultivation, and enumeration of fungi in foods and dairy products. Potato dextrose broth is a general-purpose broth medium for yeasts and molds.

Certain additives like tartaric acid, chloramphenicol, and chlortetracycline can be added as selective agents (Table 2.4). As recommended by the Food and Drug Administration, the American Public Health Association, and the Association of Analytical Chemists [15–18], PDA with tartaric acid is used for the plate count microbial examination of food and dairy products. The addition of chlortetracycline is recommended for the microbial enumeration of yeast and mold from cosmetics. PDA with chloramphenicol is recommended for the selective cultivation of fungi from mixed samples. PDA is also recommended by the U.S. Pharmacopeia for the preparation and maintenance of test strains used for microbial limit tests.

Potato infusion and dextrose promote luxuriant fungal growth, so PDA is also used for primary isolation of yeasts and molds from clinical specimens. Since it stimulates sporulation and pigmentation, it is also used for the differentiation of typical varieties of dermatophytes (species belonging to the genera *Epidermophyton*, *Microsporum* or *Trichophyton*) based on their pigment production and for the maintenance of their stock cultures [19].

2.2.3 Methods of Preparation

See Sect. 2.1.3 for general comments regarding needed materials.

Potato Dextrose Agar can be either made from individual ingredients, purchased as a dehydrated powder that must be dissolved in water, autoclaved, and dispensed, or as a prepared medium in a tube, plate, or broth format from a variety of commercial sources.

Table 2.4 Antimicrobial and other additives to Potato Dextrose Agar

Ingredient	Amount (per liter)	Purpose in medium
Tartaric acid	1.4 g	Lowers pH to 3.5; antibacterial for testing food products
Chlortetracycline[a]	40 mg	Antibacterial; for cosmetics testing
Sodium chloride	75 g	Inhibition of fast- growing molds
Chloramphenicol[a]	25 mg	Antibacterial
Copper sulfate	1 mg	Supports pigmentation

[a]See Table 2.2 for preparation information on adding antimicrobials to media

Table 2.5 Ingredients for Potato Dextrose Agar (commercial and manual)

Ingredient	Potato Dextrose Agar (commercial) (g/L)	Potato Dextrose Agar (manual) (g/L)
Potato extract*	4 g	–
Potato infusion	–	200 g
Dextrose	20 g	20 g
Agar	15 g	15 g
Final pH at 25 °C	5.6 ± 0.2	5.6 ± 0.2

*4 g of potato extract is equivalent to infusion from 200 g of potatoes

A survey of 10 companies' media found that five media sources contained insufficient copper, which reduced the pigmentation of the resulting colonies. Therefore, supplementation of the other ingredients of PDA with 1 μg mL^{-1} copper sulfate, or a trace minerals mixture, is recommended [20].

1. Suspend 39 g of dehydrated media (if commercially supplied) in 1 L of deionized water and mix thoroughly in a 2 L flask.
   Alternatively, to make medium from individual ingredients (See Table 2.5):
   - Boil 200 g of sliced potatoes in 500 mL distilled water until thoroughly cooked (about 1 h).
   - Filter infusion through cheesecloth/gauze, saving the filtrate, which is potato infusion.
   - In a 2 L flask, combine the potato infusion, dextrose (20 g), and agar (15 g), and bring the volume to 1 L.
2. Boil on a heating/stirring plate while stirring, for 1 min to dissolve the powder completely.
3. Cover the opening of the flask loosely with aluminum foil and autoclave 15 min at 121 °C under pressure of 15 lb. in^{-2}.
4. Cool to around 45–50 °C (roughly until one can support the flask underneath with an ungloved hand).
5. When modifications are needed in the standard media:
   (i) To decrease the pH of the agar medium to pH 3.5, add the specified amount of sterile tartaric acid. The amount of acid required for 1 L of sterile, cooled medium is approximately 10 mL of a 10% solution.
   Note: Do not reheat the medium after adjusting pH as it may hydrolyze the agar which can render the agar unable to solidify.
   (ii) If the antimicrobials, such as chloramphenicol or chlortetracycline or other additives like sodium chloride are to be added, aseptically add them at this point and swirl medium gently.

6. Pour into Petri dishes or tubes and leave at room temperature overnight to solidify and dry. When pouring plates, fill each Petri dish with at least 25 mL of medium to allow for medium dehydration during the longer incubation period required for fungi. If preparing tubes, slant the rack of covered tubes immediately after pouring in a slant tube rack, either at 5° or 20° slant.
7. Store prepared media away from direct light at 4 °C to 8 °C with the medium side uppermost to prevent excessive accumulation of moisture on the agar surface. Under these conditions, this medium has a shelf life of 12 weeks.

2.2.4 Methods of Inoculation and Incubation

2.2.4.1 Quality Control

After checking for correct pH, color, depth, and sterility, the following organisms are used to determine the growth performance of the completed medium: *Candida albicans* ATCC 14053 and *Aspergillus niger* ATCC 16404 will each produce growth on this medium.

2.2.4.2 Inoculation and Incubation

1. For yeast and mold counts in foods, a standard pour plate technique should be used, and the pH of the medium should be adjusted to approximately 3.5 with sterile tartaric aid.
2. For the cultivation and maintenance of pure cultures, tubed slants are used. They should be inoculated and incubated the same as a plated medium, below.
3. For other specimen processing, streak the specimen onto the medium with a sterile inoculating loop to obtain isolated colonies.
4. Plates can be incubated at various temperatures, depending on the application (molds typically use a lower temperature such as room temperature 20–25 °C while yeasts may require 25–30 °C) in an inverted position (agar side up) with increased humidity.
5. Cultures should be examined daily for fungal growth, with extended time periods up to at least one week for lower temperature incubations and several days for higher temperature incubations.

2.2.5 Results

After sufficient incubation culture plates should show isolated colonies in streaked areas and confluent growth in areas of heavy inoculation. Yeasts will grow as creamy to white colonies. Molds will grow as filamentous colonies of various colors. Further

microscopic examination and biochemical testing are required to identify the genus and species of the isolate.

The number of yeast or mold present in the particular test sample is determined by counting the colonies in pour plates and multiplying with the applicable dilution factor.

2.3 Bird Seed Agar

2.3.1 History

In 1962, Staib et al. described that incorporation of an extract of *Guizotia abyssinica* seed in a fungal medium resulted in the formation of brown colonies of *Cryptococcus neoformans* [21]. In 1966, Shields and Ajello modified Staib's *G. abyssinica* seed agar formulation by making the medium selective with the addition of the antimicrobial agent chloramphenicol [22]. In the literature, this medium is variously referred to as Staib medium, Bird seed agar, *Guizotia abyssinica* creatinine agar, niger seed creatinine agar, or thistle seed medium [23].

2.3.2 Theory

Bird Seed Agar is a selective and differential medium for the isolation of *Cryptococcus neoformans* from clinical specimens and differentiation of it from other microbes. The use of Bird Seed Agar as the primary culture medium for sputum and urine specimens from AIDS patients increases sensitivity for *C. neoformans* [24]. *Cryptococcus neoformans* produces dark colonies on this agar, unlike *Candida albicans,* which produces white colonies. *C. neoformans* is the only yeast known to produce this pigmentation [25].

The extract of *Guizotia abyssinica* seeds contains caffeic acid (3, 4-dihydroxycinnamic acid, an o-diphenol). Phenoloxidase enzyme produced by *C. neoformans* uses caffeic acid as a substrate and produces melanin. Melanin is absorbed by the yeast cell wall, yielding tan to reddish-brown pigmentation. It is also known as the phenoloxidase test.

Glucose is the energy source in the medium. *Cryptococcus* species use creatinine as their sole source of nitrogen [22]. Creatinine also enhances the melanization of some strains of *C. neoformans.* Agar is the solidifying agent. Chloramphenicol improves the recovery of *Cryptococus* species from specimens containing mixed flora by selecting against bacterial growth.

2.3.3 Methods of Preparation

See Sect. 2.1.3 for general comments regarding needed materials.

Bird Seed Agar can be prepared from individual ingredients (Table 2.6) by purchasing the seeds of *Guizotia abyssinica* (niger seed), grinding them, and adding filtrate with necessary ingredients, or by using a commercially available powder media that includes bird seed filtrate. Prepared plates or tubes are also commercially available.

The exact composition of the media supplied by different commercial suppliers varies; each mentions that they modified the original formula to suit performance parameters. Some of the modifications include a substitution of chloramphenicol for penicillin and streptomycin sulfate, preparing modified *G. abyssinicia* seed-based agar media by depleting or removing its constituents such as diphenyl, glucose, creatinine, etc. It was found that decreasing the sugar concentration resulted in the rapid development of brown pigmentation by *C. neoformans* [26].

2.3.3.1 Preparation of Bird Seed Agar

1. Suspend required quantity powder media (as per manufacturer's instruction), OR items a-c and f-g in Table 2.6, in a total of 1 l of distilled water in a 2 L flask.
2. Heat to boiling on a heating/stirring plate to dissolve the medium completely.
3. Cover opening of flask loosely with aluminum foil and autoclave 15 min at 121 °C under pressure of 15 lb. in^{-2}.
4. Cool to 45 °C before adding additives if needed (e.g., diphenyl can be added to inhibit saprotrophic fungi).
5. Mix well and pour into sterile Petri plates or tubes (see Sect. 2.1.4.1, step 6).
6. Store prepared medium at 2–8 °C with the medium side uppermost to prevent excessive accumulation of moisture on the agar surface.

Table 2.6 Ingredients for Bird Seed Agar

Ingredients	Shield's and Ajello's formulation (22)	Commercial preparation (7)
a) *Guizotia abyssinica* seeds	200 mL filtrate	50 g
b) Glucose/dextrose	10 g	1 g
c) Creatinine	780 mg	1 g
d) Chloramphenicol	50 mg	50 g
e) Diphenyl	100 mg	–
f) Monopotassium phosphate	–	1 g
g) Agar	20 g	15 g
Final volume	1 L	1 liter
Final pH at 25 °C	6.7 ± 0.2	6.7 ± 0.2

2.3.4 Method of Inoculation and Incubation

2.3.4.1 Quality Control

After checking for correct pH, color, depth, and sterility, the following organisms are used to determine the growth performance of the completed medium: *Cryptococcus neoformans* ATCC 32045 will produce brown to black pigmented colony growth on this medium. *Escherichia coli* ATCC 25922 serves as a negative control, being partial to completely inhibited in its growth on Bird Seed Agar.

2.3.4.2 Inoculation and Incubation

1. Streak the specimen onto the medium with a sterile inoculating loop to obtain isolated colonies.
2. Incubate the plates at 25–30 °C in an inverted position (agar side up) with increased humidity.
3. Cultures should be examined at least daily for fungal growth and should be held for up to 4 weeks before being reported as negative.

After inoculation of the clinical specimen, and periodically examined for brown-colored, mucoid colonies, which later turn black or brown in the case of the genus *Cryptococcus*.

2.3.5 Results

Plates inoculated with suspected samples are observed after incubation at 25–30 °C for 2 weeks. The presence of golden brown to black pigmented smooth or mucoid colonies is indicative of *Cryptococcus neoformans*. Other species like *Cryptococcus laurentii, Saccharomyces cerevisiae*, etc., produce non-pigmented colonies, and *Candida* species appear as white colonies. However, pigmented colonies arising on Bird Seed Agar should be also grown on a non-differential medium such as Sabouraud dextrose agar to rule out a naturally pigmented strain or species [19].

2.4 Dermatophyte Test Medium

2.4.1 History

Dermatophyte Test Medium (DTM) was formulated by Taplin et al. in 1969 to rapidly diagnose dermatophytic (ringworm) infections in Vietnam War soldiers,

under conditions where experienced laboratory personnel and incubators were typically unavailable [27].

2.4.2 Theory

Dermatophyte Test Medium, or DTM, is essentially a selective and differential version of Sabouraud dextrose agar designed to indicate the presence of *Epidermophyton*, *Microsporum*, and *Trichophyton* spp. that cause dermatophytic infections. Amino acids, nitrogen- and carbon-containing compounds are provided by the soy peptone, and dextrose is the energy source for fungal growth. The acidic pH 5.6 favors fungal growth.

The original formulation included chlortetracycline HCl and gentamicin as antibacterial agents [27]. Current formulations include the antibiotics chloramphenicol (due to the relative unavailability of chlortetracycline) and sometimes gentamicin, which select against a wide range of bacteria. Cycloheximide selects against saprophytic fungi. The medium is made differential through the addition of the indicator dye phenol red. At acidic pH values below 6.8, it is yellow, and at alkaline pH values of 8.2 and above, is bright pink to red. Dermatophytes produce alkaline metabolites that turn the media bright pink or red color.

2.4.3 Method of Preparation

See Sect. 2.1.3 for general comments regarding needed materials.

DTM can be either made from individual ingredients (see Table 2.7), purchased as a dehydrated powder that must be dissolved in water, autoclaved, and dispensed (after addition of antimicrobials), or as a prepared medium that can be purchased in tube or plate form.

Table 2.7 Ingredients for Dermatophyte Test Medium

Ingredient	Amount (per liter)
Soy peptone	10.0 g
Dextrose	10.0 g
Cycloheximide[a]	0.5 g
Chloramphenicol[a]	0.05 g
Gentamicin sulfate[a]	0.1 g
Phenol red	0.2 g
Agar	20.0 g

[a]See Table 2.2 for preparation information on adding antimicrobials to media

Follow steps 1–5 listed in Sect. 2.1.4.1 Standard Preparation. After cooling the sterilized media, add the appropriate antimicrobials for DTM (Table 2.7) and pour them into plates or tubes.

There is a modification of DTM, called dermatophyte isolation medium DIM [28], which uses the pH indicator dye bromocresol purple in place of phenol red, as well as penicillin and streptomycin as its antibiotics. It also uses cycloheximide, at 4 g L^{-1}, which is eight times the amount found in DTM. However, at its recommended incubation temperature of 37 °C (but not a lower temperature of 30 °C), high false-negative rates for common *Trichophyton* species were noted, perhaps due to the high levels of cycloheximide [29]. Furthermore, the dimorphic fungus *Coccidioides immitis* grew as a white mold at 37 °C that resembled the hallmark dermatophyte appearance, thereby constituting a false-positive issue with this medium [29]. Consequently, this medium formulation, as published, is not the most useful for presumptive identification of dermatophyte infections.

2.4.4 Methods of Inoculation and Incubation

Suitable samples for DTM inoculation include hair, nails, and skin scrapings. Clean the skin or body surface with alcohol before using a new toothbrush or other small brush to obtain a surface sample. Bringing the medium to room temperature helps facilitate more rapid fungal growth. Follow the instructions listed in Sect. 2.1.5. Lay the sample firmly on top of, but not into, the medium.

Loosely capped, inoculated tubes or plates should be incubated at room temperature or around 25 °C, and should be evaluated for red medium color change daily, up to 10–14 days.

2.4.5 Results

Two observations constitute a true positive reaction on DTM: white or buff-colored mold growth (containing aerial hyphae), and red-colored medium. Both the fungal growth and the medium color change to pink/red should appear at the same time, which may be as short as three to 5 days, depending on the inoculum. A red-colored medium arising after approximately 10–14 days likely represents contaminating fungal growth [27]. Various false-negative appearances are possible, such as saprophytes that grow as dark-colored molds although they turn the medium red/pink. Alternately, bacterial or yeast will grow as white- or light-colored creamy colonies, and thus easily distinguished from the mold-like appearance of dermatophytes.

DTM is primarily a screening medium and is not suitable for identification beyond presumptively belonging to the three species of dermatophytes.

2.5 Safety Notes

Fungi often produce spores that are easily dispersed into the laboratory upon the opening of plates.

Plates should be incubated with the lid on the top (as opposed to the typical practice of inverting microbiological plates for incubation) to avoid spreading spores when the plates are opened. After growth, plates should be wrapped in Parafilm to maintain them securely closed for storage and transport. Plate or tube cultures should be opened only within a class II biological safety cabinet to avoid contamination of laboratory spaces with fungal spores, possible infection of individuals by pathogenic fungi, or induction of allergic responses. See Chap. 1 for detailed procedures and guidelines.

Because the growth of large numbers of fungi can pose a potential infection hazard, measures must also be taken to prevent infection of laboratory researchers. Note that some fungi are biosafety level one (BSL-1) while most are BSL-2 [30]. The American Society for Microbiology strongly recommends that environmental enrichment experiments should only be performed in BSL-2 laboratories [31]. The following precautions apply to the use of any fungal medium:

1. Soil, water, and other materials directly obtained from the environment that typically contain infectious organisms should be handled according to the biosafety level of that infectious agent.
2. Cultures of enriched microorganisms derived from environmental samples should be handled using BSL-2 precautions.
3. Mixed, enriched, or pure cultures of microorganisms from environmental samples with a significant probability of containing infectious agents should be manipulated in a Class II biosafety cabinet if available.
4. Researchers should be aware of working in regions with endemic fungi capable of causing systemic infections and should avoid environmental isolations.

Some safe (BSL-1) fungi for student experimentation and handling include the molds *Penicillium camemberti* and *P. roqueforti* (used in making cheeses), *Rhizopus stolonifor* (used in making tempeh), *Aspergillus* species (except *A. fumigatus* and *A. flavus*), the yeasts *Saccharomyces cerevisiae, Rhodotorula rubrum,* and *Neurospora crassa*.

2.6 Additional Fungal Growth Media

Table 2.8 Other commonly used fungal isolation and growth media

Culture Media	Essential ingredients	Intended use
Inhibitory Mold agar (IMA)	Tryptone, beef extract, yeast extract, starch, dextrin, chloramphenicol, gentamicin, and saline buffer	Recovery of fungi from specimens that contain bacterial microbiota

(continued)

Table 2.8 (continued)

Culture Media	Essential ingredients	Intended use
Mycosel/ Mycobiotic agar	Papaic digest of soybean meal, dextrose, cycloheximide, chloramphenicol, agar	Highly selective medium; recommended for isolation of pathogenic fungi from materials containing a large amount of fungal and bacterial flora
Potato flake agar	Potato flakes, dextrose, agar	Primary recovery of saprophytic and dimorphic fungi, particular fastidious and slow-growing strains
Cornmeal agar/cornmeal tween agar	Cornmeal, tween 80, agar	Stimulation of chlamydospore formation in yeasts
Rice starch agar	Cream of rice, tween 80, agar	Production of chlamydospore in *Candida albicans*
Brain-heart infusion agar	Brain heart infusion, glucose, L-cysteine hydrochloride, agar	Growth of fastidious pathogenic fungi such as *Histoplasma capsulatum* and *Blastomyces dermatitidis*
Czapek-dox agar	$NaNO_3$, K_2HO_4, KCl, $MgSO_4$, $FeSO_4$, glucose, agar	Identification of *aspergillus* and P*enicillium* species
CHROMagar Candida medium	Peptone, glucose, chloramphenicol, 'chromogenic ix', agar	Selective and differential chromogenic medium for the isolation and identification of various *Candida* species

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Chapter 3
Fluorescence In Situ Hybridization of Uncultured Zoosporic Fungi

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Contents

3.1 Introduction

Molecular surveys of microbial eukaryotes have revealed overlooked uncultured environmental fungi with novel putative functions [1–3], among which zoosporic forms (i.e. chytrids) are the most important in terms of diversity, abundance, and functional roles, primarily as infective parasites of phytoplankton [4, 5] and as valuable food sources for zooplankton via massive zoospore production, particularly in freshwater lakes [6–8]. However, due to their small size (2–5 μm), their lack of distinctive morphological features, and their phylogenetic position, traditional microscopic methods are not sensitive enough to detect fungal zoospores among a mixed assemblage of microorganisms. Chytrids occupy the most basal branch of the kingdom Fungi, a finding consistent with choanoflagellate-like ancestors [9]. The above reasons may help explain why both infective (i.e. sporangia) and

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disseminating (i.e. zoospores) life stages of chytrids have been misidentified in previous studies, respectively as phagotrophic sessile flagellates (e.g. choanoflagellates, bicosoecids) and as 'small undetermined' cells. These cells often dominate the abundance of free-living heterotrophic nanoflagellates (HNFs) and are considered the main bacterivores in aquatic microbial food webs [2, 10]. Their contribution ranges from 10% to 90% of the total abundance of HNFs in pelagic systems (see review in [11]). Preliminary data have provided that up to 60% of these unidentified HNFs can correspond to fungal zoospores [12], establishing the HNF compartment as a black box in the context of microbial food web dynamics [4]. A simulation analysis based on Lake Biwa (Japan) inverse model indicated that the presence of zoosporic fungi leads to (i) an enhancement of the trophic efficiency index, (ii) a decrease of the ratio detritivory/herbivory, (iii) a decrease of the percentage of carbon flowing in cyclic pathways, and (iv) an increase in the relative ascendency (indicates trophic pathways more specialized and less redundant) of the system [13]. Unfortunately, because a specific methodology for their detection is not available, quantitative data on zoosporic fungi are missing.

Sporangia and the associated rhizoidal system are characterized by a chitinaceous wall (a common fungal structure element for many species) which can be targeted by specific fluorochromes such as calcofluor white [14]. In contrast, because the chitinaceous wall springs out after zoospore encystment, chytrid zoospores completely lack cell wall and chitin, precluding any simple use of fluorochromes for their quantitative assessment in natural environments [12]. Molecular approaches, primarily fluorescence in situ hybridization (FISH), offer an alternative for quantitatively probing both chytrid sporangia and zoospores in nature. FISH method is based on the detection of targeted nucleic acid sequences by the use of oligonucleotide probes labelled by a fluorochrome, usually Cy3 [15, 16]. One of the major limitations of FISH-based methods for natural samples is the autofluorescence interference from autotrophic organisms. During the past few years, numerous efforts have been made to improve the sensitivity of monolabelled probes for FISH assay, including the use of brightener fluorochromes [17], or of signal amplification with reporter enzymes [18]. Of particular interest is the hybridization method using horseradish peroxidase (HRP)-labelled probes activated by fluorescent tyramide (also known as catalyzed reporter deposition, CARD-FISH), which is very efficient in overcoming the interference from natural fluorescence [19]. The method is based on the fact that each HRP-labelled probe catalyzes the deposition of many labelled tyramides, so that numerous fluorescent molecules are introduced at the hybridization site, resulting in net fluorescence signal amplification, compared to the classical Cy3-monolabelled FISH probes [20].

The main objective of this chapter is to provide, in a simplified step-by-step format, classical FISH and CARD-FISH protocols for the identification and quantitative assessment of uncultured zoosporic fungi and other zoosporic microbial eukaryotes in natural aqueous environments (cf. 12), together with practical advices on how to apply the methods.

3.2 Materials

1. Gloves (should be worn when manipulating most of the materials below).
2. 0.6 μm pore size polycarbonate white filters (e.g. catalog no. DTTP02500, Millipore, Billerica, MA, USA)
3. Appropriate Cy3-labelled oligonucleotidic probe and its reverse complement stored at −20 °C (see Note 1).
4. Sodium dodecyl sulphate (SDS).
5. Formaldehyde 37%.
6. FISH hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl (pH 7.2), 0.01% SDS) containing 30% formamide and 2.5 ng μl^{-1} of Cy3-labelled probe (see Note 2).
7. Washing buffer - 20 mM Tris–HCl (pH 7.2, 5 mM EDTA, 0.01% SDS, 112 mM NaCl [21].
8. Appropriate filtration columns equipped with a peristaltic pump.
9. DAPI – 4.6-diamidino-2-phenylindole.
10. Glass slides and coverslips.
11. Non-fluorescent immersion oil.
12. Epifluorescence microscope equipped with appropriate filter sets (blue and UV) and Neofluar objective lens (optional).
13. CARD-FISH hybridization buffer: 30% deionized formamide, 0.9 M NaCl, 20 mM Tris-HCl (pH 7.5), 0.01% SDS, and 10% blocking reagent (e.g. Roche Diagnostics/Boehringer).
14. Appropriate oligonucleotide probe labelled with HRP (in our case, commercially synthesized by Biomers, Germany).
15. TNT buffer: 0.1 M Tris-Hcl (pH 7.5), 0.15 M NaCl, and 0.05% Tween 20.
16. TSA mixture: (1:1) of 40% dextran sulphate (Sigma-Aldrich, St. Louis, MO, USA) and 1X amplification diluent (PerkinElmer LAS, Waltham, MA, USA).
17. Fluorescein isothiocyanate coupled with tyramide (1×, Perkin-Elmer LAS).

3.3 Methods

3.3.1 Classical FISH Probing (See Note 3)

Fixe experimental samples with 2% formaldehyde, vol:vol final concentration. The fixation step is facultative and can be avoided when observations are made without delay.

1. Filter-collect appropriate volumes (x 3 replicates) of cultures, enriched cultures, or natural samples containing zoosporic organisms onto 0.6 μm pore size polycarbonate white filters (see Note 4) by using gentle vacuum (< 20 kPa).
2. In the dark, poor the filters with targeting fungal zoospores and sporangia and perform hybridization in the standard FISH hybridization buffer (containing 30%

formamide and 2.5 ng μl^{-1} of the Cy3-labelled oligonucleotide probe) for 3 h at 46 °C (see Note 5).
3. Use the reverse complement probe in a negative control to check for the autofluorescence interference from fungi and other natural plankton present in natural samples.
4. After hybridization, thoroughly rinse the filters in the washing buffer for 30 min at 48 °C.
5. Counterstained the filters in the dark at room temperature for 5 min with DAPI 0.5 μg ml^{-1}, and repeat the washing step.
6. Mount the filters between glass slides and coverslips using appropriate non-fluorescent immersion oil (see Note 6). At this stage, mounted filters can be conserved at −20 °C until microscopic observation.
7. In a dark room, examine the filters under an epifluorescence microscope equipped with appropriate set of filters and objective lens. Shift between blue and UV light to distinguish between Cy3 stain and DAPI, use different convenient magnifications for sporangia and zoospores, and apply a standard procedure for microscopic counting.

3.3.2 CARD-FISH Probing (See Note 7)

Perform steps 1 and 2 in classical FISH probing.

1. In the dark, poor the filters with targeting fungal zoospores and sporangia and perform hybridization in the CARD-FISH hybridization buffer (containing 30% formamide and 2.5 ng μl^{-1} of HRP labelled oligonucleotide probe) for 3 h at 35 °C (see Note 5).
2. After hybridization, thoroughly rinse the filters in the washing buffer for 2 x 20 min at 37 °C.
3. Equilibrate samples to increase enzyme activity in TNT buffer at room temperature for 15 min.
4. Perform signal amplification by 30 min incubation in TSA mixture, to which fluorescein isothiocyanate coupled with tyramide was added (1:50 vol/vol).
5. Transfer filters in two successive 5-ml TNT buffer baths at 55 °C for 20 min, in order to stop the enzymatic reaction and remove the dextran sulphate.
6. Follow steps 6 and 8 in classical FISH probing.

3.4 Notes

1. We propose to use a probe named Chyt1061 (sequence 5′ > 3′: CATAAGGTG CCGAACAAGTG), because of the sequence position (1061 base pairs) on Saccharomyces cerevisiae small-subunit rDNA molecule (GenBank accession no. J01353). According to Behrens and collaborators [22], this position provides

good accessibility for FISH probing. There were two mismatches in the middle of the probe with sequences of Chytridiales species (cf. 12), which did not result in loss of positive signal. Chyt1061 was designed in silico for targeting fungal species in the order Chytridiales, i.e. the largest order of the division Chytridiomycota (chytrids) mainly represented by phytoplanktonic parasites in aquatic environments [12]. The design was based on the alignment of rDNA sequences of Chytridiales obtained from GenBank, together with 106 sequences derived from18S rDNA PCR surveys of freshwater picoeukaryotes conducted in French Lakes Pavin, Godivelle, and Aydat [12]. Distinct rDNA sequence unique to target organisms was localized and imported in Primer3 software (http://fokker.wi.mit.edu/primer3/input.htm) in order to design a probe with a size between 18 and 27 bases, probe melting temperature (Tm) between 57 °C and 63 °C, and GC percentage at about 50%. The probe was analyzed for potential complementarities, and no dimers or hairpins were found using Netprimer software (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html). The probe was commercially synthesized by MWG-biotech Company (Germany) and labelled with the fluorochrome Cy3 for classical FISH or application to environmental samples using the CARD-FISH approach [12].

2. In the case you design your one probe because of the increasing availability of sequences in the database, hybridization stringency should be tested and validated using appropriate positive and negative cultures, before application to natural samples. In the absence of laboratory cultures, our probe Chyt1061 was evaluated from an adaptation of an alternative approach called clone-FISH, known from prokaryotes [23]. This approach is based on the genetic modification of a clone of *Escherichia coli* by inserting a plasmid vector containing the target rDNA sequence. In our adaptation of the approach, cells of *E. coli* clone BL21 star were genetically transformed by a inserting plasmid vector containing rDNA sequence from several different target fungal cells. Specific plasmid inserts came from freshwater lake surveys of picoeukaryote 18S rDNA and fungal 18S-ITS rDNA as well (cf. 12).
3. The specificity of the designed probe should be checked both in silico by using a basic local alignment search tool (e.g. BLAST) [24] and in vivo by screening clone libraries with classical FISH (the clone-FISH approach can be used here). In our case, clones containing rRNA gene inserts from different eukaryotes closely related to microorganisms of interest and negative controls as well were FISH-targeted following the protocol described in this chapter (i.e. with 30% formamide in the hybridization buffer). In addition, the in vivo transcription of the 18S rRNA gene insert was induced with IPTG (1 mM) for 1 h. The designed probe or its reverse complement probe was used, depending on the orientation of insertion into the vector, i.e. $3' \rightarrow 5'$ or $5' \rightarrow 3'$ way downstream the T7 promoter (cf. 12).
4. In vivo tests using the clone-FISH approach yield the best fluorescence signal when hybridization was performed at 30% formamide concentration in the hybridization buffer. Assuming an increase of the effective hybridization temperature of 0.5 °C per 1% of added formamide, the melting temperature (Tm) of the probe Chyt1061 was experimentally calculated at 61 °C [12].

5. This protocol is suitable for cultures and enriched cultures (i.e. concentrates of targeted zoosporic organisms during host blooms) (cf. 12). However, the FISH resolution for fungal images and species identification based on sporangium features is poor, compared to the calcofluor approach which is more appropriate for the identification of zoosporic organisms [14]. In addition, the Cy3-monolabelled FISH probing of natural samples clearly showed that the fluorescence of targeted chytrid zoospores may be quite similar to the autofluorescence from natural picoautotrophs. That is why CARD-FISH is more appropriate for environmental samples.
6. For natural waters, the appropriate volume depends on the trophic status of the natural waters, whether the sampling period corresponds to a bloom period, and on the nature of the phytoplankton species. Zoosporic parasites are much more abundant when large-size phytoplankton hosts such as diatoms or filamentous cyanobacteria develop [5, 14]. In oligotrophic waters, concentration of natural samples could be required before harvesting targeted organisms onto polycarbonate filters [15].
7. One filter corresponding to one sampling time point can be cut in pieces before hybridization when several probes are used.
8. The mounting medium should not be fluorescent and will minimize the fading of fluorochromes. An example of mountant is a solution composed of 50% glycerol, 50% phosphate-buffered saline (0.05 M Na_2HPO_4, 0.85% NaCl, pH 7.5), and 0.1% p-phenylenediamine (made fresh daily from a frozen 10% aqueous stock solution; Sigma-Aldrich, St. Louis, MO, USA).
9. The CARD-FISH protocol is suitable for natural samples, primarily in oligotrophic waters where its application improves the detection and the recognition of chytrid, because of the enhanced signal conferred by HRP-labelled probes, compared to monolabelled oligonucleotides. In addition, the choice of fluorescein as a stain (emission in the green spectrum at 520 nm) significantly reduces the interference from natural fluorescence of autotrophic organisms, thereby preventing the use of the deductive approach based on double counting of the same sample, i.e. with and without hybridization [25]. However, similar to the simple FISH approach, the CARD-FISH resolution for fungal images and species identification based on sporangium features is poor, compared to the calcofluor approach which is more appropriate for the identification of zoosporic organisms [14].

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Chapter 4
Technique for Identifying and Counting Infective Chytrid Sporangia Using the Chitinaceous Fluorochrome Calcofluor White

Télesphore Sime-Ngando, Serena Rasconi, and Mélanie Gerphagnon

Contents

4.1 Introduction

Fungal infections are recurrent in aquatic ecosystems [1, 2]. The most described aquatic fungi in freshwater ecosystems belong to Chytridiomycota (or chytrids). Chytrids infect a wide variety of hosts, including fish, eggs, zooplankton, and other aquatic fungi but especially phytoplankton. Typical phytoplankton hosts include prokaryotes and eukaryotes, primarily large-size diatoms and filamentous species [3]. Associated chytrids are external eucarpic parasites which produce a specialized rhizoidal system within host cells, i.e. the diet conveying system that leads to the formation of the chitinous fruit bodies: the sporangium. This parasitic stage produces

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numerous uniflagellate spores: the zoospores, which constitute the dissemination phase of the life cycle [4].

Various approaches have been used to study fungal parasites, but routine techniques for reliably identifying and counting these organisms are missing in the context of aquatic microbial ecology [5, 6]. So far, observations of parasitic fungi were obtained by using phase contrast light microscopy with live or Lugol's iodine preserved samples [7–9]. Such conventional microscopy allows observation of fungal sporangia or similar forms (especially in laboratory cultures) but is a poor approach for characterizing chytrid parasites in natural samples, at the complex community level. For example, a simple light microscopy observation of fungal rhizoidal systems, i.e. a pertinent criterium for identifying chytrids [4, 10, 11], is very difficult. This situation may help explain the confusion of chytrids with protistan flagellates such as choanoflagellates or other bacterivorous flagellates in the group of Bicosoeca, which are attached to phytoplankton but do not harm their host [5–7].

Earlier studies on chytrids were restricted to morphological descriptions and focused on few species [12–15]. Electron microscopy was used to describe different life stages and the ultrastructural cytology of fungal zoospores and spore differentiation [16–18], providing the basis for chytrid taxonomy [19, 20]. Studies on pelagic chytrids started in the British lakes [21], and different authors have provided descriptions of morphological characters [22–26]. However, few attempts have been made to include the related parasitism pathway in the aquatic food web dynamics and to understand environmental factors that trigger epidemics as well [27]. Some authors have also investigated the effects of parasitism on the growth of algal host species and on the genetic structure of infected populations [28]. Parasites are thus considered relevant not only for the evolution of their hosts but also for the population dynamics such as successions of phytoplankton communities and for structuring microbial communities in general [9, 29]. Moreover, chytrids can represent interesting key intermediates in the food chain [30, 31]. The nutrients from infected large-size algae which could not be directly fed by zooplankton can be transferred from sporangium to grazers via fungal zoospore production. Fungal zoospores have suitable dimensions and represent a valuable food source for zooplankton [32]. The activity of zoosporic fungi and the related biogeochemical processes can thus be crucial in matter and energy transfer in aquatic systems [29]. Methodological limitations for the study of the ecological dynamics of chytrid populations can be overcome with epifluorescence microscopy coupled to a specific fluorochrome targeting molecular tracers (i.e. some types of polysaccharides) of the fungal chitinous structures, including sporangium and the rhizoidal system.

The protein stain fluorescein isothiocyanate (FITC) and, in particular, the chitin stain calcofluor white (CFW) were suggested as good markers that offer useful tools for the investigation of fungal dynamics in aquatic samples [33]. CFW binds to β1–3 and β1–4 polysaccharides such as those found in cellulose or in chitin which commonly occur in the fungal cell wall [1, 2]. It fluoresces when exposed to UV light and is currently used in clinical mycology for direct microscopic examination of skin scrapings, hairs, nails, and other clinical specimens for fungal elements

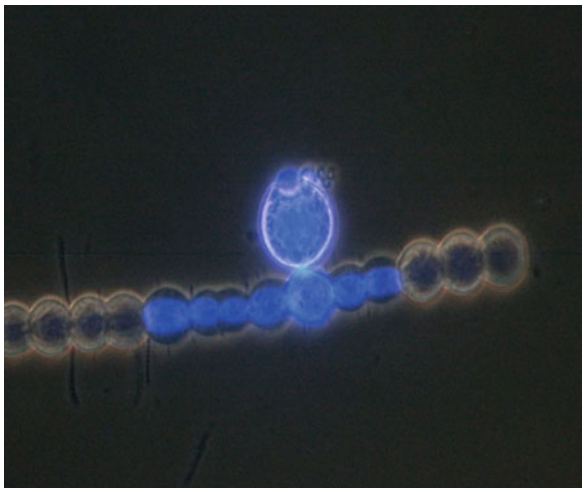

Fig. 4.1 Calcofluor white (CFW) staining of a chytrid infected filamentous cyanobacteria observed under an epifluorescence microscope using the proposed protocol. CFW penetrates well into infected host cells and is more efficient for the observation of both the fungal chitinaceous sporangium and the complete rhizoidal system within the host

[34, 35]. In contrast to FITC, CFW penetrates into infected host cells and is more efficient for the observation of the complete rhizoidal system of parasites (Fig. 4.1), i.e. a pertinent criterion for chytrid identification [4, 10, 11].

The main objective of this chapter is to provide, in a simplified step-by-step format, a routine protocol based on size fractionation of pelagic samples and the use of the fluorochrome calcofluor white for diagnosing, identifying, and counting chitinous fungal parasites (i.e. sporangia of chytrids) within phytoplanktonic communities [3], together with practical advice on how to apply the method.

4.2 Materials

1. 25 µm nylon filter
2. 0.2 µm filters
3. High-performance concentration/diafiltration system. As an example, we use the system Amicon model DC 10LA (Epernon, France) equipped with a reusable hollow fibre cartridge (0.2 µm cutoff, surface area of 0.45 m^2).
4. 36% Formaldehyde.
5. Calcofluor white ($C_{40}H_{44}N_{12}O_{10}S_2$ fluorescent brightener 28; Sigma catalog no. F3543).
6. 10 N NaOH
7. Balance.
8. Distilled water.
9. 15 ml and 0.2 ml tubes
10. Glass slides and coverslips.
11. Epifluorescence microscope equipped with appropriate UV filter sets and Neofluar objective lens (optional).

4.3 Methods

4.3.1 Concentrations of Cells (See Note 1)

1. Pass the sample (ca. 20 L) through the 25 μm pore size nylon filter (*see Note 2*).
2. Collect large phytoplankton cells in the >25 μm size fraction by washing the filter with 40 mL of 0.2 μm filtered natural sample.
3. Fix the concentrate sample with formaldehyde (2% final conc.), before staining and analysis.
4. Concentrate nanoplanktonic cells in the <25 μm size fraction (i.e. the 20 L filtrate) ca 20x by ultrafiltration to a volume of approximately 1 L, entry pressure 0.9 bar.
5. Fix about 180 mL of the ultrafiltrate retentate with formaldehyde (2% final conc.), before staining and analysis.

4.3.2 Preparation of Calcofluor Stock Solution

1. Weigh 35 mg of calcofluor white into a 15 mL tube.
2. Add 7 mL of sterile distilled water and 2–3 drops of 10 N NaOH (to increase pH to 10–11). Calcofluor does not dissolve well in neutral solutions.
3. Dissolve the calcofluor.
4. Adjust the volume to 10 mL by adding sterile distilled water.
5. Distribute the stock solution in 0.2 ml tubes and store in a lightproof tube at −20 °C.

4.3.3 Staining and Visualization

1. In the dark, stain aliquots (about 200 μl) of concentrated and fixed materials by adding 1–2.5% (vol/vol) of CFW stock solution directly in solution for 10 min.
2. Mount drop (5–10 μl) of the stained samples between glass slides and coverslips for observations and counting.
3. In a dark room, examine the slides under an epifluorescence microscope equipped with an appropriate set of filters and objective lenses. Shift between white and UV light to visualize and determine parasites and phytoplankton cells, and check the viability of the host cell, e.g. presence of chloroplast.
4. Applied a standard procedure for microscopic counting (*see Notes 3 and 4*).

4.4 Notes

1. Different approaches were tested to concentrate samples: the total community approach and the size-fractionated community approach. For the former approach, 180 ml of experimental samples were fixed with formaldehyde (2% final conc.) and aliquots concentrated in three different ways: (i) by simple gravity following Utermöhl's [36] method before staining the chyrids, (ii) by vacuum pressure on two different filters before staining directly onto filters, and (iii) by vacuum pressure on the same two types of filters but after staining in solution.
   For the Utermöhl method, 100 ml of fixed samples were settled for at least 24 h. For each of the two filter-vacuum pressure methods, 10 ml × 2 of fixed samples were filtered onto polycarbonate white filters (pore size 0.6 µm, catalog no. DTTP02500, Millipore) and nuclepore polycarbonate black filters (pore size 0.8 µm, catalog no. 110659, Whatman), by using gentle vacuum (< 0.2 bar or 20 kPa). For the total community approach using the classical Utermöhl [36] method, visualization of fungal parasites was very difficult and most of the time practically impossible for all the stain concentrations tested. The main reason was that staining directly in the Utermöhl chamber resulted in very poor-quality specimens of parasites observed in any given sample. Other disadvantages of the procedure include the relatively long sedimentation time and the difficulty of increasing the volume analyzed.

   The alternative total community approaches based on vacuum pressure concentrations on polycarbonate filters, i.e. white (0.6-µm-pore-size) and black (0.8-µm-pore-size) filters, yielded similar quality images of fungal parasites, either when CFW staining was done before (i.e. in solution) or after (i.e. on filters) concentrating phytoplankton host cells onto filters. However, substantial differences were noted depending both on the type of the filter and on the concentration of the stain. In general, for the two types of filters, high levels of background noises were obtained when using CFW at final concentrations of 3%, 10%, or 20%, precluding any accurate assessment of numerical and phenotypic characteristics of both host cells and their fungal parasites. Staining with 1% CFW final concentration substantially improved the viewing of chytrids on filters, with an increasing contrast from the white DTTP Millipore to the black Whatman filters. However, none of the membrane-retaining approaches yielded satisfactory images of morphological and cellular features of the host cells, e.g. presence of chloroplast and viability of the host cell. Accordingly, the proposed protocol is based on the size-fractionation approach using 1% to 2.5% vol/vol CFW final concentration (from the stock solution), which substantially enhanced the observational results.
2. The approach is efficient since it is based on the concentration of large initial volumes and size partitioning of samples, a step that we judged necessary in order to yield good analytic images of infectious sporangia for an accurate diagnosing and identification of parasites. In addition, this approach yielded satisfactory images of morphological and cellular features of the host cells, for phytoplankton

identification based on phenotypic features and viability of the host cell, through the integrity of cell wall and the presence of chloroplasts, which are fundamental parameters to assess the intensity of the disease. We consider this protocol as an optimal for the diagnosis and quantitative assessment of phytoplanktonic chytrid infections in natural samples. Finally, the approach was designed to freeze-conserved particulate DNA samples for quantifying the propagule stages (i.e. zoospores) of chytrids via FISH targeting of specific rRNA oligonucleotide probes (see Chap. 3).

3. To estimate the infectivity parameters of ecological interest in the phytoplankton population, several algorithms are used according to the formula proposed by Bush et al. [37]. These parameters include the prevalence of infection (Pr), i.e. the proportion of individuals in a given the phytoplankton population having one or more sporangia or rhizoids, expressed as $Pr\ (\%) = [(Ni/N) \times 100]$, where Ni is the number of infected host cells and N the total number of host cells. The second parameter is the mean intensity of infection (I), calculated as $I = Np/Ni$ where Np is the number of parasites and Ni is the number of infected individuals within a host population.
4. We propose a third parameter concerning the prevalence of infection of cells in colonial (or filamentous) species (Pr_{CF}). $Pr_{CF}\ (\%) = [(Ni/N) \times 100]$ where Ni is the number of infected host cells in parasitized colonies (or filaments) and N the total number of parasitized host colonies (or filaments).

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Chapter 5
Assessment of Host Immune Responses to Fungal Pathogens

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5.1 Introduction

Fungal infections (mycoses) to animals and humans have increased substantially in the last few decades [1]. Fungi cause not only superficial infections such as dermatophytosis but also tissue and organ invasions and disseminated infections [2, 3]. Owing to the rise of immunocompromised patients, the morbidity of invasive fungal infections has increased remarkably worldwide [2]. The clinical outcome of fungal infection is linked to the pathogen virulence level, the immune responses of hosts, as well as the interaction between pathogens and hosts [4–6].

Our immune system is mainly composed of immune organs, immune cells, and immune active substances, which performs immunological surveillance, defense, and regulations [7]. The primary immune organs include the bone marrow and the thymus, which provide the sites for cytogenesis, differentiation, and maturation of immune cells. Peripheral immune organs like the spleen, lymph nodes, and mucosa-associated lymphoid tissue are the colonization sites in which immune responses are induced. Fungi are recognized by cells of the innate immune system (e.g. dendritic cells and macrophages) that bind to the components of fungal cell walls. Mononuclear phagocyte system, granulocytes, lymphocytes, dendritic cells, and mast cells have different roles in defense against fungal infection [6, 8].

Host defense mechanisms range from innate immunity to adaptive immunity when exposed to fungi [7, 9]. Innate immunity is considered the first-line defense and plays an important role in antifungal immunity. The physic barriers, innate immune cells, and related immune active factors work together to protect the hosts from fungal invasion [10]. Immune cells such as dendritic cells function as a linkage between innate immunity and adaptive immunity, inducing the differentiation of adaptive immune cells. Detecting the immune cells numbers, function, and production of related cytokines or chemokines is useful to the understanding of immune response [11].

Immune response varies with fungal species, morphologies, and the immune condition of the host. Impaired host factors can lead to increased susceptibility to unusual, sporadic fungal pathogens, particularly during subcutaneous and systematic infections. When constructing the infection model in vitro and in vivo, both the host and pathogen conditions should be taken into consideration for research [12]. As the infection is a dynamic process, the infection time and host status should be also taken into account. Here, we will describe a few frequently used methods for investigating the immune responses to fungal pathogens, particularly to *A. fumigatus*.

5.2 Methods

5.2.1 Analysis of the Fungal Burden

Fungal burden is an important parameter that reflects the ability of the host to eliminate the pathogen. In general, the colony-forming unit (CFU) per unit weight of infected organ or tissue is calculated; for bronchoalveolar lavage fluid (BALF), CFU per unit of volume is calculated instead. CFU detection is more suitable to quantify yeast than filamentous fungi since the number of yeast colonies is simpler to estimate. Nowadays, the quantitative polymerase chain reaction (qPCR) approach is commonly used to determine the fungal burden. qPCR assays targeting the 18S rRNA gene [13] and *FKS* gene [14] in *A. fumigatus* have been frequently reported in recent research. Here are two protocols for determining the fungal burden in infected animals.

5.2.1.1 CFU

1. Remove the infected organ/tissue from the infected hosts in sterile condition. Weigh the infected samples in 1.5 ml Eppendorf tubes.
2. Homogenize the tissue in saline (e.g. unilateral lung in 2 ml saline). Sterile mortar and pestle are conventional tools (precaution: grind the organs first before adding saline), but homogenizers with glass beads at 4 °C in 10–20 Hz/s for 30s in the 5 ml sterile tubes with a screw top are recommended for a larger number of samples. (precaution: reduce or increase the grinding time according to the sample types until the tissue is completely homogenized).
3. Dilute the homogenate with saline and plate onto Sabouraud dextrose agar (SDA, Difco, NJ, US) plates in duplicate. A serial diluting concentration (such as 1:10, 1:100, 1:1000) is recommended (30–300 CFU/plate).
4. Count the CFU on the SDA of different dilution ratios after incubation at 35 °C for 48 h.
5. Calculate the fungal burden in the infected organ/tissue (CFU/g).

5.2.1.2 qPCR[13] Targeting the 18S rRNA Gene

1. Weigh the infected organ in sterile Eppendorf tubes after dissection.
2. Add 3.5 times the volume of saline to the tissue in tubes. Homogenize the tissue with beads (4 °C, 10–20 Hz, 30s). Centrifuge at 1500 rpm at 4 °C for 5 min. Collect the homogenate for DNA extraction.
3. Extract the total DNA with DNeasy Blood and Tissue kit (Qiagen, CA, US) according to the manufacturer's instructions.
4. Perform the PCR with primers and probes targeting the 18S rDNA of *A. fumigatus* in optical 96-well reaction plates (Applied Biosystems, MA). The

Table 5.1 Sequences of primers and probe targeting the 18S rDNA of *A. fumigatus* [13]

Primer name	Sequence
Sense amplification primer	5'-GGCCCTTAAATAGCCCGGT-3'
Antisense amplification primer	5'-TGAGCCGATAGTCCCCCTAA-3'
Hybridization probe	5'-FAM-AGCCAGCGGCCCGCAAATG-TAMRA-3'

Table 5.2 qPCR system [13]

DNA sample	5uL
Sense amplification primer	900 nM
Antisense amplification primer	900 nM
Hybridization probe	200 nM
TaqMan universal PCR master mix(2X)	25uL
H_2O	–
Total volume	50uL

recommended PCR conditions are 10 min at 94 °C, 40 cycles of 15 s at 95 °C, 1 min at 60 °C (Tables 5.1 and 5.2).

5. Generate a standard curve by using various known amounts of *A. fumigatus* conidia into naive, uninfected kidneys. Calculate the conidial equivalents (CE) according to the standard curve, with fungal burden shown as CE/g of tissue.

5.2.2 *Isolation of the Immune System Organs, Infected Organs, and Immune Cells*

The immune system organs consist of the central and peripheral immune organs [15]. Hematopoietic stem cells proliferate and differentiate into B lymphocyte and T lymphocyte in the central immune organs and are transferred to the peripheral immune organs [16, 17]. Peripheral immune organs include blood circulation and lymphatic circulation, which are interconnected into a network and supply the area where immune cells aggregate and immune responses occur [18, 19]. To investigate the immune responses in the hosts, it is important to separate the immune organs and immune cells from the hosts. The size and status of organs and constitution of immune cells in the organs could reflect the immune responses in local or general infection after fungi invasion [20, 21]. If the separated immune cells need to be cultured for further experiment, all the procedures should be performed in sterile condition. Here, we use the mouse model as an example to show the process of immune organs separation.

5.2.2.1 Thymus

The thymus is an organ in the central immune system for cytogenesis, differentiation, and maturation of different T cells [22]. The thymus consists of thymus cells and thymus stromal cells, and stromal cells include thymus epithelial cells, macrophages, dendritic cells, and fibroblasts. Thymus epithelial cells secrete thymosin and thymulin, which activates the function of immune cells [23].

1. After euthanasia is performed, disinfect the mice's skin on the chest and abdomen.
2. After dissection, cut and lift the ribs and sternum. The thymus of mice is located in the anterior superior thorax. The white lobes above the heart are the thymus.
3. Remove the thymus and put it in the complete medium (DMEM or RPMI1640, 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate) (Gibco, MA, US) or PBS buffer (1%FBS + 2 mM EDTA) (Gibco, MA, US) for further experiment.

5.2.2.2 Bone Marrow

Bone marrow is the main hematopoietic organ that produces erythrocytes, granulocytes, monocytes, lymphocytes, and platelets [24]. Bone marrow contains cells with the ability to differentiate into all blood cell types. Bone marrow-derived macrophages (BMDM) represent the primary macrophages and have been established as important inflammation host cells in vitro in the immunological study [25, 26]. The bone marrow of mice is mainly obtained from the tibia and femur. The procedure for BMDM should be performed under sterile condition.

1. Disinfect the skin of mice with 75% alcohol. Remove the skin and muscles from the tibia and femur.
2. Cut the epiphyses of the tibia and femur and wash the bone marrow using an injection syringe with a complete medium for a few times.
3. Resuspend the bone marrow cells in BMDM medium (Iscove's modified. Dulbecco's medium (IMDM)) supplemented with 10% fetal calf serum, 100 u/mL penicillin, 100 μg/mL streptomycin, or phosphate buffer saline (PBS) for further experiment.

5.2.2.3 Spleen

The spleen is the largest peripheral immune organ in the body, which accounts for 25% of the total lymphoid tissue in the whole body. It contains a large number of lymphocytes and macrophages and is the center of cellular immunity and humoral immunity [27]. It also filters the blood of pathogens and abnormal cells and works as a hemopoietic organ during embryonic development [28].

1. Disinfect and cut the skin on the abdomen.
2. The spleen is next to the fundus of the stomach with the shape of a sickle.
3. Remove the spleen and put it in a complete medium or PBS for further experiment.

5.2.2.4 Lymph Nodes

Lymph nodes (LNs) are filters of lymphatic fluid and supply sites where T and B lymphocytes migrate, proliferate, and differentiate into immune effector cells after being stimulated by antigens [29]. In fungal infection, it performs the function of filtering, killing, and clearing pathogens [30].

The LNs are easy to isolate and commonly used in research and mainly include cervical, axillary, inguinal, and mesenteric LNs [31, 32]. The superficial cervical LNs are located on the superficial and lateral sides of the left and right submandibular glands, often surrounded by adipose tissue. Axillary LNs are located in the adipose tissue of the axilla. Inguinal LNs are located in the left and right gluteus muscles lacuna. Mesenteric lymph nodes are located in mesenteric adipose tissue. During infection, the draining lymph nodes of the infected site would be larger than the normal ones and easy to see and separate. Remove the lymph nodes and put them in a complete medium or PBS for further experiment.

5.2.2.5 Blood

Blood cells are derived from the hematopoietic pluripotent stem cells in the bone marrow [33]. Neutrophils, eosinophils, basophils, monocytes, and lymphocytes are the main immune cells in the blood. Neutrophils play an important role in the prevention of invasive fungal infections [34]. Peripheral blood mononuclear cells (PBMCs) are indispensable in immunological diagnostics and research. PBMCs include lymphocytes (T cells, B cells) and monocytes in blood. A few methods for isolation of PBMCs are available, among which density centrifugation using a commercial kit is frequently used. The procedures could be referred to references or kit instructions [35, 36]. The number and function of these immune cells in blood determine the results of infection and are considered as the indicator for evaluating the infectious status of the host. Here, we only describe the protocol for whole blood cells isolation.

1. Collect blood in tubes containing heparin for cells separation.
2. Centrifuge at 4 °C at 500 × g for 5 min, and the supernatant is discarded.
3. Collect the cells for erythrocyte lysis following the session 2.8.4–2.8.7.

5.2.2.6 Bronchoalveolar Lavage Fluid (BALF)

The contents of BALF include the immune cells and immunological materials [37]. During fungal infection in the lung, the constitution and number of immune cells, level of cytokines, and CFU could reflect the level of immune responses in infected lung [38]. CFU in BALF could also be used as a sign of fungal burden. CFU per unit of volume is calculated. Galactomannan in BALF could be used in the diagnosis of pulmonary aspergillosis [39, 40].

1. The airway contents are recovered by the instillation and retrieval of 1 ml of sterile PBS through a tracheotomy tube. Cells are collected by a total of 3 lavages and pooled.
2. Centrifuge at 1500 rpm for 5 min at 4 °C. After centrifugation, the supernatant is used for biochemical and immunological tests for the pulmonary infection model.
3. Resuspend the cells in complete medium. Centrifuge at 1500 rpm at 4 °C for 5 min.
4. Perform gentle operations in the whole process. If there are erythrocytes in the cells, collect the cells for erythrocyte lysis following 2.8.4–2.8.7.

5.2.2.7 Infected Organs

In fungal infection, the immune cells migrate to the infected organs. Analyzing the immune cells in infected organs or cytokines in tissue homogenate can reflect the degree of inflammation and the immune responses after infection.

Here, we use a lung tissue sample to illustrate the methods for tissue digestion. Adjust the constitution of digestion buffer according to the types of tissue or organ [41].

1. Wash the lung sample with sterile PBS to remove the blood on the surface of the organ. Dissect the tissue into small pieces with ophthalmic scissors.
2. Transfer the unilateral lung tissue into the tube containing 2 mL digestion buffer (1.7 ml DMEM+0.2 ml FBS + 100ul 10 mg/ml collagenase IV + 4 μl 0.05 mg/μl DNase) (ThermoFisher Scientific, MA, US).
3. Incubate the tube for 30 min at 37 °C, mixing at 260 r/min in the shaker. The parameters for enzymatic digestion vary with tissue pieces size and enzymatic activity.
4. Collect the digested lung tissue and filtrate with 100 μm-pore-size screens.
5. Centrifuge the filtrated cell suspension at 1500 rpm for 5 min.
6. Discard the supernatant. Resuspend the cells in a complete medium for erythrocyte lysis.

5.2.2.8 Separation of Immune Cells and Erythrocyte Lysis

For the immune solid organs isolated in 2.1–2.4, they need to be processed in the immune cell suspension for further experiment especially in the flow cytometry. For the immune organs with a rich blood supply, erythrocyte lysis needs to be performed before further study of the immune cells. Blood cells, BALF cells, and bone marrow can be directly used for erythrocyte lysis and start from 2.8.4 [42].

1. Weigh the immune organs (spleen, LNs, thymus) in Eppendorf tubes after dissection of the euthanized animals.
2. Grind the immune organs using the blunt end of the syringe.
3. Filter the cells through a 100 μm-pore-size screen to separate the cells from the connective tissue.
4. Add Tris-NH_4Cl (tris(hydroxymethyl)aminomethane-ammonium chloride) erythrocyte lysis buffer (3.735 g NH_4Cl, 1.3 g Tris in 500 ml ddH_2O) into the cells to lyse the erythrocytes. Volumes are adjusted for different cells (e.g. 5 times the volume for normal immune organs; 10 times volume for blood cells).
5. Wash the cells with a complete medium, and centrifuge and resuspend the cells in the complete medium.
6. If the erythrocyte lysis process is incomplete, repeat steps 4 and 5 again.
7. Resuspend the cells in a complete medium for cell counting and flow cytometry.

5.2.2.9 Histology

Histopathology of the infected tissue and immune organs would demonstrate directly the extent of inflammation. It is helpful to study the inflammation type and to evaluate the immune condition. The immune organs and infected tissue are fixed by inflation with 4% paraformaldehyde. After paraffin embedding, 5 μm-thick sections are cut and stained according to different staining methods for different objectives. Hematoxylin and eosin (HE) stain is usually used to evaluate the inflammation in the infected sites and observe the immune cells [43]. Periodic acid-Schiff (PAS) stain and Gomori's methenamine silver (GMS) stain are used for fungi detection. These two methods could dye the fungal pathogen in red and black, respectively [44, 45]. Also, immunohistochemistry (IHC) using specific markers is useful to show the different immune cells in tissue and to compare the relative expression of different cytokines and receptors [46].

5.2.3 Detection and Quantification of Immune Cells

Flow cytometry is commonly used for characterizing different immune cells [47]. Since different immune cells have different specific markers, choosing suitable cell surface markers with a fluorescence color scheme is important. For instance,

Table 5.3 Cell markers commonly used in flow cytometry

	Cells	Marker in mice peripheral blood and lymphoid organ	Marker in human peripheral blood and lymphoid organ
Innate immunity	**Monocyte**	$CD11b^+CD115^+Ly6C^+$	$CD14^+$
	Macrophage	$CD45^+$ $CD11b^+$ $F4/80^+$	$CD11b^+CD68^+$
	Neutrophil	$CD11b^+Ly6G^{++}$	$CD11b^+CD16^+CD15^+CD32^+$
Adaptive immunity	**T cell**	$CD3^+$	$CD3^+$
	Th cell	$CD3^+$ $CD4^+$	$CD3^+$ $CD4^+$
	Treg cell	$CD3^+$ $CD4^+$ $CD25^+FoxP3^+$	$CD3^+$ $CD4^+$ $CD25^{high}$ $PD\text{-}1^{high}$
	Cytotoxic T cell	$CD3^+CD8a^+$	$CD3^+CD8a^+$
	B cell	$B220^+$	$CD19^+$ $CD20^+$ $CD79a/b^+$
	Plasmacyte	$B220^+CD19^+$	$CD19^+$ $CD20^-$ $CD38^{high}$
	NK cell	$CD49b^+CD3^-$	$CD56^{bright}$ $CD16^+/CD56^{dim}$ $CD16^+$
	NKT cell	CD1d-$tetramer^+TCR\beta^+$	$CD3^+$ $TCRV\alpha24^+$

some immune cells have different surface markers in humans and animals. Here is a list of common markers for innate and adaptive immune cells (Table 5.3).

5.2.3.1 Cell Detection Using Flow Cytometry

1. Choose the suitable surface marker for targeting immune cells and design the multicolor plan.
2. Measure and add about 10^6 cells in the Eppendorf tube.
3. Preincubate cell suspension with purified CD16/CD32 mAb ($\leq$ 1 μg/million cells in 100 μl) (BD, NJ, US) at 4 °C for 5 min or dilute the antibodies in PBS containing 2% bovine serum albumin (BSA) to reduce Fc receptor-mediated binding.
4. Add 50 μl antibodies for staining. Cells are stained for 30 min at 4 °C and protected from light.
5. Centrifuge at 1500 rpm for 5 min at 4 °C.
6. Wash the cells with PBS, and centrifuge and resuspend the cells in 1 mL PBS containing 0.1% BSA.
7. Perform the flow cytometry in a few hours according to the protocol published before [48] or the protocol provided by the flow cytometer.
8. Fix the cells in paraformaldehyde if they are detected in a timely manner. Keep the samples in the dark after fixation.
9. Analyze the flow cytometry data using FlowJo (BD, New York, USA) or other software package.

5.2.3.2 Cell Counting

1. Weigh the immune organs. Prepare blood sample or cell suspension for erythrocyte lysis. Resuspend the cells in a complete medium in 2.1.
2. Count the cells using a hemocytometer or automated cell counting instrument.
3. Calculate the number of cells per organ or volume of blood.
4. Calculate the number of specific immune cells using the total number and the percentages generated from flow cytometry.

5.2.4 Detection and Quantification of Cytokines

Cytokines are small molecular proteins with extensive biological activities, and they work together as a network in the innate and adaptive immunity systems in fungal infection [49, 50]. Cytokines are usually protein or polypeptide molecules which have antigenicity. Based on the high specificity of the antigen-antibody reaction, immunoassays are the most common methods for detecting cytokines. The common methods include flow cytometry, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), enzyme-linked immunospot assay (ELISPOT), and Western blot.

5.2.4.1 Intracellular Cytokine Detection Based on Flow Cytometry

1. Pipette 2×10^6 cells in the 48-well cell culture plate.
2. Suspension cells are for stimulation in a complete medium with protein transport inhibitor according to the cells and cytokines types. 20ug/ml PMA (phorbol myristate acetate), 1ug/ml ionomycin, and 1ug/ml lipopolysaccharide (LPS) are commonly used as stimulatosr; 3.0 ug/ml brefeldin A and 2uM monensin are commonly used as protein transport inhibitors.
3. Collect the cells for surface marker staining (refer to cell detection steps 3.1.1–3.1.5).
4. Add 100 μL Cytofix/Cytoperm solution and incubate at room temperature protected from light for 30 min.
5. Add 500 μL Perm/Wash solution, and centrifuge at 4 °C at 5000 rpm for 5 min. Wash the cells twice.
6. Dilute the cytokine antibody in 100 mL Perm/Wash solution. Add the antibody in the tubes. Cells are stained for 30 min at 4 °C and protected from light.
7. Add 500 μL Perm/Wash solution, and centrifuge at 4 °C at 5000 rpm for 5 min.
8. Resuspend the cells in 1 mL PBS for flow cytometry.
9. Analyze the flow cytometry data using (Flowjo) or other suitable software. The relative expression of cytokines is shown in percentages.

5.2.4.2 ELISA

ELISA is a qualitative or quantitative test that uses antibodies to bind and measure a molecule of interest. It remains the method of primary choice and one of the most widely used methods for detecting cytokines for its specificity and simplicity in many laboratories.

1. Select the antigen-coated plates and enzyme (take horseradish peroxidase (HRP) as an example).
2. Add 100 μL of standards and samples (select appropriate dilution ratio) to wells. Incubate the samples at room temperature for 2 h.
3. Discard the solutions from wells.
4. Wash the plates four times.
5. Add 100 μL HRP enzyme-labeled antibody to wells and incubate at room temperature for 30 min to 1 h according to the manufacturer's instructions.
6. Discard the solutions from wells.
7. Wash the plates four times.
8. Add 100 μL chromogenic substrate to wells.
9. Develop the plate at room temperature in the dark for 30 min.
10. Add 100 μL stop solution to wells.
11. Evaluate the results in 30 min after stopping the reaction. Read the plates at 450 nm and 550 nm. Subtract 550 nm values from 450 nm values to correct for optical imperfections in the microplate.
12. Calculate the results according to the standard curve.

5.2.4.3 Other Methods

There are other methods important in immunological research not covered in this chapter. Several traditional methods including radioimmunoassay (RIA), enzyme-linked immunospot assay (ELISPOT), and Western blot are commonly used for cytokine detection. Most of these methods are based on antigen/antibody reaction; the antibodies are incorporated with a radioisotope or biotin-labelled secondary antibody for detection. Molecular methods are also used for cytokine detection through the gene probes of cytokines to detect the expression level. The detection methods include Northern blot, real-time PCR, and in situ hybridization.

With the advent of novel technologies in laboratory diagnosis, multiple cytokine detection methods have been developed recently, such as flow cytometry and electrochemiluminescence. Based on flow cytometry platform, technologies such as cytometric bead array (CBA) [51], FlowCytomix, LEGENDplex, and Luminex Assay have been developed. Briefly, fluorescent microbeads are coated with specific antibodies. When the beads are mixed with the biological sample, biomolecular binding interaction occurs between the specific antibodies and cytokines antigens, the binding activity/analyst can be detected by signals generated by means of flow cytometry. In the electrochemiluminescence assay, electrochemistry-induced

specific chemiluminescence reaction is occurring on the surface of the electrode, and the light signal is detected and used for analysis. These methods have higher sensitivity and can detect a series of cytokines at the same time with a smaller sample volume [52, 53].

5.3 Summary

The interaction between the host and fungus pathogens are complicated; human hosts having different immune status may lead to contrasting clinical outcome. Early diagnosis and treatment contribute to the prognosis of fungal infections. Better understanding of antifungal immune response contributes to the improvement of diagnosis and therapy of fungal infections. The protocols described above are fundamental and useful in immunological research.

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Chapter 6
Recent Advances in Applications of Support Vector Machines in Fungal Biology

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6.1 Introduction

Machine learning has recently been employed successfully in fungal biology. The methodologies used include both supervised and unsupervised learning. Supervised learning employs prior domain knowledge to build models. The models built can be used to derive information for new examples. Classification and regression form major supervisory learning paradigms used in fungal biology. Unsupervised learning, on the other hand, is used to discover structured information without prior domain knowledge. Several classification and regression algorithms are available in machine learning which is routinely employed for quick and low-cost acquisition of invaluable information. Support Vector Machines is a valuable classification and regression tool employed in different fields of science and engineering for high-performance prediction tasks. Support Vector Machines have been used profusely in a fungal domain for the identification of plant diseases, characterization of fungal keratitis, and other fungal infections. Support Vector Machines have also been successfully used to predict potential antifungal peptides. In this chapter, after a brief discussion on SVM, we will be discussing various case studies where SVM has been used in fungal biology. Apart from that, we have also presented our own unpublished work on the prediction of antifungal peptides. This is based on a recent method on the distributed representation of protein sequences which has been used for extracting embedding vectors using unsupervised learning. The informative embedding vectors are further employed as input attributes to SVM classifier to find whether a given peptide sequence has antifungal activity or not.

6.1.1 Support Vector Machine

Support Vector Machine is a hyperplane-based classifier [1]. Support Vector Machine employs a linear maximum margin hyperplane for classifying examples into two groups. As an illustrative example assume that we are given a set of peptide sequences with experimental proven antifungal activity. We also have been given sequences with no known antifungal activity. Support Vector Machine finds a hyperplane which maximizes the distance between the hyperplane and the closest sequences belonging to both classes of sequences (Fig. 6.1).

If the sequences are linearly classifiable, the accuracy of classifications will be high and acceptable. If the classification accuracies are not good, then the sequences may not be linearly classifiable. For the non-linearly classifiable tasks, SVM

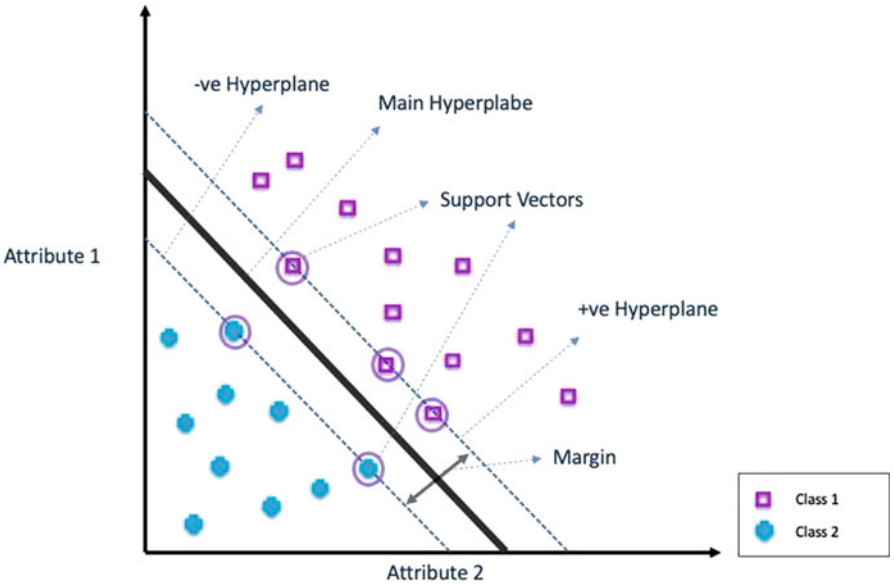

Fig. 6.1 Cartoon of SVM as a maximum margin classifier. Examples on the margin are Support Vectors

transforms the examples to a higher dimensional attribute space. After this, SVM employs a linear maximum margin classifier to separate these classes of sequences. Further, it reduces complexity by defining different kernel functions. These include RBF, Polynomial, Gaussian, and Neural network kernels. These are domain-dependent kernels like mismatch kernel, Fischer kernel, and string kernels. Detailed discussions on the working of the SVM algorithm along with diverse types of the algorithm have been provided in Modak et al. [2].

6.2 Domain Features

Several classes of domain features are extracted for predictions and classifications. These include sequence-based features, image-based features, and spectral features. Sequence-based features include (1) amino acid frequencies, (2) dipeptide & tripeptide, and higher-order K-mer frequencies; (3) Physiochemical properties (4) Position specific Scoring Matrix (PSSM) profile-based attributes (5) Pseudo-amino acid composition-based features etc. Image-based features include pixel-based features, shape descriptors, frequency domain-based features like wavelet features, and other descriptors extracted from leaf images and solid phase microextraction/gas chromatography-mass spectrometry (SPME/GC-MS) based volatility markers. Once the features are extracted, feature selection algorithms can

be employed for the identification of the most informative features. Feature selection algorithms include filter, wrapper, and embedded methods. A detailed discussion of diverse types of feature selection algorithms has been provided in Modak et al. [2].

6.3 Review of Some Recent Fungal Bioinformatics Applications Using SVM

In this section, we outline few important problems in bioinformatics where SVM has been applied with interesting results on many real-world fungal biology case studies.

6.3.1 Haar Wavelets Features for Fungal Disease Detection in Maize Leaves

Agriculture is the most vital part of the economy of any of the countries in the world. That is why the diseases which affect the agricultural crops are serious concerns since they affect the economy of the country. One of the most common crops cultivated throughout the globe is maize. The importance of this crop is not only limited to being food for humans and cattle, it is also the source of a lot of commercial products like baby corn, corn starch, and corn oil. Thus, the research community has been exploring the avenues to detect such diseases in maize crops in the preliminary stages to minimize the loss. From the range of the diseases affecting maize crops, Desai et al., proposed an image-based method to detect diseases in maize plants [3].

For this study, the authors targeted fungal diseases along with common rust and northern leaf blight in the maize leaf. The very first step was the collection of images, which was carefully executed by making sure that all the images collected over the period are consistent. This was achieved by capturing images; all pictures are taken at 11:00 AM and by keeping a consistent distance of two feet from the leaves. The final dataset of images consisted of 200 images, which were distributed in the following categories (a) 50 healthy, (b) 50 common rust, (c) 50 northern leaf blight, and (d) 50 images with both diseases present in it. All these images were preprocessed to make sure that the illumination is uniform throughout all the images. Once the images were converted to RGB colour space, red, blue, and green channels were separated for feature extraction. The first-order histogram features were considered for this study which are estimated properties of individual pixel values by ignoring the spatial interaction between image pixels [4]. One important aspect of image analysis is texture analysis, which was incorporated in this work by wavelet representation. The wavelet transformation for feature extraction involves Haar transform [5]. Haar wavelet is applied to RGB images to obtain horizontal, vertical, and diagonal coefficients. Later, Gray-Level Co-occurrence Matrix (GLCM) is

constructed in two directions (0 and 90°) for each of the coefficients. Finally, k-NN and SVM classifiers were employed for classification. k-NN yielded the highest accuracy of 85% for k = 5 and while SVM-based classification yielded 88% of accuracy.

6.3.2 *Quality Detection of Pomegranate Fruit Infected with Fungal Disease*

The hazards of fungal infections are not only restricted to common crops like maize but also some varieties of fruits that are well known and widely consumed for their known benefits. Pomegranate fruit is one such example because of its several health-promoting effects including positive effects on the cardiovascular system, human immune system, menopausal complaints, and diabetes mellitus [6]. Like most crops, the harvest and transportation of these fruits involve the risk of contamination of pathogenic fungi. Alternaria specie or Aspergillus specie is known which infects pomegranate throughout all its stages of life, from the early stage of ripeness through its development [7, 8].

Even with current advances in technologies, a lot of time and effort will be needed to develop a non-destructive quality assessment of Alternaria fungi. To address this problem, Rafiee S. et al., worked on developing a non-destructive, inexpensive, and fast technique to evaluate the quality of pomegranate [9]. Metal Oxide Semiconductor (MOS) biosensors were employed using linear and non-linear methods for quality assessment. In this elaborative work, sampling was done using 60 Pomegranates of the 'Robab' genotype, which were similar in shape and size. These samples were classified into different amounts of Alternaria specie infection, ranging from 0%, 25%, 50%, 75%, and 100%. The methods which were employed for linear and non-linear analysis methods for detection were (a.) Back Propagation Neural Network (BPNN), (b.) Discriminant Analysis (LDA), and (c.) Support Vector Machine (SVM). Finally, the results from each different method were compared with each other. In this study, authors concluded that 100% detection accuracy between healthy and infected samples was achieved by the LDA method, by using only two MOS sensors. Back Propagation Neural Network proved to be the most efficient prediction method with an accuracy of 100% in the detecting of 0%, 25%, 50%, 75%, and 100% infected pomegranates. In this comparison study, the BPNN method performed better than LDA and SVM analysis, in terms of accuracy, in detecting fungal infestation in various stages. The authors concluded that this technique can be reliably used to effectively evaluate the quality of the pomegranate with high precision.

6.3.3 Support Vector Machines Enabled Fungal Rust Disease Detection in Pea Plant (Pisam Sativam)

Another prominent example of the dreadful consequences of agricultural diseases is the fungal rust disease of the Pea plant. Uromyces fabae (Pers.) de Bary fungus cases Pea rust, which is characterized by mutilation of upper and/or lower surfaces of leaves, spotting in leaves, and rust coloured spotting on petioles, pods, and stem [10]. Such plant diseases harm the economy by reducing production. Thus, the research community has been working towards cost-effective and rapid solutions where such diseases can be detected as early as possible [11, 12]. There are some notable studies where Image processing-based tools were effectively used to screen crops for diseases [13, 14]. Out of all these studies, specific attention has been given to digital imaging at spore germination or penetration level for fungal diseases identification [15–17] since the plants can be rarely saved after such infection.

Thus, Kaur P et al. aimed to prove the early detection of rust diseases of Peas at the microscopic level by using image processing techniques [18]. In this work, authors tried to develop a technique which helps identification of symptoms at an early stage of infection of rust disease. Image data was gathered by collecting leaves of affected plants. Staining techniques and biochemical methods were applied to the leaves as part of preprocessing [19]. The images obtained were subjected to further preprocessing, which included (a) image smoothing, (b) noise/background removal, (c) image enhancing, (d) image format changing, and (e) image segmentation. After preprocessing, features could be extracted from the region of interest (ROI) for classification. In this work, the authors employed Discrete Wavelet Transform which is a technique for 2-D decomposition of the image with respect to Haar wavelet. Once the features were extracted, an SVM classifier was used to build the model for leaf disease detection in Pea Plant. Authors were able to reach an accuracy of 89.60% in disease detection in this study, which could be further explored to develop efficient, rapid, and cost-effective technology for plant disease identification.

6.3.4 Volatomic Visualization for Fungal Infection Detection on Storage Jasmine

Fungal contamination in grains can not only affect the economy but also can cause danger to human health since some fungal strains such as Aspergillus can produce harmful mycotoxins [20–24]. Siripatrawan U. et al., with the aim of preventing infected rice from entering the food chain by detecting the fungal infection in rice at an early stage [25]. In this study, the detection unit, i.e. the electronic nose when coupled with chemometrics, proved to be a fast and non-destructive detection method for fungal contamination in Jasmine brown rice grain. For the prediction model, the source of data was the artificially infected Jasmine brown rice, which was

achieved by storing Aspergillus infected rice at 30 °C and 85% relative humidity. Solid phase microextraction/gas chromatography-mass spectrometry (SPME/GC-MS) was employed in this study for the identification of volatile markers in the infected rice.

An electronic nose (described thoroughly in the reference) was used to analyze the profiles generated from the identification, i.e. Volatomic profiles. Principal component analysis (PCA) was employed to explore the multivariate electronic sensor response data and to reduce the data dimension. Machine learning techniques, SVM and Linear discriminant analysis (LDA) were employed for the classification of samples, contaminated at a different level over the time series of storage. Finally, the prediction of fungal growth on brown rice was formulated by implementing the Partial least squares (PLS) regression model. The PLS regression model combined with the electronic nose predicted fungal growth precisely. It reached a coefficient of determination (R2) value of 0.969, and root mean square error (RMSE) value of 0.31 Log CFU/g.

In the work, the authors demonstrated the use of PCA reducing the data, but this technique was not able to classify the sample groups in different categories, while both SVM and LDA were not only able to perform well identifying and classifying fungal infections in samples, but it also detected the level of infection with respect to storage time. The authors concluded that the electronic nose described in this study can be effectively used for fast and non-destructive detection of rice grain samples with fungal infection.

6.3.5 Hyperspectral Imaging Enabled Electronic Nose for Fungal Contamination Identification in Strawberries

E-nose coupled with hyperspectral imaging (HSI) was also used by Tu K. et al. to show a non-destructive method for detection of quality and microbial content of strawberries during decay [26]. The spectral data and images of strawberries were acquired using a lab-scale visible/near-infrared HSI system. In the proposed method, PCA was employed to reduce the dataset dimensionality and for extracting features from the HSI and E-nose data.

First, the aroma, spatial, and spectral information was acquired by HSI and E-nose during the decay of strawberries. Multivariate calibration models were developed using the sensor and spectral signals to detect fungal contaminations in strawberries. For the development of calibration models, E-nose and HSI data was integrated for improving the decay detection of strawberries. In this study, one of the important conclusions was the correlation between the interior compositions of the fungi-infected strawberries and changes in exterior appearances was highly significant. Colony-forming units were found with a correlation coefficient value of 0.925 and root mean square error of 0.38. In conclusion, the authors recorded and

recommended that the two sensing techniques can be combined for the detection of the quality of strawberries.

6.3.6 *Rapid Discrimination of Fungal Species by the Colony Fingerprinting*

In continuation with series of examples where agricultural goods are affected by fungal contamination, this example deals with addressing the same problem with another approach. Tanaka T. et al., used a bioimage informatics approach for fungal discrimination in the samples [27]. For this work, the concept of 'colony fingerprinting' was used which was already proven in earlier studies [28, 29]. Colony fingerprinting is an imaging technique where the colonies of the microorganism are visualized using a lens-less imaging system. This was followed by the extraction of quantitative features having discriminative power needed for processing by machine learning techniques.

The authors aimed to prove the effectiveness of the colony fingerprinting approach in fungal specie discrimination since its high performance for bacterial identification was already reported in earlier studies [29]. In contrast to bacterial discriminative parameters (e.g. roundness, solidity, doughnut-ness etc.), seven discriminative distinct parameters for fungal specie were extracted as colony fingerprints. Some of the parameters were as simples as the number of hyphae (fungal filaments), while other complex characters include the number of branches of hyphae. The overall idea was to extract the count of hyphae and their branches, along with information on distributions of intensities on the images. Based on this approach, authors were able to successfully discriminate five distinct species of fungi belonging to Fusarium, Eurotium, Alternaria, Aspergillus, and Penicillium genre. This work was further extended by discriminating six closely related Aspergillus specie by introducing more parameters in the feature set. The most exciting discovery in the work was the turnover time required for deriving the colony fingerprinting from generated fungal colonies, which was just 48 h. This time is less than that of other methods used for discrimination like MALDI-TOF-MS.

6.3.7 *SVM Classifier Based Grape Leaf Disease Detection*

India is one of the major producers of grapes, which adds up to the agro-economy of the country. The annually major percentage of this important crop is affected by the diseases due to microbial infections; thus, technological solutions aiding the increase in the yield of this crop has been one of the focused areas of the research. Plant diseases are mostly caused to leaves by attacks of viruses, fungi, and bacteria. As discussed in the above examples, one simple approach is to find the disease in the

early stage, so that necessary control steps could be taken to minimize the hazard. There are several successful studies proving the use of image processing to efficiently detect and classify leaf diseases in plants [30]. Yadav et al. worked on the same approach, using image processing coupled with SVM to detect and classify diseases in grape leaves [31].

For this study, the images data of healthy leaves were combined and leaves infected by Powdery Mildew and Downy Mildew. In such a method, it is important to preprocess the image data to filter any noise in the background and suppress distortions to make all images consistent. After preprocessing, the authors performed image segmentation by separating images into homogenous regions as per certain features. K-means clustering was employed for image segmentation. Clustering methods group similar data from large sets of data into much smaller clusters. Once the diseased region in the leaves is detected using segmentation by K-means clustering, colour, and texture features were extracted. Total nine texture features were considered for this study, namely; (a) contrast, (b) uniformity, (c) maximum probability, (d) homogeneity, (e) diagonal variance, (f) difference variance, (g) entropy, (h) inverse difference, (i) and nine colour features. Finally, Linear Support Vector Machine (LSVM) was employed for the classification of leaf diseases. Classification accuracy of 88.89% was achieved for examined diseases.

6.3.8 Hyphae Detection in Fungal Keratitis Images

Some fungal organisms also cause infection in the human cornea and that results in conditions like Fungal Keratitis [32]. This inflammatory infection can cause blindness in most cases, thus accurately diagnosing Fungal Keratitis is critical [33]. Fungal Keratitis can be clinically diagnosed by methods like microscopic examination of cornea scrapings, slit-lamp examination, polymerase chain reaction (PCR), tissue biopsy, fungal culture, and confocal microscopy [34]. Out of all these techniques, confocal microscopy is based on the optical imaging approach, where hyphae detection can be done by analyzing the cornea images from confocal microscopy. The challenging part is to accurately find the abnormal cornea images with hyphae from the normal cornea images with nerves. To address this issue, Ren J. et al., proposed a novel method to aid physicians in diagnosing this condition by automatically detecting hyphae after processing images from confocal microscopy [35].

The method is composed of two important steps: (a) classification of texture in the input images and (b) detection of hyphae. Adaptive robust binary pattern (ARBP) is evolved as a contrast stretching algorithm and is sub-regional in character. This method is used to extract the texture features from the images, which are eventually fed into the SVM classification model to classify between abnormal and normal images. In the downstream pipeline, the targets are further enhanced by binarization, where hyphae detection was achieved by a line segment detector algorithm. Finally, the severity of infection is quantitatively evaluated by the hyphal density. The

authors were able to show the effectiveness of improvement of the AMBP in the extraction of texture features from images.

6.3.9 *Plant Diseases Classification Using SVM and ANN (Artificial Neural Network)*

With the advent of computational techniques, there has been a sudden increase in its application in areas of agriculture and horticulture. In simple words, the computational techniques are explored to develop a decision support system which can be used as an expert system for decision making for agricultural yield or plant protection. Byadgi AS. et al. showed a study where colour and texture features were used to build a classification model to recognize diseases in plants [36]. The method proposed by authors in this work starts with acquisition of images, followed by preprocessing the images, features extraction and selection, and finally building the classification model.

Because most of plant diseases are caused by five factors, namely: (a) viral, (b) fungal, (c) bacterial, (d) nematodes, and (e) deficiency [37], authors used 900 sample images as test data. This set consisted of 150 images for each category mentioned above, and 150 images from normal plants which were not affected, thus resulting in six classification categories. In the work, colour and texture features were extracted, where colour features were based on RGB and HSI colour models, while the texture features were based on Gray level cooccurrence matrices (GLCM) [38, 39]. For classification, authors used studied the behaviour of SVM and ANN in the context of the suitability of classifiers by employing ANN-based classifiers using multilayer feed-forward with back propagation and SVM based classifiers for recognizing plant disease in the images. In this comparative study, SVM yielded maximum accuracy of 98% using joint colour and texture features, showing that SVM classifiers are more suitable for the identification and classification of plant diseases that affect agricultural and horticulture crops.

6.3.10 *A Hybrid Combination of Multiple SVM Classifiers for Automatic Recognition of the Damages and Symptoms on Plant Leaves*

Benazoun A. et al. described in their work how image recognition was addressed to automatically recognize the disease symptoms and damages in plant leaves using a hybrid combination of three SVM classifiers [40]. The proposed system was based on the serial combination of two classifiers, which is used in parallel with an individual classifier [41].

In the work, the test dataset consisting of images were composed of two categories; symptoms of three fungal diseases namely, (a) Early blight, (b) Late blight, and (c) Powdery mildew and damage by three pest insects namely, (d) Leaf miners, (e) Thrips, and (f) Tuta abosluta. To improve the quality of the data, images were preprocessed, which included filtering and resizing. This is a crucial step for efficient segmentation and analysis, which are further steps in the proposed method. In the feature extraction step, segmented images were represented as the feature vector. Texture, colour, and shape of plant leaves were features included in this study to classify damage and symptoms in the image data. The serial architecture handled the colour-based classification of images. It dealt with the damages and/or symptoms, taking into consideration the similarity or nearest colour belonging to the same class. While the second classifier further classified similar colours based on texture and shape. In this comparative study, an overall recognition rate of 93.9% was achieved, which was better than other existing methods. In conclusion, the authors recorded that the proposed method can easily overcome the classification problems by other methods and it can be strategically used for phytosanitary and diagnosis for recognition of infected plants using image data.

6.3.11 Machine Learning of Protein Interactions in Fungal Secretory Pathways

Magnaporthe grisea (M.grisea) causes excessive destruction of rice crops. Protein-protein interactions (PPIs) of rice and M.grisea is the key factor of this disease. Protein-protein interaction study and machine learning based prediction would be most useful in finding ways to counter this destruction. Biswajit Karan et al, applied the machine learning algorithms to address the problem of PPI predictions [42]. From whole rice data base these authors [42] extracted a total 12091 positive candidates and rest 54062 negative candidates. For the blast Fungi M.Grisia they filtered 6151 positive examples and 5236 negative examples. For domain information they used 20 amino zacid features and seven conjoint triad (CTD) features. Employing SVM as the classifier they obtained 89% accuracy with CTD features and equally good accuracy of 88% with amino acid feature.

6.3.12 Fungal Adhesins and Adhesin Like Proteins Predictions

The cell surface interacts with the outside environment, which is important to carry out cellular functions. This interaction involves macromolecules such as Adhesins. They are present in a wide variety of microorganisms such as fungi, bacteria etc. The key role of adhesins is to help adhesion of pathogens to host cells, which is followed

by colonization of the microorganisms in the hosts. One of the most significant roles played by adhesins includes the formation of a biofilm, which acts as a defensive shield against hazardous surroundings and aids in prolonging the infection [43]. Identification and characterization of fungal adhesins have been explored less in contrast with bacterial adhesins. Insights on sequence characterization of the fungal adhesins might open new avenues to understand the process where they play a critical role in pathogenesis. Thus, research efforts are directed towards exploring computational methods to develop a rapid and effective approach to characterize and find fungal adhesins.

Nath A. proposed a method to accurately predict the fungal adhesins [44]. His work also includes the identification of consensus molecular signatures present in fungal adhesins. Dataset for this study consisted of 75 fungal adhesins and 341 non-adhesin protein sequences, which were processed to extract sequence-based and evolutionary features. These features include Amino acid composition (AAC), Dipeptide counts (DPC), Property group composition (PGC), Physicochemical-2-grams (P2G), Atomic composition (ATC), Physicochemical properties (PCP) and features based on evolutionary information. In this comparative study, different algorithms were employed to find the best performing machine learning algorithms which can effectively predict adhesin protein. These methods include (a) Naïve Bayes, (b) SVM with Sequential minimization optimization implemented with Poly-K, RBF, and PuK kernels, (c) Bagging with REPTree, (d) Real Adaboost, and (e) Adaboost with decision stumps. The author noted that the SVM kernel-based approaches viz., RBF, PuK, and SMO-PolyK, performed better than other algorithms. Based on the leave, one outperformance measure accuracy of 94.9% was obtained on cross validation procedure and a validation set accuracy was achieved up to 98.0%.

6.3.13 Computational Prediction of Antifungal Peptides

Most of the living organisms show innate shield immunity gotten during lifecycle and its strength depends on serval factors. In humans, patients with weak immunity are prone to fungal infections, which can lead to dreadful consequences. Invasive fungal infections cause high morbidity and mortalities. Despite tremendous advances in the field of antibiotics research, Candida, Aspergillus, Pneumocystis, and Cryptococcus spp. [45] cause more than a million deaths every year [46]. To combat the twin problems of fungal infections and drug resistance, peptide-based therapeutics are actively under consideration. Antimicrobial peptide-based therapeutics have gained momentum in recent years with several formulations available in the market. For fungal infections, more specific actions can be expected from antifungal peptides. It is thus contingent to focus research on the discovery of novel antifungal peptides with high activity.

Employing machine learning based methods is a cost-effective way of identification of antifungal peptides. Several methods have been developed in the past for

the identification of antifungal peptides; these include template-based methods, docking simulations, and sequence-based methods. These have been listed in the work of Agrawal et al. [47]. In sequence-based methods, it is possible to extract several fixed-length attributes which include amino acid, dipeptide and K-mer frequencies. Apart from this, several physiochemical properties can be employed as attributes. Information about Position specific scoring matrix (PSSM) profiles can also be included in the feature list.

Agrawal et al. [47] have developed different models for the prediction of antifungal peptides. These include amino acid & dipeptide composition features, binary profile, and terminal residues-based attributes. Their Support Vector Machines compositional features-based method yielded 88.78% training accuracy and independent test accuracy of 83.33%. Further, their terminal residues based binary patterns features yielded 84.88% and 84.64% accuracies respectively on training and test sets and validation dataset. They have developed a mobile app, a standalone and a user-friendly web server.

6.3.14 A Novel Method of Annotation of Antifungal Peptides Based on Distributed Representation of Protein Sequences

One of the methods in classical Natural Language Processing (NLP) in text mining jobs is to convert the text into frequency vectors. While this is quite successful, it has its own drawbacks. This representation is unable to capture the meaning of the words under a given context, as all vectors are orthogonal to each other. The introduction of distributed representation of words has introduced a revolutionary change in text mining. The main idea being the meaning of a word under a given context is given by surrounding words that often occur close to it. In this method, a dense representation of words of a predetermined size is created to capture the meaning of the word and words with similar meanings will appear closely in a higher dimensional embedding space. An extremely popular algorithm knowns skip-gram model by Mikilov et al. [48] is the front runner of the distributed representation of words. The idea behind skip-gram representation is to predict the context words surrounding it by using the word as input in a shallow network. Using this unsupervised learning, embedding vectors of certain dimensions can be created for a large vocabulary of words. The most informative embedding vectors are obtained the prediction loss. Asgari and Mofrad [49] first applied the word2vec model for distributed representations annotating protein sequences and named it as ProtVec (Protein Vectors) model. They first extracted embedding vectors from the entire SWISSPROT experimentally verified list of sequences using the skip-gram model. They used these embedding vectors for handling multiple protein annotation tasks. They reported excellent accuracy. This method uses only sequence information and achieves superior performance.

6.3.15 *Identification of Antifungal Using Distributed Representation of Sequences*

As a first step, the complete set of experimentally verified sequences are first converted to K sets of shifted non-overlapping K-grams. The skip-gram embedding model is then used to learn the embeddings with a setting like that of Asgari and Mofrad [49]. We employed word sizes of three and five and a context size of 25 to build the unsupervised learning model to create embedding vectors; the size created varied from 100 to 500 vectors. We used the same dataset used by Agrawal et al. [47] which has 1168 antifungal peptides as positive dataset obtained from the DRAMP database and 1168 antimicrobial peptides other than antifungal as negative dataset obtained from the DRAMP database. We used the embedding vectors specific to the training set as input to the SVM classifier and built the training model. The training model was used to validate a set of test sequences having 291 antifungal peptides as positive dataset obtained from DRAMP database and 291 antimicrobial peptides other than antifungal as negative dataset obtained from DRAMP database.

Our results show that the ProtVec model with a vector size of three hundred, K-mer word size of three and context vector size of 25 achieved particularly reliable performance with 86% validation accuracy. Our results show that the distributed representation of Antifungal peptides is a very efficient way of robust annotation of antifungal peptides.

6.4 Illustration of Use of SVM in Fungal Bioinformatics

The below table enlists all the examples that illustrate the use of SVM in Fungal bioinformatics:

Title	Method	Dataset	Performance
Haar wavelet features based fungal disease detection [3] (Published in 2018)	K-NN classifier and SVM classifier were used for classification Features used: First-order features Haar wavelet based GLCM features	Images of maize leaf are captured using digital camera keeping uniform brightness and a constant distance of two feet. All images were in PNG format. Totally, two hundred images are captured: 50 healthy 50 common rust 50 northern leaf blight 50 images with both diseases present in it	k-NN for k = 5 yielded 85%accuracy and SVM-based classification gave 88%

(continued)

Title	Method	Dataset	Performance
Quality detection of pomegranate fruit infected with fungal disease [9] (Published in 2020)	Linear Discriminant Analysis (LDA), Back Propagation Neural Network (BPNN) and Support Vector Machine (SVM) methods were applied and compared as linear and non-linear analysis methods for detection Features used: Metal Oxide Semiconductor (MOS) biosensors data, which watch and record the changes in different food products during storage time and assessment of shelf life and spoilage	Data was collected after screen 100 pomegranates of 'Robab' genotype, similar in size and shape. Based on the amount of infestation samples were divided into five groups including 0 (healthy samples), 25%, 50%, 75%, and 100% infected with Alternaria specie of fungi	As a prediction method, Back propagation neural network showed higher accuracy of 100% in the detection of 0%, 25%, 50%, 75%, and 100% infected pomegranates
Fungal rust disease detection in Pea Plant (Pisam sativam) [18] (published in 2018)	The fungal disease symptoms were segmented using Gaussian filters, long transform and 2D-discrete wavelet transform used for feature extraction of infected leaves Features used: Discrete wavelet transform which is a technique for two dimensional decomposition of image with respect to Haar wavelet	The leaves samples were collected from the field and prepared for the dataset	Finally, support vector machine classifier yielded an accuracy of 89.60% to detect the leaf disease of pea plant
Volatomic visualization for fungal infection detection on storage Jasmine [25] (Published in 2020)	Chemometric methods including PCA, LDA, SVM, and PLS regression were used to analyze the complicated data qualitatively and quantitatively from the electronic sensor array Features used: Volatomic profiles and volatile metabolic	Fungal infected rice was used in this study. The number of yeast and mould in brown rice before inoculation was less than2 log CFU/g. A non-pathogenic aspergillus strain was used to avoid the occurrence of mycotoxins in the laboratory environment which can be	All methods achieved quite good and robust performance

(continued)

Title	Method	Dataset	Performance
	compounds of fungal infected Jasmine brown rice	harmful to students and researchers in the laboratory	
Rapid discrimination of fungal species by the colony fingerprinting [27] (Published in 2019)	Fungal Species classification using SVM and Random Forest Features used: Hyphae and their branches count and distribution of intensity across the hyphae on the lens-less images	Deployment of lens free imaging system to capture micro sized fungi colonies	Rapid identification and fingerprinting within 2 days and faster than MALDI-TOF-MS. and related methods
SVM classifier based grape leaf disease detection [31] (Published in 2016)	The diseased region is found using segmentation by K-means clustering, then both colour and texture features are extracted. Finally, the classification technique is used to detect the type of leaf disease Features used: Nine texture features-contrast uniformity maximum probability homogeneity diagonal variance difference variance entropy inverse difference and nine colour features	Images data was gathered of healthy leaves and leaves infected by Powdery Mildew and Downy Milde	Classification accuracy of 88.89% was achieved for examined diseases
Hyphae Detection in Fungal Keratitis Images [35] (Published in 2018)	SVM model was used to classify the normal and abnormal images Features used: Adaptive Robust Binary Pattern (ARBP)	183 normal images and 195 abnormal images, which were collected in vivo using Heidelberg engineering HRT-3 confocal microscopy	With SVM classifiers, texture analysis methods can achieve accuracies more than 90%
Plant Diseases classification Using SVM and ANN [36] (Published in 2016)	SVM and ANN classifiers Features used: Colour features based on RGB, HSI colour models and texture features based on Gray level cooccurrence matrices (GLCM)	900 sample images: 150 samples of each class mentioned below: Fungal, bacterial, viral, nematodes, deficiency and normal (not affected)	SVM yielded maximum accuracy of 98% using colour and texture features

(continued)

Title	Method	Dataset	Performance
A hybrid combination of multiple SVM classifiers for automatic recognition of the damages and symptoms on plant leaves [40] (published in 2016)	Hybrid combination of three SVM classifiers: A serial combination of two classifiers, which is used in parallel with an individual classifier Features used: Texture, colour, and shape of plant leaves	Images were gathered based on two categories: Symptoms: (a) early blight, (b) late blight, and (c) powdery mildew Damages: (d) leaf miners, (e) Thrips, and (f) Tuta abosluta	Overall accuracy of 93.9% for disease recognition was seen
Hyperspectral imaging enabled electronic nose for fungal contamination identification in strawberries [26] (Published in 2019)	Kernel function of SVM was radial basis function (RBF) and important parameters 'cost' and 'λ' were optimized by the grid search procedure Features used: Spectral profiles	The spectral data and images of strawberries were acquired using a lab-scale visible/near-infrared HSI system	Colony forming units were effectively found with a correlation coefficient value of 0.925
Fungal adhesins and adhesins like proteins prediction [44] (Published in 2019)	Naïve Bayes (NB), SVM with sequential minimization optimization (implemented with Poly-K (SMO-PolyK), RBF (SMO-RBF) and PuK (SMO-PuK) kernels), Bagging with REPTree as the base learner, Real Adaboosting(RAB) and Adaboosting (AB) with decision stumps as the base learners Features used: Sequence-based and evolutionary features: Amino acid composition (AAC) Dipeptide counts (DPC) Property group composition (PGC) Physicochemical-2-grams (P2G) Atomic composition (ATC) Physicochemical properties (PCP) Features based on evolutionary information	75 fungal adhesins and 341 non-adhesin protein sequences	Obtained robust performance with leave one out error 0f 5.9% and test error of only 2%

6.5 Concluding Remarks

In this chapter, we have discussed in detail the utility in the usefulness of SVM in fungal biology. We have highlighted the robustness of the algorithm in several predictions and case studies. These include among others recognition of damage and disease symptoms in plant leaves, detection of fungal infections, and contaminations in fruits prediction of antifungal peptides etc. In these recent applications, it is evident that SVM has performed very well and can be used as a reliable and robust paradigm not only in fungal biology but also in other bioinformatics applications.

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Chapter 7
Real-Time Quantitative PCR Assay for the Assessment of Uncultured Zoosporic Fungi

Télesphore Sime-Ngando and Marlène Jobard

Contents

7.1 Introduction

Molecular surveys of microbial eukaryotes have revealed overlooked uncultured environmental fungi with novel putative functions [1–3], among which zoosporic forms (i.e., chytrids) are the most important in terms of diversity, abundance, and functional roles, primarily as infective parasites of phytoplankton [4, 5] and as valuable food sources for zooplankton via massive zoospore production, particularly in freshwater lakes [6–8]. However, due to their small size (2–5 μm), their lack of distinctive morphological features, and their phylogenetic position, traditional microscopic methods are not sensitive enough to detect fungal zoospores among a mixed assemblage of microorganisms. Most chytrids occupy the most basal branch of the kingdom Fungi, a finding consistent with choanoflagellate-like ancestors [9]. The above reasons may help explain why both infective (i.e., sporanges) and disseminating (i.e., zoospores) life stages of chytrids have been misidentified in

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previous studies, respectively, as phagotrophic sessile flagellates (e.g., choanoflagellates) and as "small undetermined" cells. These cells often dominate the abundance of free-living heterotrophic nanoflagellates (HNFs) and are considered the main bacterivores in aquatic microbial food webs [2, 10]. Their contribution ranges from 10% to 90% of the total abundance of HNFs in pelagic systems (see review in [11]). Preliminary data have provided that up to 60% of these unidentified HNFs can correspond to fungal zoospores [12], establishing the HNF compartment as a black box in the context of microbial food web dynamics [4]. A simulation analysis based on Lake Biwa (Japan) inverse model indicated that the presence of zoosporic fungi leads to (i) an enhancement of the trophic efficiency index, (ii) a decrease of the ratio detritivory/herbivory, (iii) a decrease of the percentage of carbon flowing in cyclic pathways, and (iv) an increase in the relative ascendency (indicates trophic pathways more specialized and less redundant) of the system [13]. Unfortunately, because a specific methodology for their detection is not available, quantitative data on zoosporic fungi are missing.

Molecular approaches have profoundly changed our view of eukaryotic microbial diversity, providing new perspectives for future ecological studies [3]. Among these perspectives, linking cell identity to abundance and biomass estimates is highly important for studies on carbon flows and the related biogeochemical cycles in natural ecosystems [11]. Historically, taxonomic identification and estimation of in situ abundances of small microorganisms have been difficult. In this context, our inability to identify and count many of these small species in the natural environment limits our understanding of their ecological significance. Thus, new tools that combine both identification and quantification need to be developed. Fluorescent in situ hybridization (FISH) has been an assay of choice for simultaneous identification and quantification of specific microbial populations in natural environments [14, 15]. However, this technique is limited because of (i) the relatively low number of samples that can be processed at a time and (ii) its relatively low sensitivity due to background noise and the potentially low number of target rRNA molecules per cell in natural environments [16]. In contrast, real-time quantitative PCR (qPCR) which has been widely used to estimate prokaryotic and eukaryotic population abundances in natural ecosystems, allows the simultaneous analysis of a high number of samples with a high degree of sensitivity [15].

The main objective of this chapter is to provide, in a simplified step-by-step format, a qPCR assay for the quantitative assessment of uncultured zoosporic fungi and other zoosporic microbial eukaryotes in natural environments (cf. [15]), together with practical advice on how to apply the method. QPCR was recently used to estimate fungal biomass in a stream during leaf decomposition [17] and in biological soil crusts [18]. The interpretation of the semiquantitative data obtained in these studies was relatively difficult because the whole fungal community was targeted (including unicellular, multicellular, and multinuclear fungal species). Thus, an estimation of fungal density or even fungal biomass was not possible. In the following protocol, the primary targets are zoospores in liquid suspensions. Because zoospores are unicellular, qPCR data could be directly converted into cell density estimates, i.e., by multiplying semiquantitative data by the number of rDNA copies

per cell. Moreover, we designed primers targeting Rhizophidiales taxon to limit quantification bias generated by the variability in the number of rDNA copies within the eukaryotic ribosomal operon.

7.2 Materials

1. Gloves (should be worn when manipulating most of the following materials).
2. Sterile distilled water.
3. 0.6 μm pore size polycarbonate filters.
4. Filtration columns.
5. Sodium dodecyl sulfate (SDS).
6. Proteinase K.
7. TE buffer – 10 mM Tris–HCl pH 7.5 (25 °C), 1 mM EDTA.
8. NucleoSpin Plant kit® (Macherey-Nagel) with silica-membrane columns and the materials for running the provided protocol from the manufacturer.
9. Molecular-biology-grade agarose.
10. Ethidium bromide: because suspected as a mutagen, particular care should be taken when handling (consult safety data sheet).
11. Calf thymus DNA (Sigma).
12. Oligonucleotidic primers resuspended in sterile distilled water and stored at −20 °C (*see Note 1*).
13. SYBR Green (Sigma).
14. dNTPs: a mixture of dATP, dCTP, dGTP, and dTTP (10 mM of each), stored at −20 °C.
15. Thermostable DNA polymerase and reaction buffer supplied by the manufacturer. To avoid nonspecific amplicon, use hot-start (e.g., HotStarTaq, Qiagen).
16. Vortexer.
17. Centrifuge.
18. Water bath.
19. Horizontal electrophoresis machine.
20. TBE buffer: 50 mM Tris, 50 mM boric acid, 1 mM EDTA, diluted when needed from a 50x stock solution.
21. Spectrophotometer – we use NanoDrop (NanoDrop Technologies, Inc., Wilmington, USA).
22. Disposable conical tubes (1.5 ml); PCR tube strips or plate with adhesive film and cap adapted for real-time quantitative PCR assay.
23. Thermal Cycler – we use e.g., Mastercycler ep realplex detection system (Eppendorf).
24. UV transilluminator equipped with a camera suitable for photographing agarose gels.

7.3 Methods

7.3.1 DNA Extraction and Purification

Collect zoosporic organisms onto 0.6 μm pore size polycarbonate filters (after the removal of the algal host by prefiltration when only zoospores are targeted) (*see Note 2*).

1. For cell disruption, incubate the filters in 560 μl of a buffer containing 1% SDS and 1 $mg.ml^{-1}$ proteinase K in TE buffer for 1 h at 37 °C in a water bath (*see Notes 3 and 4*).
2. For DNA purification, use the silica-membrane columns provided with the NucleoSpin Plant kit® (Macherey-Nagel), following the instructions from the manufacturer (*see Note 4*).
3. Visualized the integrity and yield of the extracted genomic DNA in a 1% agarose gel stained with 0.3 μg ml^{-1} of ethidium bromide solution (Sigma), using a UV transilluminator and a photograph. For this, (i) heat (45 s using a microwave oven) a mixture of agarose in 1x TBE buffer, (ii) leave it to cool on the bench for 5 min down to about 60 °C before adding ethidium bromide (i.e., to avoid vapor formation), (iii) mix and pour into suitable gel grey with a comb and leave to set for at least 30 min, (iv) remove the comb and submerge the gel to 2–5 mm depth in an electrophoresis tank containing 1x TBE buffer, (v) transfer DNA sample aliquots (i.e., 2 volumes of sample and 1 volume of loading buffer), marker, and the serial dilution of 5–10 ng of calf thymus DNA (Sigma) to the wells of the agarose gel, and (vi) start the electrophoresis migration for about 30 min at 100 V.
4. Calculate DNA extract concentrations from dilutions of calf thymus DNA (Sigma), using a standard curve of calf thymus DNA versus band intensity.

7.3.2 Real-Time qPCR Assays

1. PCR mix contained SYBR Green (Sigma), 200 μM of each dNTPs, 10 pM of each primer, 2.5 units of *Taq* DNA polymerase, the PCR buffer supplied with the enzyme, and 1.5 mM $MgCl_2$. Vortex briefly (less than 10 sec) and centrifuge the mix before distributing aliquots in suitable PCR tubes (strips or plates) and place on ice.
2. Add variable quantity of DNA (we used 5 ng for our environmental freshwater samples and 10 ng for DNA from appropriate PCR negative control strains) used as template in a final volume of 25 μl (*see Note 5*).
3. Standard curve of Ct (*see Note 4*) versus DNA copy number required to calculate target copy numbers (*see Note 6*) in each reaction is generated using triplicates of PCR reactions of tenfold dilutions of linear plasmid (containing Rhizophidiales 18S rDNA insert; PFB11AU2004) ranging from 100 to 1×10^8 $copy.\mu l^{-1}$ (*See Note 7*). This number of copies was calculated using the equation:

molecules.μl^{-1} = a / (b x 660) x $6.022.10^{23}$, where a is the plasmid DNA concentration ($g.\mu l^{-1}$), b the plasmid length in bp, including the vector and the inserted 18S rDNA fragment, 660 the average molecular weight of one base pair, and $6.022.10^{23}$ the Avogadro constant [15, 19].

4. Place all tubes (i.e., samples, controls, and standards) in the real-time qPCR cycler and run the appropriate cycling program: initial HotStarTaq activation at 95 °C for 15 min, 35 cycles with denaturation at 95 °C for 1 min, annealing at 63.3 °C for 30 sec with Fchyt/Rchyt primers pair (*See Note 1*), elongation at 72 °C for 1 min, and a final additional elongation step at 72 °C for 10 min.
5. Using SYBR Green molecule, melting curves analysis can be performed immediately following each qPCR assay to check the specificity of amplification products (to confirm the absence of primer dimers or unspecific PCR products) by increasing the incubation temperature from 50 to 95 °C for 20 min.
6. Analyze the real-time PCR result with suitable software. Check to see if there is any bimodal dissociation curve or abnormal amplification plot (*see Note 5*) before calculating the initial concentration of the targeted uncultured fungal 18S rDNA (copies.ml^{-1}) in the environmental genomic DNA (*see Note 6*).

7.4 Notes

1. Consensus (universal) primers can be used to amplify regions of fungal ribosomal RNA genes. For natural waters, we have designed primers specific to chytrids using a database containing about a hundred 18S rDNA environmental sequences recovered from surveys conducted in different lakes and sequences belonging to described fungi (cf. 15). Sequences were aligned using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and the resulting alignment was corrected manually. A great proportion of the environmental chytrid sequences recovered from lakes was closely affiliated to the Rhizophidiales. Thus, Rhizophidiales-specific primers F-Chyt (sequence 5′ > 3′: GCAGGCTTACGC TTGAATAC) and R-Chyt (sequence 5′ > 3′: CATAAGGTGCCGAACAAGTC) were designed in order to fulfill three requirements: (1) a GC content between 40% and 70%, (2) a melting temperature (Tm) similar for both primers and close to 60 °C, and (3) PCR products below 500 bp (i.e., between 304 and 313 bp depending on the species considered). The absence of potential complementarities (hairpins and dimers) was checked using Netprimer (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html) and confirmed by inspection of the melting curve following the qPCR assay.
2. For targeting uncultured zoosporic fungi, zoospores are discarded from other environmental microorganisms by successive prefiltrations through 150, 80, 50, 25, 10, and 5 μm filters before being collected onto 0.6 μm polycarbonate filters. Filters can be conserved at −80 °C until DNA extraction in appropriate tubes (2 ml).

3. Other enzymes such as lyticase can be used for cell disruption, with no significant difference compared to proteinase K. However, the one-step proteinase K yields a higher amount of genomic DNA than the lyticase method and has a better reproducibility. Physical disruption procedures such as sonication or thermal shocks (i.e., freezing in liquid nitrogen and thawing) are to be avoided because of the possible degradation of DNA.
4. A standard phenol–chloroform purification procedure can also be used, but when the genomic DNA extracts are used as templates in PCR reactions, the DNA purification method using the commercial kit gave significantly better results (based on Ct, the threshold cycle during PCR when the level of fluorescence gives signal over the background and is in the linear portion of the amplified curve) than the phenol–chloroform method. Consequently, the DNA extraction method using Proteinase K and the commercial kit was selected and considered the best overall.
5. In the case of novel designed primers (*see Note 1*) for uncultured fungi, DNA from both positive and negative plasmids and different mixtures (e.g., 5%, 10%, 25%, and 50% of the positive plasmids) will be used for the optimization of the conditions (annealing temperature, cycling), cross-reactivity, the detection limit (using serial tenfold dilutions of the positive plasmids, *see Note 7*), and the amplification efficiency of the qPCR essays which should be at least 80%. Poor primer quality is the leading cause of poor PCR efficiency. In this case, the PCR amplification curve usually reaches a plateau early, and the final fluorescence intensity is significantly lower than that of most other PCRs. This problem may be solved with resynthesized primers.
6. The initial concentration of target 18S rDNA (copies.ml^{-1}) in environmental samples can be calculated using the formula: [(*a*/*b*) x *c*] / *d,* where *a* is the 18S rDNA copy number estimated by qPCR, *b* is the volume of environmental genomic DNA added in the qPCR reaction, *c* is the volume into which the environmental genomic DNA was resuspended at the end of the DNA extraction, and *d* is the volume of sample filtered from which environmental DNA was extracted.
7. In the absence of cultures, plasmids used in qPCR to construct standard curves and to optimize qPCR reactions come from genetic libraries constructed during previous environmental surveys [2]. Briefly, the complete 18S rRNA gene was amplified from environmental genomic DNA extracts using the universal eukaryote primers 1f and 1520r. An aliquot of PCR products was cloned using the TOPO-TA cloning kit (Invitrogen) following the manufacturer's recommendations. The plasmid containing the insert of interest was extracted with NucleoSpin® plasmid extraction kit (Macherey Nagel) following the manufacturer's recommendations. The 18S rRNA gene was sequenced from plasmid products by the MWG Biotech services using M13 universal primers [M13rev (−29) and M13uni (−21)]. Phylogenetic affiliation of sequences acquired was established using neighbor joining and Bayesian methods. In our case, positive plasmids contain insert affiliated to target chytrid (i.e., Rhizophidiales species) and displaying less than 2 mismatches with primers F-Chyt and R-Chyt sequences (*see Note 1*). The plasmid PFB11AU2004 (Genbank accession number

DQ244014) was selected to construct the standard curve required for qPCR. Linearized plasmids were produced from supercoiled plasmids by digestion with restriction endonuclease one-time cutting into the vector sequence. Linear plasmid DNA concentration can be determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer.

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Chapter 8
Assays for the Quantification of Antioxidant Enzymes in Fungi

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8.1 Introduction

Molecular oxygen (O_2) was introduced to the anoxic atmosphere as a by-product of photosynthesis from cyanobacteria. Additionally, O_2 generation allowed organisms to settle on the land by providing a protective filter from ultraviolet radiation, which is known as the ozone layer. In general, organisms employed O_2 in their metabolism and oxidative metabolism could be considered an evolutionary advantage of aerobic life, mainly because it yields a higher amount of ATP per catabolized sugar molecule via the glycolysis, Kerbs Cycle, and oxidative phosphorylation pathways. However, there is a detrimental side effect of oxidative metabolism, which is the generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) [1]. In physiological conditions, the concentration of ROS/RNS is maintained low by endogenous antioxidants and antioxidant enzymes, while the condition when these defenses fail to decompose ROS/RNS is referred to as oxidative stress. Oxidative stress has been implicated in several physiological phenomena such as aging, fungal metamorphosis [2], and pathological conditions (i.e., neurodegenerative diseases) [3].

Antioxidant enzymes are important parameters of the cellular antioxidant defense mechanisms, and their role focuses on (i) the removal of ROS, (ii) the detoxification of xenobiotic substances, and (iii) the repair of cellular damages (i.e., regeneration of reduced thiols and reducing power). In relation to the elimination of ROS, the primary component of oxidative stress is superoxide radical ($O_2^{\cdot -}$). The enzyme that specifically scavenges this reactive species, superoxide dismutase (SOD), was discovered by Fridovich [4]. SODs are highly conserved enzymes that rely on manganese, zinc, iron, or nickel [5] to catalyze the dismutation reaction of two $O_2^{\cdot -}$ molecules to generate one O_2 molecule and one molecule of hydrogen peroxide (H_2O_2). Hydrogen peroxide is well known for its signaling role as well as being a radical initiator via the metal-catalyzed Fenton reaction, which effectively leads to the generation of hydroxyl radical, another potent ROS. Therefore, scavenging H_2O_2 is another major task for the enzymatic antioxidant defense, and this is achieved by the catalase enzyme, which reduces one molecule of H_2O_2 to water and oxidizes a second one to O_2. Another category of enzymes that decompose H_2O_2 and organic hydroperoxides, in general, are the peroxidases, which consume reducing power from a proton donor. Catalases and peroxidases are also conserved across species, and their activity relies on metal catalytic centers.

As mentioned earlier, oxidative stress is not only mediated by the generation of ROS but also by imbalances triggered in relation to redox balances such as the status of thiol groups. This balance is better known as the thiol redox state, and when oxidized moieties overpower the reduced forms, antioxidant enzymes may replenish their reduced forms at the expense of reducing power. Consumption of reduced thiols (i.e., glutathione, cysteine) may be related to detoxification of toxicants by glutathione-S-transferases, which inactivate xenobiotics by coupling them with glutathione or via glutathione peroxidases which consume glutathione as reducing power to decompose hydrogen and other peroxides. This expense of reduced glutathione is regenerated by glutathione reductase which consumes NADPH

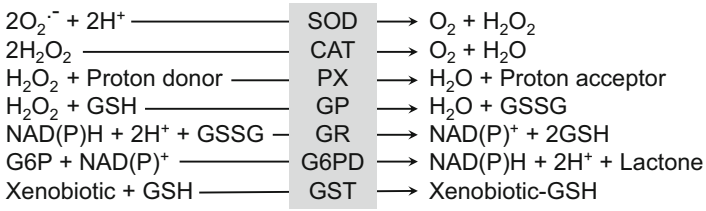


Fig. 8.1 Reactions catalyzed by antioxidant enzymes. SOD: superoxide dismutase, CAT: catalase, PX: peroxidase, GP: glutathione peroxidase, GR: glutathione reductase, G6PD: glucose-6-phosphate dehydrogenase, GST: glutathione-S-transferase

which in turn is regenerated by glucose-6-phosphate dehydrogenase an enzyme of the pentose phosphate pathway.

All the aforementioned network of enzymatic reactions reveals the complexity (Fig. 8.1) of enzymatic defense mechanisms and the necessity of specific protocols to quantify all these parameters. This chapter will present simple biochemical methods for their accurate quantification and discuss the impact of oxidative stress in fungal physiology. Additionally, an initial protocol for homogenization and protein quantification of fungal tissues has also been included.

8.2 Materials and Methods

8.2.1 Materials

3-amino-1,2,4-triazole, ammonium sulfate, bovine serum albumin (BSA), Coomassie Brilliant Blue G-250 (CBB), cummene hydroperoxide (CumOOH), glucose-6-phosphate, disodium hydrogen phosphate (Na_2HPO_4), ethanol, ethylenediaminetetraacetic acid (EDTA), hydrochloric acid (HCl), horseradish peroxidase, hydrogen peroxide, 1-chloro-2,4-dinitrobenzene (CDNB), NADH, NADPH, NAD+, NADP+, *o*-dianisidine, oxidized glutathione (GSSG), reduced glutathione (GSH), riboflavin, phenylmethylsulfonyl fluoride.

8.3 Methods

8.3.1 Fungal Tissue Homogenization

In general, for biochemical assays, it is preferred to avoid substances that may interfere in the assays to be performed afterward. Therefore, avoiding detergents, thiol reductants (e.g., β-mercaptoethanol or dithiothreitol), metals, organic solvents, acids, or keeping their concentrations to the minimum is advised. Using metal

chelators, such as EDTA (at 1 mM) and protease inhibitors (e.g., 0.5 mM phenylmethylsulfonyl fluoride; PMSF), are also potential precautions for the preservation of the samples to avoid artificial oxidation. A phosphate (50 mM) buffer, pH 7.2, supplemented with the aforementioned is a preferable homogenization buffer. Furthermore, hard fungal tissues contain rigid cell walls and are preferably first homogenized in liquid nitrogen (e.g., using a mortar and a pestle) to generate a fine powder form. Following, phosphate buffer is added, and the tissues are homogenized on ice using a Potter-Elvehjem type glass-glass or a Teflon homogenizer or any other mechanical disruption available. The crude homogenate obtained needs to be cleaned from cellular debris by centrifugation at 20,000 *g* for 10 min at 4 °C, and the clear supernatant is collected for analysis. At this step, it is possible to concentrate proteins by precipitation. In this case, proteins are precipitated in their native (functional) form with ammonium sulfate by bringing the clear supernatant to 90% ammonium sulfate (by dissolving solid ammonium sulfate) and incubating overnight at 0 °C. Proteins are precipitated by centrifugation at 20,000 *g* for 10 min at 4 °C and solubilized in a minimum volume of buffer or water. If needed, ammonium sulfate can be removed by dialysis in another step.

Homogenization buffer: 50 mM Na_2HPO_4, 1 mM EDTA, 500 μM PMSF, 0.5% ethanol, pH 7.2: Dissolve 0.73 g $Na_2HPO_4.2H_2O$, 0.03 g EDTA, in 80 ml ddH_2O and adjust pH to 7.2. Dissolve 7 mg PMSF in 0.4 ml ethanol and add it to the phosphate buffer solution.

8.4 Protein Quantification

The assay is based on the electrostatic reaction of proteins with the Coomassie Brilliant Blue G-250 (CBB) reagent and is presented in a rapid sensitive microplate method [6].

2 M HCl: Dilute the 37% (or 12 M) HCl six-fold with ddH_2O, by mixing 250 ml ddH_2O with 50 ml 12 M HCl under stirring.

CBB: Dissolve 60 mg CBB in 100 ml 2 M HCl. After stirring for 40 min, centrifuge at 20,000 *g* for 5 min at room temperature or alternatively, filter under vacuum or with a syringe to remove any undissolved dye particulates. This solution is stable for 2 months kept light-protected.

CBB:2 M HCl (1:1): Prepare fresh by mixing 1 volume of CBB with 1 volume of 2 M HCl.

Bovine serum albumin (BSA): Prepare a stock of 1 mg BSA/ml ddH_2O and dilute with ddH_2O to 20 μg BSA/ml. Prepare a series of dilutions of standards for the linear standard curve according to Table 8.1.

Samples (and standards, Table 8.1) are assayed for protein concentration according to Table 8.2.

Table 8.1 BSA linear standard curve. The volumes are in μl

BSA final μg/ml	2	4	6	8	10	12	14	16	18	20
20 μg BSA/ml	50	100	150	200	250	300	350	400	450	500
ddH_2O	450	400	350	300	250	200	150	100	50	0

Table 8.2 Protein quantification assay conditions. The volumes are in μl

Reagents	RB	S
Sample appropriately diluted in ddH_2O or BSA standard in ddH_2O	–	200
ddH_2O	200	–
CBB:2 M HCl	50	

Incubate mixtures for 10 minutes at room temperature and measure absorbance at 610 nm. The net absorbance is derived from the absorbance difference of Sample (S) minus Reagent Blank (RB), and the following is converted to protein (BSA) concentration equivalents using the corresponding standard curve (Table 8.1).

8.5 Antioxidant Enzymes Related to the Decomposition of ROS

8.5.1 Catalase (CAT) Activity

The activity of catalases is measured by kinetics for either the decomposition of hydrogen peroxide photometrically or the production of oxygen using an oxygenometer [7]. For confirmation that the measured kinetics is a result of only catalase activity, its specific inhibitor aminotriazole can also be included as an additional control (not showing any kinetic change) for the oxygenometric measurements (since aminotriazole absorbs).

50 mM Na_2HPO_4 pH 7.2: Dissolve 0.71 g Na_2HPO_4 in a final volume of 80 ml and adjust pH at 7.2.

5xH_2O_2: Dilute the concentrated 11 M (or 30%) with phosphate buffer appropriately in order to have an absorbance of ~0.3 when using 50 μl in 250 μl reaction volume. Use this solution for the photometric version as most spectrophotometers have a linear absorbance range to perform kinetics to monitor even small changes.

2 mM H_2O_2: Dilute the concentrated 11 M (or 30%) solution to 2 mM with phosphate buffer. Use this solution for the oxygenometer version.

0.4 M 3-amino-1,2,4-triazole: Prepare fresh by dissolving 33.6 g aminotriazole in 1 ml phosphate buffer.

For the photometric version, analyze samples by continuous kinetics for the decomposition of hydrogen peroxide follow Table 8.3.

Table 8.3 Photometric quantification of catalase activity. The volumes are in μl

Sample appropriately diluted in 50 mM phosphate buffer, pH 7.2	200
H_2O_2	50

Table 8.4 *o*-dianisidine standards. The volumes are in μl

o-dianisidine final μM	1	2	3	4	5	6	7	8	9	10
μl from 10 μM *o*-dianisidine stock	50	100	150	200	250	300	350	400	450	500
50 mM phosphate buffer, pH 7.2	450	400	350	300	250	200	150	100	50	0

Incubate at room temperature and measure absorbance at 340 nm with continuous kinetics to calculate the rate. The final result will be expressed as the rate per mg protein of the sample. When performing the assay photometrically, the production of oxygen will generate bubbles which will account for absorbance, therefore, only the linear part of the measurements should be used. Finally, for the oxygenometer version, catalase activity can be also confirmed by its specific inhibitor aminotriazole by preincubation with 10 mM aminotriazole before the addition of hydrogen peroxide to initiate the reaction and measure oxygen production.

8.5.2 Peroxidases (Px) Activity

Nonspecific peroxidase activity is measured by the production of a colorful oxidized product resulting from the oxidation of its reduced form upon consumption of hydrogen peroxide. There are several substrates for peroxidases, and here, we present a photometric method based on the enzymatic oxidation of *o*-dianisidine [7].

50 mM Na_2HPO_4 pH 7.2: Dissolve 0.71 g Na_2HPO_4 in a final volume of 80 ml and adjust pH at 7.2.

10 mM *o*-dianisidine: Prepare fresh by dissolving 3.2 mg *o*-dianisidine in 1 ml phosphate buffer. The solution must be kept light protected because *o*-dianisidine is photosensitive. Prepare a series of dilutions of standards for the linear standard curve according to Table 8.4.**5 mM H_2O_2:** Dilute the concentrated 11 M (30%) solution to 5 mM with phosphate buffer.

Horseradish peroxidase: Dissolve 1 mg in 5 ml and use it to create a standard curve of oxidized *o*-dianisidine.

Perform the reaction with HRP to generate the standard curve of *o*-dianisidine as follows (Table 8.5):

Table 8.5 *o*-dianisidine linear standard curve. The volumes are in μl

Reagents	RB	St
50 mM phosphate buffer, pH 7.2	200	–
o-dianisidine standard in 50 mM phosphate buffer, pH 7.2	–	200
Horseradish peroxidase	25	
5 mM H_2O_2	25	

Table 8.6 Peroxidase activity assay. The volumes are in μl

Sample appropriately diluted in 50 mM phosphate buffer, pH 7.2	200
5 mM H_2O_2	25
10 mM *o*-dianisidine	25

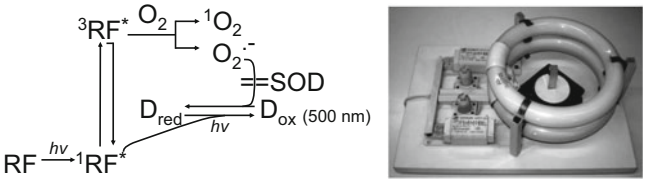


Fig. 8.2 Mechanism of SOD-inhibited reduction of oxidized *o*-dianisidine and light apparatus used when volumes are scaled up for cuvettes

Incubate at room temperature for 15 min and measure absorbance at 560 nm. Express the standard curve in moles of oxidized *o*-dianisidine acid in 200 μl sample volume.

For analyzing samples, use end point kinetics (at 15 minutes' incubation) following Table 8.6.

Incubate at room temperature for 15 min and measure absorbance at 560 nm. Calculate the moles of oxidized *o*-dianisidine using the corresponding standard curve and following calculate the rate. The final result will be expressed as a rate per mg protein of the sample.

8.5.3 Superoxide Dismutase (SOD) Activity

Superoxide dismutase activity assays use a superoxide radical generator and a scavenger molecule which is used to monitor the SOD-inhibitable scavenging activity of the sample [8]. The assay presented is based on the SOD-inhibited reduction of oxidized *o*-dianisidine. The latter results from the reaction of its reduced form by the photochemically sensitized riboflavin. This reaction is in competition with the reaction of the formation of reduced dianisidine from oxidized dianisidine and superoxide radical generated photochemically by riboflavin (Fig. 8.2) [9]. Thus, SOD increases the rate of the photooxidation of dianisidine due to the catalytic

Table 8.7 Superoxide dismutase activity assay. The volumes are in μl

Reagents	B1	B2	SB	S
Sample appropriately diluted in 50 mM phosphate buffer, pH 7.2			200	200
50 mM phosphate buffer, pH 7.2	225	200	25	
10 μM riboflavin	25	25	25	25
10 mM *o*-dianisidine		25		25

scavenging of superoxide radicals that would, otherwise, nullify the overall dianisidine photooxidation by reducing an intermediate dianisidine oxidation product (absorbing at 500 nm).

50 mM Na_2HPO_4 pH 7.2: Dissolve 0.71 g Na_2HPO_4 in a final volume of 80 ml and adjust pH at 7.2.

10 mM *o*-dianisidine: Prepare fresh by dissolving 3.2 mg *o*-dianisidine in 1 ml phosphate buffer. The solution must be kept light protected because *o*-dianisidine is photosensitive.

10 μM riboflavin: Prepare fresh by a 60 μM solution by dissolving 1.2 mg riboflavin in 50 ml phosphate buffer. Dilute the 60 μM solution six-fold with ddH_2O, by mixing 50 ml ddH_2O with 10 ml 60 μM riboflavin.

For analyzing samples, use end point kinetics (after incubation for 5 minutes) following Table 8.7.

Incubate at room temperature under light for 5 min and measure the absorbance of the samples and blanks at 500 nm. The final sample net absorbance is derived from the absorbance difference of Sample (S) minus Sample Blank (SB) and subtracted the difference of Blank 2 (B2) minus Blank 1 (B1), and the following is converted to moles of oxidized *o*-dianisidine using the corresponding standard curve (from the peroxidase assay) and following the rate. The final result will be expressed as a rate per mg protein of the sample.

8.6 Thiol Redox State Related Enzymes

8.6.1 Glutathione-S-Transferase (GST) Activity

Glutathione-S-transferases are enzymes that detoxify xenobiotic compounds by coupling them with reduced glutathione (GSH) and their activity can be measured by continuous kinetics of the product formed (S-DNP-GS) measured at 340 nm (Fig. 8.3) [10, 11].

50 mM Na_2HPO_4 pH 7.2: Dissolve 0.71 g Na_2HPO_4 in a final volume of 80 ml and adjust pH at 7.2.

1.5 mM 1-chloro-2,4-dinitrobenzene (CDNB): Prepare fresh by dissolving 6.2 mg CDNB in 1 ml ethanol. Dilute 30-fold to 1.5 mM with phosphate buffer.

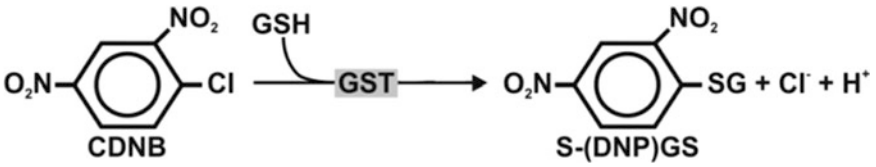
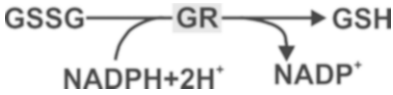


Fig. 8.3 Kinetic determination of GST activity

Table 8.8 Glutathione-S-transferase activity assay. The volumes are in µl

Sample appropriately diluted in 50 mM phosphate buffer, pH 7.2	150
750 µM CDNB 1.5 mM GSH	100

Fig. 8.4 Kinetic determination of GR activity



3 mM glutathione (GSH): Prepare fresh by dissolving 1.9 mg GSH in 1 ml phosphate buffer. Dilute two-fold to 3 mM with buffer.

0.75 mM CDNB:1.5 mM GSH (1:1): Prepare fresh by mixing equal volumes of 1.5 mM CDNB and 3 mM GSH.

Analyze samples by continuous kinetics for the production of S-DNP-GS following Table 8.8.

Incubate at room temperature and measure absorbance at 340 nm with continuous kinetics to calculate the rate. The final result will be expressed as a rate per mg protein of the sample.

8.6.2 Glutathione Reductase (GR) Activity

Glutathione reductase activity is estimated by the decrease of NAD(P)H measured by absorbance at 340 nm with continuous kinetics (Fig. 8.4) [12, 13].

50 mM Na_2HPO_4 pH 7.2: Dissolve 0.71 g Na_2HPO_4 in a final volume of 80 ml and adjust pH at 7.2.

5 mM oxidized glutathione (GSSG): Prepare fresh by dissolving 3.3 mg GSSG in 1 ml phosphate buffer.

NAD(P)H: Prepare fresh an NADH or NADPH stock in phosphate buffer. Dilute appropriately in order to have an absorbance of ~0.3 when using 25 µl in 250 µl reaction volume as most spectrophotometers have a linear absorbance range to perform kinetics to monitor even small changes.

Analyze samples by continuous kinetics for the decomposition of NAD(P)H following Table 8.9.

Table 8.9 Glutathione reductase activity assay. The volumes are in μl

Sample appropriately diluted in 50 mM phosphate buffer, pH 7.2	200
1 mM GSSG	25
NAD(P)H	25

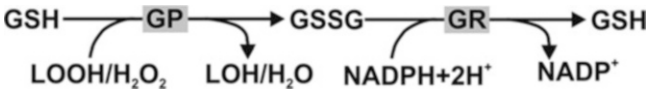


Fig. 8.5 Kinetic determination of GR activity

Incubate at room temperature and measure absorbance at 340 nm with continuous kinetics to calculate the rate. The final result will be expressed as a rate per mg protein of the sample.

8.6.3 Glutathione Peroxidase (GP) Activity

Glutathione peroxidase activity is measured by the decrease of NAD(P)H photometrically at 340 nm by continuous kinetics. The assay is using as a detection of the reaction catalyzed by glutathione reductase. Therefore, it is a system of two reactions coupled together, where the latter is in excess rate, while the reaction catalyzed by glutathione peroxidase regulates the system via the generation of GSSG by endogenous GP (Fig. 8.5) [13]. As there are two categories of enzymes with glutathione peroxidase activity, those with selenium and those without selenium in their active site, the assay detects the selenium GP when hydrogen peroxide is used as substrate and the non-selenium GP when cummene hydroperoxide is used as substrate.

50 mM Na_2HPO_4 pH 7.2: Dissolve 0.71 g Na_2HPO_4 in a final volume of 80 ml and adjust pH at 7.2.

20 mM GSH: Prepare fresh by dissolving 6.3 mg GSH in 1 ml phosphate buffer.

20 mM sodium azide: Prepare fresh by dissolving 1.32 mg sodium azide in 1 ml ddH_2O. Sodium azide can be used to block possible existing catalase (if expected to be present) in the sample when hydrogen peroxide is used as the substrate of the reaction, while it is omitted when cummene hydroperoxide is used as substrate.

1 mM cummene hydroperoxide (CumOOH): Dilute the concentrated solution of 5.5 M with ethanol to 10 mM. Dilute the 10 mM to 1 mM in phosphate buffer.

1 mM H_2O_2: Dilute the concentrated 11 M (30%) solution to 1 mM with phosphate buffer.

4 units ml^{-1} GSSG reductase: Prepare fresh by diluting the original stock reagent 100x with phosphate buffer.

NAD(P)H: Prepare fresh an NADH or NADPH stock. Dilute appropriately in order to have an absorbance of ~0.3 when using 25 μl in 250 μl reaction volume (this is

Table 8.10 Glutathione peroxidase activity assay. The volumes are in µl

Sample appropriately diluted in 50 mM phosphate buffer, pH 7.2	125
20 mM GSH	25
20 mM sodium azide/H_2O	20
1 mM H_2O_2/CumOOH	25
GSSG reductase	30
NAD(P)H	25

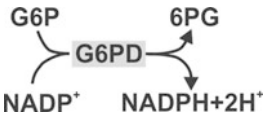


Fig. 8.6 Kinetic determination of G6PD activity

Table 8.11 Glucose-6-phosphate dehydrogenase activity assay. The volumes are in µl

Sample appropriately diluted in 50 mM phosphate buffer, pH 7.2	200
4 mM $NAD(P)^+$	25
10 mM glucose-6-phosphate	25

to allow you to perform kinetics and monitor even small changes in the linear part of your spectrophotometer).

Analyse samples by continuous kinetics for the decomposition of NAD(P)H following Table 8.10.

Incubate at room temperature and measure absorbance at 340 nm with continuous kinetics to calculate the rate. The final result will be expressed as a rate per mg protein of the sample.

8.6.4 Glucose-6-Phosphate Dehydrogenase (G6PD) Activity

Glucose-6-phosphate dehydrogenase activity is quantified by the generation of reducing power in the form of NAD(P)H by continuous kinetics at 340 nm from the reduction of $NAD(P)^+$ and glucose-6-phosphate (Fig. 8.6) [14].

50 mM Na_2HPO_4 pH 7.2: Dissolve 0.71 g Na_2HPO_4 in a final volume of 80 ml and adjust pH at 7.2.

4 mM $NADP^+$: Prepare fresh an NAD^+ or $NADP^+$ stock.

10 mM glucose-6-phosphate: Prepare fresh by dissolving 3.3 mg glucose-6-phosphate (304.31) in 1 ml phosphate buffer.

Analyze samples by continuous kinetics for the production of NAD(P)H following Table 8.11.

Incubate at room temperature and measure absorbance at 340 nm with continuous kinetics to calculate the rate. The final result will be expressed as a rate per mg protein of the sample.

8.7 Conclusions

The aforementioned protocols are optimized to be universal and can be applied to any type of homogenate. In relation to fungal physiology, which is the interest of this topic, we have previously demonstrated and provided substantial bibliography over the impact of oxidative stress in the physiology of filamentous fungi [3]. In this section, we discuss results we have obtained thus far based on these methods.

In a recent study, we explored the proliferating and differentiating roles of H_2O_2 by altering its levels in cultures of *Sclerotium rolfsii* and *Sclerotinia sclerotiorum*. Hydrogen peroxide was administered extracellularly (in the media) or alternatively, aminotriazole, the specific inhibitor of catalase, was added to the media to inhibit the intracellular decomposition of H_2O_2 [15]. Results showed that the externally added H_2O_2 caused an increase in endogenous catalase activity, while aminotriazole administration resulted in a significant decrease of the enzyme. These changes were also accompanied by an increase in proliferation in both fungal species (by approximately 50%) and reduced the production of sclerotia, hence, dedifferentiated the fungi. These results are in accordance with previous studies where we compared the sclerotiogenic and non-sclerotiogenic mutants of the aforementioned fungal species [16], which implies that a high concentration of intracellular H_2O_2 is associated with sclerotial differentiation in filamentous fungi. We verified this conclusion by another study showing that endogenous SOD activity was higher in non-sclerotiogenic mutants [17] while using SOD mimetics (artificially increasing superoxide radical dismutation); we also decreased fungal sclerotial differentiation [18]. In relation to the role of thiol redox state modulating enzymes, we have extensively studied their impact on filamentous fungal growth. For *Sclerotinia sclerotiorum*, we have detected a decrease in glutathione reductase as the fungus differentiates. This finding was also accompanied by an increase in oxidized thiols [19]. Additionally, administration of certain thiol redox state modulators resulted in dedifferentiation of the tested fungi and resulted in changes of certain thiol redox status parameters [20].

Acknowledgments This work was financially supported by the internal funds of Dublin City University and the Greek Ministry of Education, University of Patras, Greece.

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Chapter 9
Cellulomics of Live Yeast by Advanced and Correlative Microscopy

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Contents

9.1 Introduction

Atomic force microscopy (AFM) is a fundamental tool to investigate the morphology and ultrastructural properties of biomaterials and cells at the micro-nanometer scale, and to evaluate the real-time relationship between their physicochemical properties and biological response. AFM is becoming one of the gold standards for detailing biological interfaces, including the surfaces of microbes.

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AFM is capable of acquiring surface images with nanometer (nm) scale resolution or any type of physical interaction between a sharp tip and a surface, for example, mapping interaction forces from a few pN to hundreds of nN for probing single molecule interactions to overall cellular mechanics, respectively. Ultrastructure, roughness, viscoelasticity and macromolecular interaction forces measured by AFM are informative parameters that describe biological characteristics and processes such as cell morphology, adhesion, transport and motility, and signalling [1–4].

Traditional modes of AFM data collection are tricky for challenging samples with steep edges, as well as those that are fragile, soft, sticky, or loosely attached to the surface [5, 6]. Recently developed AFM operating modes, like Quantitative Imaging™ (QI), enables not only the study of sample surface architecture with nm-scale resolution, but also the mapping mechanical and adhesive properties of a sample in situ. QI uses pN forces to quickly image immobilised samples at high resolution, resulting in force-curve data at each pixel that can be analysed by any model [7]. The major advantage of QI is that it does not impart any lateral forces on the sample and provides precise force control during scanning, preserving the integrity of soft biological samples. QI also allows for kinetic studies on difficult biological samples and is not limited by either sample geometry or environment. Any standard cantilever can be used for QI. Very sensitive samples (i.e. biological cells) or the detection of small interaction forces requires a low spring constant (0.01–0.6 N/m) cantilever. In comparison with AFM imaging, QI can record up to 512 × 512 (262144) QI force curves, which contain much more information than only surface topography. The force curves can be further analysed to obtain additional information, for example, stiffness, using the Heinrich Hertz formulated theory [8] or any other appropriate model. Decreasing either the pixel resolution or imaging area reduces the time per image, which enables the study of dynamic processes.

Recently, correlative AFM-QI and laser scanning confocal microscopy (LSCM) has been used to develop a multiplexed assay of living cells (bacteria, fungi, human) in response to a common herbicide, with broad application to other xenobiotics. In addition to determining surface architecture, AFM in QI mode probes surfaces biochemistry and cellular mechanics, while LSCM is a window into the cell to simultaneously track fluorescently tagged macromolecules, as many as can be optically separated. Thus, correlative AFM-QI-LSCM generates multiplexed data for the extensive characterisation of specimens ranging from single molecules or nanoparticles to living cells [9–11].

Sample preparation for AFM is governed by a combination of overall cell morphology and cell wall surface characteristics. The dimorphic fungus *C. albicans*, able to grow both as fission yeast and filamentous (hyphal) cells, is an opportunistic pathogen, whereas the budding yeast, *Saccharomyces cerevisiae*, has been domesticated for culinary uses (i.e. brewing and baking) [12]. We have previously detailed methods for imaging *Aspergillus nidulans* hyphal cells [13], so this chapter will focus on the fission yeast form of *C. albicans* and budding yeast form of *S. cerevisiae*.

S. cerevisiae is one of the most highly studied organisms based on its role in food production, and the detailed research on its cell wall became the basis for cell wall models of other yeast such as *C. albicans* [14]. Similar to *S. cerevisiae,* the cell wall of *C. albicans* consists of three main components: mannoproteins (~ 39%), β-glucans (~ 59%), and chitin (~ 2%), to which external cell wall proteins (CWPs) are attached. The cell walls contribute to a highly dynamic molecular architecture, which is continuously remodelled in response to cell surface interactions. Moreover, the cell wall surface is decorated with homogenously dispersed mannoproteins called adhesins that play pivotal roles in cell communication, adhesion and microbial infection. Various factors such as cell wall stress, temperature variation, exposure to antifungal agents, host interaction, and biofilm formation can alter the cell's adhesive properties [15–19].

This chapter will detail the methods required to successfully acquire AFM-QI and AFM-QI-LSCM data for *Candida* and *S. cerevisiae*, including sample preparation techniques and imaging.

9.2 Materials

Unless otherwise specified, all solvents and acids were reagent grade.

Acetone (HPLC Grade, Fisher Scientific).

Cantilever Probes for QI™-AFM (AppNano Hydra4V-100NG, spring constant 0.039–0.184 N/m, resonance frequency 31–58 kHz; Bruker MLCT probes, spring constant 0.01–0.6 N/m, resonance frequencies 7–125 kHz; nanosensors qp-BioAC, spring constant 0.06–0.3 N/m, resonance frequencies 30–90 kHz).

Cell-Tak (corning).
Deionised water (18 MΩ NanoPure water).
Glass coverslips (22 mm x 22 mm, Fisher Scientific for fixed cells; 18 mm x 18 mm, Carl Zeiss™ cover glasses, high performance for live cells).
Hydrochloric acid (HCl) (EMD Millipore).
Hydrogen peroxide 30 wt % in H_2O (H_2O_2, Sigma-Aldrich).
Kim wipes (Kimberly Clark).
Methanol (HPLC Grade, Fisher Scientific).
PDMS stamps (Laboratory for Analysis and Architecture of Systems) [20].
Petri dish (100 mm x 15 mm, VWR International).
Phosphate Buffered Saline (0.01 M PBS, Sigma Aldrich).
Sodium bicarbonate (Fisher Scientific).
Sodium hydroxide (Caledon laboratories).
Sulphuric acid (H_2SO_4) (Caledon laboratories).
PLL (Poly-L-lysine) (Sigma Aldrich).
Triton-X 100 (Sigma Aldrich).
YPD (Yeast, Peptone, Dextrose) medium (Sigma Aldrich).

9.3 Experimental Methods

9.3.1 Preparing Coverslips

The methods described below detail the preparation of materials required for immobilising fixed and live cells, which is crucial for successful AFM imaging.

9.3.1.1 Cleaning Coverslips

Coverslips must be cleaned prior to coating.

1. Dip coverslips (22 × 22 mm) in 1 M HCl for 2 min.
2. Wash coverslips in deionised water and let air dry (it is helpful to have a rack that separates the coverslips for processing many at a time).
3. Soak coverslips in Piranha solution (5 mL of 30% H_2O_2 + 15 mL of 18 H_2SO_4) for 1 h.
4. Remove the coverslip, immerse and wash with a copious amount of deionised water.
5. Dip coverslip in methanol for 2 min and air dry.
6. Dip coverslip in acetone for 2 min and air dry.
7. Store the clean coverslips in a dust-free container (Petri dish) until ready to coat.

9.3.1.2 Coating Coverslips

Poly-L-Lysine (PLL)

1. Make a 1:10 dilution of the PLL solution (1 part PLL to 9 parts deionised water).
2. Add 200 µl PLL onto the coverslip.
3. Incubate at room temperature for 30 min.
4. Wash twice with 200 µl deionised water.
5. Place the coverslip in a dust-free container and dry in a fume hood for 10 min or let dry overnight (O/N) if convenient.

Cell-Tak

Coverslips were coated with Cell-Tak with a procedure slightly modified from Louise Meyer et al. [21]

1. Prepare a solution of Cell-Tak by mixing 3.5 µl of Cell-Tak with 140 µl of buffer (NaOH (1 M) and $NaHCO_3$ (0.1 M pH 8)).
2. Spread 40 µl of the Cell-Tak solution onto a clean coverslip and incubate for 30 min at room temperature.

3. Wash 3 × with deionised water (0.2–0.5 mL, depending on coverslip size) and air dry.

   Coated coverslips can be stored for up to 2 weeks at 4 °C in a desiccator.

9.3.2 Sample Preparation

Samples can first be fixed or imaged live, but both require immobilisation.

9.3.2.1 Fixed Samples

1. Culture cells in YPD (Yeast, Peptone, Dextrose) O/N at 30 °C in a shaker at 200 rpm (*Candida*) or 165 rpm (*Saccharomyces*).
2. Add 200 µl of the culture to PLL-coated coverslips and incubate at room temperature for at least 30 min, up to 1 h.
3. Wash 3 times with 100 µl of phosphate buffered saline (PBS) and air dry.
4. Add 200 µl of fixative solution (3.7% formaldehyde 0.2% Triton X-100 in 10 mM phosphate buffer saline) and incubate for 10 min.
5. Wash 5–10 × with 100 µl deionised water.
6. Air dry and store in a dust-free container (Petri dish) at 4 °C until ready for AFM imaging.

9.3.2.2 Live Samples

Live cell imaging requires immobilisation, and here, we describe two such methods using biochemical [20] and physical methods [22], respectively.

1. Culture cells in YPD media overnight at 30 °C in a shaker.
2. Dilute the culture to an OD_{600} of 0.2 in pre-warmed YPD and incubate at 30 °C with shaking until it reaches exponential phase ($OD_{600} = 1$).
3. Continue to prepare samples as per 9.3.2.2.1 or 9.3.2.2.2

Physical Immobilisation of Cells with PDMS Stamps

1. Using a scalpel, cut a small section of the PDMS stamp containing the pattern multi-patterned PDMS stamp and place it onto a clean glass microscope slide with the pattern side up.
2. Place an O-ring on the slide to secure the PDMS stamp and pipette approximately 100 µL of cell culture onto the PDMS stamp so it covers the entire stamp.
3. To capture cells in the PDMS wells, manually drag a coverslip across the PDMS stamp, ideally with an advancing and receding contact angle of approximately

96 and 84 degrees respectively [23], pulling the droplet of the cell suspension along with the stamp several times.
4. Check to see if cells have entered the wells under a bright-field microscope at 400 × resolution.
5. Once the wells contain cells, add phosphate buffered saline (PBS) or 1:1 YPD: PBS to cover the entire stamp and proceed to liquid AFM (Fig. 9.1b).

Biochemical Trapping of Cells with Cell-Tak

A circular hole (18 mm) was cut at the bottom of a 100 X 15 mm polystyrene Petri dish and sealed with a Zeiss high precision coverslip using epoxy resin. The coverslip was pre-cleaned [22] and then coated with Cell-Tak as described in 9.3.1.2.2.

1. Add 500 µl of the culture to Cell-Tak coated coverslip attached to the polystyrene Petri dish, as described above, and incubate at 30 °C for 30 min in the dark.
2. Rinse with filtered (0.2 um filter) and pre-warmed 1:1 diluted YPD: PBS (0.01 M, pH 7) and add 500 µL of the same solution for imaging and maintained at 30 °C prior to use).
4. Place the Petri dish with the sample in a heated AFM sample holder maintained at 30 °C.

9.3.3 Pros and Cons of Immobilisation Techniques

Since AFM is a surface scanning technique, live cells must first be immobilised on solid substrates prior to imaging, using methods such as those outlined above [20].

PLL and Cell-Tak both biochemically immobilise cells onto a surface of choice. PLL coating effectively immobilises both live and fixed cells of bacteria and fungi, and it is less expensive than other methods. PLL works through electrostatic interactions between the cationic (Poly-L-Lys^{+}) thin film on a substrate which creates a positively charged surface that attracts negatively charged microbes. The method works well with highly charged cells, but the interaction is easily reversible by shear forces during sample rinses or liquid imaging [23, 24]. Furthermore, PLL is antimicrobial and directly hinders bacterial cell division and physiology, especially when used at higher concentrations [14, 25] which can sometimes induce cell death [11]. So based on all of the above considerations, we only use PLL for fixed cells. For live cells, we have found Cell-Tak to be the better alternative since it non-specifically attaches yeast and bacteria to glass surfaces. Cell-Tak is a formulation of the polyphenolic proteins extracted from the marine mussel *Mytilus edulis*, the key components of the glue secreted that allows the mussel to anchor itself to solid structures in its natural environment [21].

PDMS stamps are an efficient way of physically immobilising cells and other samples by convective or capillary deposition as described above for live cell imaging [23]. The surface layer of the PDMS pattern needs to be sufficiently thin so that cells are not trapped too deeply, which would prevent the AFM tip from reaching the sample and preclude imaging [18, 26].

Immobilisation techniques are not limited to those we describe above, and other methods exist including the use of optical tweezers and coating with gelatin [20]. Gelatin-coated surfaces used to immobilize cells can bias AFM images through adhering to the AFM tips [20] or by coating the sample and changing its topography [16, 24]. Optical tweezers can be used to accurately trap cells in 3D by focusing a laser to a single diffraction-limited location [16, 24], however, these cells are easily detached in comparison to those immobilised on glass surfaces [20, 27]. Furthermore, the laser used for optical tweezers can damage cells [16, 24].

9.4 Imaging Modes

For all experiments, a JPK NanoWizard 3 or 4 AFM was used in quantitative imaging (QI) mode. We have found that yeast is best imaged with silicon nitride cantilevers with calibrated spring constants ranging from 0.01 to 0.6 N/m. For QI adhesion data, a cantilever with a lower spring constant may be preferable for greater sensitivity, but QI viscoelasticity data from yeast, which has a relatively rigid cell wall, requires a cantilever with a slightly higher spring constant. So, choosing an AFM cantilever can often be a trade-off between the two. Furthermore, the choice of the cantilever will be governed by whether or not the sample is fixed or live, the latter requiring a cantilever with a lower spring constant. When imaging at a higher speed to reduce imaging time, there are shorter cantilevers which combine a low spring constant with a high resonant frequency.

9.4.1 Imaging Fixed Cells in QI Mode

1. Turn on the AFM, followed by the AFM imaging software on the computer connected to the AFM.
2. Place the coverslip with the sample to be imaged on the microscope slide and secure it on both sides using tape, and mount the slide onto the AFM stage (Fig. 9.1a).
3. Use the CCD camera or optical microscope to locate the specimen.
4. Once the specimen has been located, place the AFM head on the sample stage.
5. Using the z-stepper motors, bring the AFM cantilever within a few hundred micrometres of the sample surface.
   Caution: If the tip hits the sample surface, the cantilever will likely break

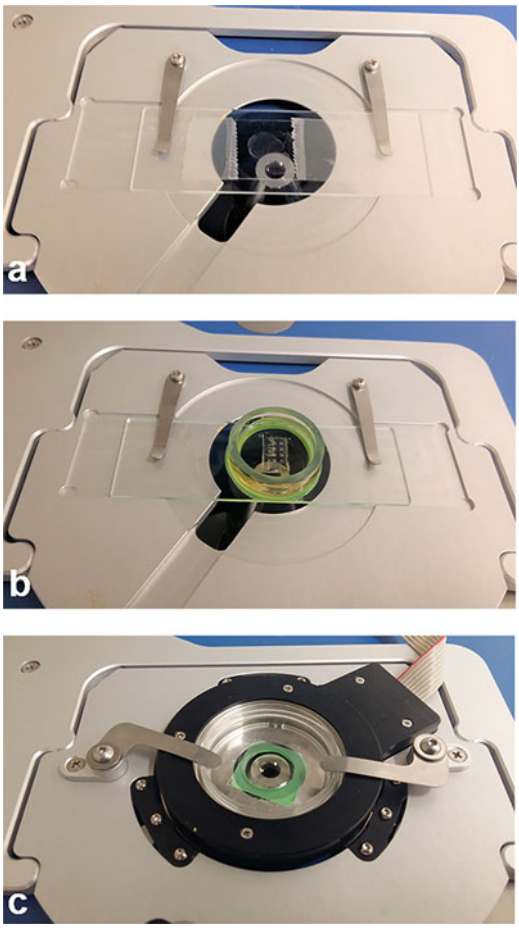

Fig. 9.1 Common immobilisation strategies for yeast cells to be imaged by AFM using several different methods: (**a**) fixed cells deposited onto PLL-coated coverslips, (**b**) live yeast cells deposited into a polydimethylsiloxane (PDMS) stamp by convective capillary deposition, and (**c**) live yeast cells firmly immobilised onto a Cell-Tak coated coverslip glued to the bottom of a Petri dish

6. Align the laser onto the cantilever portion directly above the AFM tip and optimize to get the maximum sum signal (>1 V) on the four-quadrant photodetector. In general, a higher sum indicates a good feedback signal from the cantilever.
7. Adjust the lateral and vertical deflection and if needed, the mirror, to bring the laser to the centre of the photodiode.
8. Approach the cantilever to the surface, using the automatic approach. Retract the cantilever by approximately 10–20 μm and calibrate the AFM tip position against the sample using the direct overlay optical calibration.

   Note: Following tip-sample calibration (optical overlay), it is important to only move the sample in x and y directions, rather than the AFM head, which would render the calibration void and would require recalibration of the sample position in relation to the tip

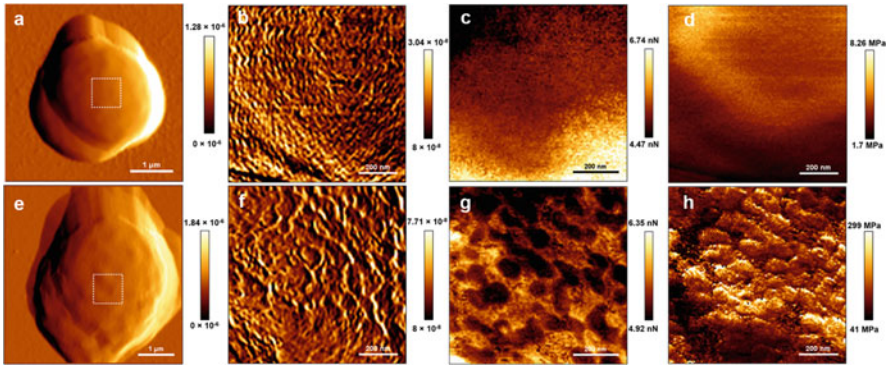

Fig. 9.2 QI™ images of *C. albicans* cell surface, adhesion and elasticity. (**a**, **b**) height images (10 µm × 10 µm, 128 × 128 pixels) of whole fixed cells deposited on PLL-coated coverslips, for (**a**) untreated cells and (**b**) those treated with cinnamon bark essential (CNB) oils at 0.5 × MIC (62.5 µg/ml). High-resolution QI AFM images recorded (128 × 128 pixels) on a 1 μm^2 area (white boxed squares) on the top of cells reveal (**f**) increased surface roughness, (**g**) altered adhesion patterns and (h) greater viscoelasticity for treated compared to (**b**, **c**, **d**) untreated cells, respectively

9. Calibrate the tip:
   (a) Change to force spectroscopy mode, collect a force curve on an area of the coverslip with no cells using a z-length of 1 µm. Now, obtain the sensitivity from the force curve and the spring constant from the thermal noise to calibrate the tip (thermal noise or contact-based method).
   (b) For rectangular cantilevers, the tip calibration can be done without a force curve using the dimensions of the cantilever and density and viscosity of the medium (Sader or contact free method).
10. Change the AFM to force mapping mode and bring the tip into feedback.
11. Select a 50 × 50 µm scan area at low resolution (128 × 128 pixels), and quickly collect a force map to determine the precise location of cells.

    Note: Areas with bright pixels indicate the presence of cells available for centering and QI imaging.
12. Select the area around the cell to be imaged by QI, and change the imaging setting to QI mode.
13. Choose appropriate imaging parameters, such as speed, resolution, Z-length and set-point.

    Note: It is better to start with a lower speed and larger set point (z-length). For *Candida albicans*, the starting Z-length was generally in the range of 3.5–6 µm with a scan speed 100 µm/s. This is then optimised for shorter imaging times with shorter z-length and higher scan speed (up to 300 µm/s).

    14. Choose a specific area of interest on the cell surface for further high-resolution (512 x 512 pixels) imaging (Fig. 9.2b-d, f-h).

    Note: With a raster scanning method, there is a balance between image resolution and image speed, with very high-resolution images taking longer to collect.

9.4.2 *Imaging Live Cells in QI Mode*

1. Insert a Petri dish heater designed to hold a polystyrene Petri dish and connect it to the controller.
2. Turn on the controller and set it to the desired temperature.
3. Place the polystyrene Petri dish containing the sample onto the AFM stage and secure both sides using the steel clamps (Fig. 9.1c).
4. Using the CCD camera or optical microscope, locate the specimen using a low magnification objective such as 10–20 ×.
5. Slowly and carefully put one drop of the imaging media onto the cantilever glass block mount, tilt the mount, and allow the drop to slowly cover the cantilever.
6. Place the AFM head into the sample stage.
7. Using the z-stepper motors, carefully lower the AFM cantilever until the drop on the cantilever surface fuses with the sample imaging media.
   Caution: Again, avoid touching the sample surface, which could break the cantilever
8. Once the cantilever is under liquid and within a few hundred microns of sample surfaces, align the laser onto the cantilever portion directly above the AFM tip to optimize the sum (>1).
9. When switching from air to liquid the mirror needs to be adjusted to account for the refraction of the laser as it passes through the liquid–glass interface.
10. Calibrate the tip position and sample using the optical overlay according to 9.4.1.
11. Determine the precise location of the cells using fast force mapping (Fig. 9.3), switch to QI mode, set imaging parameters, and begin imaging (Fig. 9.4) as described in 9.4.1.

    Note: Live cells can be AFM or QI-AFM [26] imaged using cells immobilised in PDMS stamps (see Sect. 9.3.2.2.2) under PBS, 1:1 PBS/YPD or YPD media, by following steps 4–11 above. If the exact density and viscosity of the media are unknown, use water and the Sader method [27] to determine the spring constant.

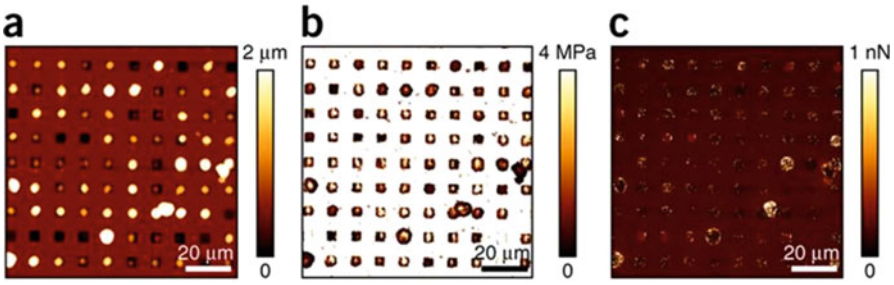

Fig. 9.3 AFM images of *Candida albicans* array on a PDMS stamp. (**a**) 2-dimensional AFM height image on 10 × 10 PDMS wells, corresponding (**b**) elasticity and (**c**) adhesion maps. Taken from [20]

9.4.3 Tracking Dynamic Processes in QI Mode

When the pixel resolution is reduced and z-length and speed are optimised, it is possible to study dynamic processes on the order of minutes using QI mode. Short cantilevers, with a low spring constant (0.06–0.3 N/m) and a high resonant frequency (30–90 kHz), allow a high z-speed and reduced acquisition times.

It is imperative that samples are well immobilised, including both the cells and the substrate, so the PDMS stamp itself was glued to the glass substrate. This particular method has been used to examine changes to the *S. cerevisiae* cell wall in response to enzymatic degradation (Fig. 9.4).

1. Immobilize the PDMS stamp on a glass slide using epoxy resin.
2. Grow and immobilize cells in PDMS stamps according to 9.3.2.2.2.
3. Glue the imaging ring to the glass slide around the PDMS stamp and add 1.5 mL of the desired medium (see Fig. 9.1b).
4. Follow steps 4–11 in Sect. 9.4.2.

9.4.4 Correlative AFM-QI-LSCM Live Cell Imaging

1. Replace the LSCM stage with the AFM sample stage.
2. Mount the AFM on the inverted LSCM and attach an additional camera onto the front port of LSCM and connect it to the AFM ECU.
3. Set the AFM-LSCM according to the instrument schematic in Fig. 9.5, and mount a specimen with a suitable fluorescent label for simultaneous imaging by LSCM.
4. Mount a cantilever and place the AFM head on the stage as described in 9.4.2 for live cell imaging.
5. Focus the sample from below using a 20 × objective to assess the distance between the specimen and the cantilever, but avoid bumping the sample with the objective.
6. Lower the cantilever until its blurry image is visible under the 20 × objective.
   Caution: Do not lower the cantilever completely into the focal plane, since at this point it will be too close to the sample with the associated risk of breaking the cantilever.
7. Align the laser on the cantilever and maximize the sum as described for live cell imaging (9.4.2).
8. At this point, the objective can be carefully switched to 40 × or oil immersion, a fluorescence image collected and the LSCM gains optimised.

   Caution: When imaging in oil immersion, it is important that the sample focus is not changed while the tip is in feedback. When the objective is in oil immersion, it is in physical contact with the sample and moving the objective will cause the AFM tip to crash into the sample.

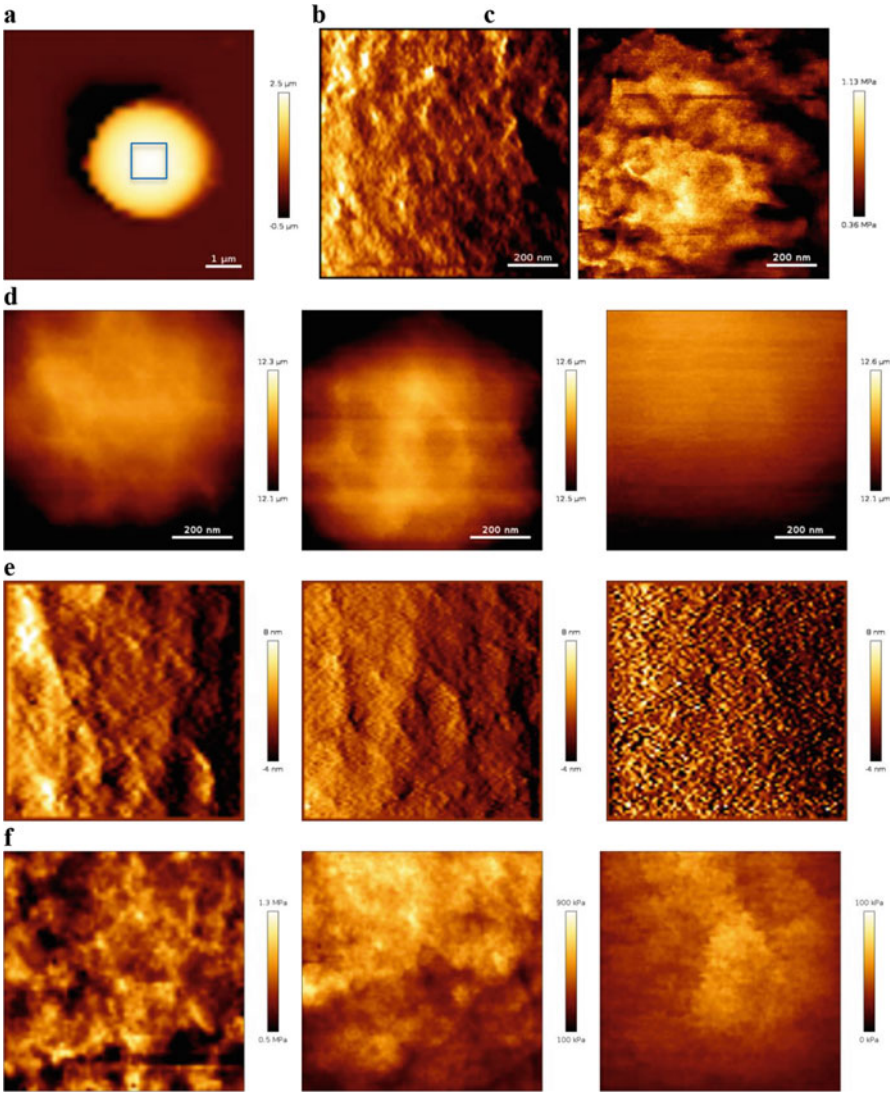

Fig. 9.4 AFM QI images a *S. cerevisiae* cell as (**a**) height, (**b**) surface topography as pixel difference, and (**c**) viscoelasticity at high resolution. The sequence of changes in (**d**) height, (**e**) pixel difference, and (**f**) elasticity maps at 0, 16, and 29 min exposure to lyticase show dramatic changes to the surface properties

9. The cantilever should be calibrated on a clean glass surface free of specimen to obtain a force constant, as previously described for live cell imaging (9.4.2 and 9.4.3).

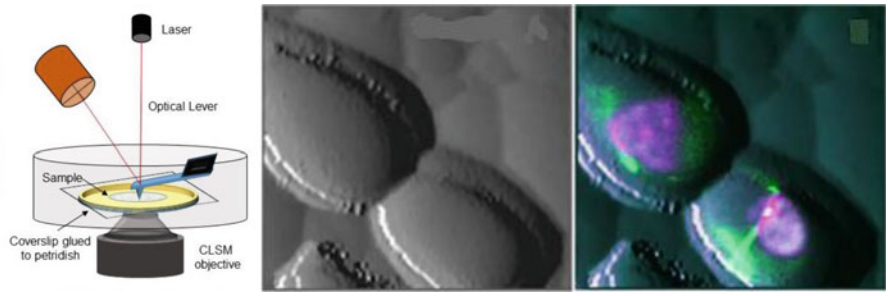

Fig. 9.5 AFM-QI-LSCM schematic illustration of the inverted LSCM objective focused onto the sample from below, with the AFM cantilever and tip imaging from above. The live sample is firmly immobilised to a Cell-Tak coated coverslip mounted in a Petri dish (left). Image of living *Candida albicans* AFM-QI™ surface topography (middle), and overlay for AFM-QI™-LSCM confocal data (right) that were simultaneously collected. In addition to surface ultrastructure, shown on the right is the localisation of tubulin2-GFP (Tub2-GFP, green) and histone protein B-RFP (Htb-RFP, purple). (Adapted from [11])

10. Calibrate the AFM tip position in x-y using the direct overlay and bring the AFM tip into feedback mode (9.4.2).
11. Select a desired location on the sample for imaging and determine the precise location using force mapping (9.4.2).

    Note: It is important to select a cell which is immobile, has a height in an expected range and shows bright fluorescence signals in the expected regions of the cell. It is also important to have cells sparsely distributed since overcrowding negatively affects imaging quality.
12. LSCM imaging parameters need to be adjusted prior to initiation of the AFM-QI scan.

    Note: It is important not to change any confocal parameters that will move large components such as filters and lasers, which will cause large noise and could cause the AFM tip to crash into the surface during imaging. The best practice to collect an LSCM image directly prior to and after the AFM scan to ensure similar results during simultaneous scanning.
13. For multicolour LSCM imaging, select a suitable excitation wavelength for each fluorophore that will prevent bleed [11].
14. LSCM imaging is much faster than AFM-QI, therefore it is possible to obtain multiple LSCM scans during QI imaging, provided LSCM settings are not changed during imaging.

    Note: With the advent of high-speed AFM, it is possible to better match the data collection speeds of AFM and LSCM.
15. Since LSCM has a much wider field of view than AFM, match, as much as possible, AFM and LSCM image resolution and size to vastly simplify image overlay (Fig. 9.5).

9.5 AFM QI Mode Image/Data Processing

1. Using the JPK data processing software retrieve QI force curves at each pixel of the raster scan.
2. Select a single reference force curve in the middle of the specimen and define the operations for batch processing such as spring constant, sensitivity, baseline correction, tip-sample separation, then measure the adhesion and apply the Hertz or other appropriate model to estimate the Young's modulus.
3. Apply the same processing parameters for all force curves in the image using the batch processing module.
4. Batch process all force curves and export to excel, plot the data using Origin and use the Gaussian distribution to place a fit on the histograms, or use a statistical analysis package (i.e. GraphPad Prism).
5. Determine the surface roughness by selecting 200 × 200 nm squares at the centre of the specimen using the QI™ height images.

9.5.1 AFM Image Processing and Overlay

1. Process images using the data processing software (DPS), ImageJ or photoshop, for adjusting brightness and contrast, for which the former allows roughness and subunit measurements.
2. Multi-colour LSCM images should be processed using linear unmixing to separate different channels, along with other downstream analyses, including intensity measurements and particle counts.
3. AFM-QI and LSCM images collected at the same time with similar resolution parameters for the same specimen at the same position can be manually overlaid using Photoshop or ImageJ.
   Note: In the majority of cases, LSCM images need to be digitally enlarged to correspond with the higher resolution AFM height image.

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Chapter 10
Molecular Taxonomy and Multigene Phylogeny of Filamentous Fungi

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Contents

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10.1 Introduction

The second largest kingdom "Fungi" includes rusts, yeasts, mildews, molds, oomycetes, and smuts. They are generally classified into four types: Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota; however, they have recently been reclassified as into nine phylum-level clades: Opisthosporidia, Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Zoopagomycota, Mucoromycota, Glomeromycota, Basidiomycota, and Ascomycota [104]. It has been reviewed that the estimated number of species comprise of 2.2–3.8 million with only 3%, named on the basis of data acquired from environmental fungi DNA sequences [105]. Hence, to identify fungi at species level is the fundamental approach for researcher to carry any type of study, be it basic or applied. Most of the studies on plant pathogens start from identification of the pathogen and then to find its cure as it can have economical effect on commercial scale [57, 98]. The pathogenic behaviour of fungi is not only limited to plants; they can also infect, human health. Various fungal infections and diseases require the specific identification of causal agent before its treatment [11, 73]. The recognition of fungi from the positive aspect is also very important as their vast industrial application increases the need to discover more efficient species for commercial purposes.

The initial morphological basis of fungal classification basically applies only for the cultivable fungus at laboratory scale. They are observed, macro-morphologically (colony characteristics), micro-morphologically (spore characteristics) [81] and on the basis of life cycle [32] (e.g. pleomorphic fungi) which can occur in both forms, i.e. anamorph (asexual form) and teleomorph (sexual form). These dual life forms of fungi had followed a dual nomenclature system according to Article 59 of ICBN (International Code of Botanical Nomenclature), thus creating more confusion while working on them. The recent amendment in Article 59 (at 18th International Botanical Conference in Melbourne), implied effectively from 1 January 2013, states the rule of "one fungus – one name" irrespective of its alternative life forms (mitotic or meiotic). Then there comes polyphyletic genera (same genus appearing at different clades or places in phylogenetic tree) which have either lost some characteristics which resemble to one ancestor or gained some characters resembling another ancestor, for example, *Mycosphaerella* was earlier considered as a genus for 30 different anamorphic genera [23, 38], but the sequence data of 28S nuclear rRNA made it clear that it is an assembly of various genera belonging to different families [20, 23]. Thus, as much as it is important to study any fungi residing at vast environmental conditions, morphologically, it is difficult to completely rely on

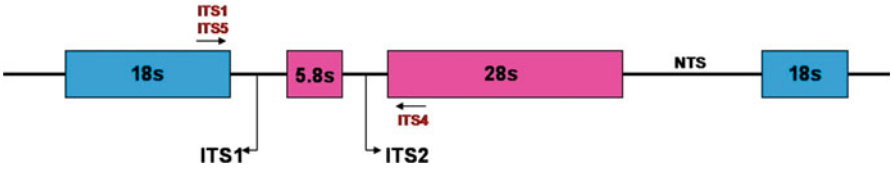

Fig. 10.1 A ribosomal RNA (rRNA) gene cluster

them; therefore, the need of molecular approach is utmost for accuracy. Various new molecular tools have been developed in last few decades, which allow efficient evolutionary inferences, wherein one or more gene sequences can be used for accurate fungal species identification. In general, the first level molecular identification involves single-gene sequence-based identification, and for a reliable phylogenetic placement in fungal tree of life, multigene-based phylogenetic approach is employed to define and recognize new fungal species.

In the 1980s, the initial molecular approach for phylogenetic study used polymerase chain reaction (PCR) of nuclear internal transcribed spacer sequences (ITS) region of ribosomal DNA (rDNA) [28]. These sequences are consensus regions and occur in long tandem repeats, and the universal primers could be designed for fungi [97]. The nuclear ribosomal RNA (rRNA) gene is divided into smaller subunit (18S or SSU) and larger subunit (28S or LSU) with two internal transcribed spacers (ITS1 and ITS2) between them linked by 5.8S region (Fig. 10.1). The 18S region helps to narrow down the classification from kingdom level to phyla including phyla, class, order, and family if the primer pair employed is NS1–NS4 [97], whereas LSU region resolves intermediate level of taxonomy, i.e. family and genera with primer pair LROR-LR6 [72, 94]. For species level identification, ITS is the most appropriate region as this portion, though transcribed it does not code for any protein, hence more prone to evolutionary mutation. It not only discriminates interspecific but also helps in detecting intraspecific variations if studied combinatorial with hypervariable domains of LSU (D1/D2 domain) in some group of fungi [79].

Buée had found 1000 molecular taxonomic operational units based on approximately 30,000 reads for ITS1 region from pyrosequencing 4 grams of forest soil sample [17]. Schoch has also mentioned that approximately 172,000 ITS region sequences have been deposited to GenBank including 25,000 genera and 15,500 species. These studies highlight the general acceptance and wide utility of ITS region for fungal identification. Unfortunately, this region has limitation when there are huge numbers of species for a particular genus (*Fusarium*, *Aspergillus*, *Penicillium*, etc.) [4, 49, 75, 79, 82, 87] or for cryptic species.

This drawback changes into advantage when we incorporate protein-coding genes (secondary molecular markers) along with ITS, i.e. multigene approach for superior resolution. The combined analysis of two or combination of more than two genes or even whole genome has become very popular in molecular phylogenetic studies [25]. These approaches yield unequivocal results [47]. Combined gene analyses, in support with morphological information, resolves the conflicts arose

from single-gene analyses and enhances phylogenetic resolution [30]. The leverage of using these additional gene markers (1) include faster evolving intronic region than ITS [28], (2) provide better resolution at the level of higher taxonomy [80], (3) occur in single copy, therefore prone to less mutations, and (4) include easy understanding of homology and converging aspect [10, 27]. Now the question arises, which protein coding genes should be used? Assembling the Fungal Tree of Life, a National Science Foundation project, which has supported extensive studies on fungal systematics using protein coding genes and designing molecular phylogeny in the field of systematic and taxonomy [13, 34, 43, 53, 80]. The genetic markers are *RPB1* and *RPB2* (RNA polymerase – largest and second largest subunit) [15, 50, 70, 88], *tef1* (translational elongation factor 1-alpha) [71], *tub2/BenA* (beta-tubulin) [29, 60], and *MCM7* (mini chromosome maintenance protein), which provides superior resolution in association with other genes, e.g. with LSU for Ascomycota members [2, 68]. In some lineages of Eurotiales (includes genus *Aspergillus*and *Penicillium*), species level identification involves *CaM/cal* (calmodulin) gene along with *tef1, RPB2, tub2/BenA*, and *RPB1*. Visagie and colleagues have recommended the use of *RPB2* or *CaM* genes to avoid any ambiguity based on gene *tub2/BenA* for *Penicillium spp.* [95]. Similarly, for molecular phylogeny of *Aspergillus sp.*, *CaM* gene is recommended, but *RPB2* and *tub2/BenA* are also preferred and can be used for classification [75]. Torbati suggested the use of *CaM*, *tef1*, *tub2*, and *RPB2* secondary markers along with ITS and LSU to resolve the phylogenetic study of *Fusarium spp.*, which exhibit immense morphological plasticity [92]. A detailed list of commonly used gene targets/markers has been given in Table 10.1, which is an easy guide to select the right gene targets/markers for some specific fungal genera.

In order to determine phylogenetic relationship, DNA sequences of different fungi can be aligned and compared to establish variability among themselves and with ancestors. This data is generally represented as "phylogenetic/relationship tree" where the branch's length and position explain the similarity and distant resemblance between different fungi. The variation at the intra- and inter-specific levels for a specific locus can be used for identification among all the closely related species, thus giving a possibility to make a decisive prediction regarding new species [74]. However, nowadays a molecular method using several appropriate gene targets/markers instead of only one, has made the phylogenetic study more authenticated to describe particular unknown species [47]. This method facilitates comparisons with already present taxonomic information of related species.

Initially, the use of phylogenetic trees was solely in the field of systematics and taxonomy to depict the relation among species. But nowadays, the advent of DNA sequencing technology it can be applied to all the branches of biology. Phylogeny has also explained the relationships between paralogues of gene family along with evolutionary and epidemiological dynamics of pathogens and histories of populations, etc. [100]. The methods required for phylogenetic analysis, includes parsimony, likelihood distance, and Bayesian methods. The detailed information about these methods is vast and very exhaustive that cannot be accommodated at one place; however, some basic information regarding phylogeny has been mentioned in the pretext of phylogenetic tree construction section of this chapter.

Table 10.1 Gene targets/molecular markers for different fungi and their respective primer pairs and sequences

S. N.	Genus of Fungi	Genes for molecular phylogeny	Primer name		References
			Forward primer (Primer Sequence 5′-3′)	Reverse primer (Primer Sequence 5′-3′)	
1.	*Alternaria*	ITS	**V9G:** TTACGTCCCTGCCCTTTGTA	**ITS4:** TCCTCCGCTTATTGATATGC	[99]
		GAPDH	**gpd1:** CAACGGCTTCGGTCGCATTG	**gpd2:** GCCAAGCAGTTGGTTGTGC	
		RPB2	**RPB2-5F2:** GGGGWGAYCAGAAGAAGGC	**fRPB2-7cR:** CCCATRGCTTGYTTRCCCAT	
		OPA10-2	**OPA10-2L:** TCGCAGTAAGACACATTCTACG	**OPA10-2R:** GATTCGCAGCAGGGAAACTA	
		Alt a 1	**Alt-for:** ATGCAGTTCACCACCATCGC	**Alt-rev:** ACGAGGGTGAYGTAGGCGTC	
		endoPG	**PG3:** TACCATGGTTCTTTCCGA	**PG2b:** GAGAATTCRCARTCRTCYTGRTT	
		TEF1-α	**EF1-728F:** CATCGAGAAGTTCGAGAAGG	**EF1-986R:** TACTTGAAGGAACCCTTACC	
2.	*Aspergillus*	β-tubulin	**Bt2a:** GGTAACCAAATCGGTGCTGCTTTC	**Bt2b:** ACCCTCAGTGTAGTGACCCTTGGC	[85]
		ITS	**ITS1:** TCCGTAGGTGAACCTGCGG	**ITS4:** TCCTCCGCTTATTGATATGC	
		TEF1-α	**983F:** GCYCCYGGHCAYCGTGAYTT	**2218R:** ATGACACCRACRGCRACRGTYTGYAT	
		Calmodulin	**CF1L:** GCCGACTCTTTGACYGARGAR **CF1M:** AGGCCGAYTCTYTGACYGA **cmd5:** CCGAGTACAAGGAGGCCTTC	**CF4:** TTTYTGCATCATRAGYTGGAC **cmd6:** TTTYTGCATCATRAGYTGGAC	
		RPB2	**fRPB2-5F:** GAYGAYMGWGATCAYTTYGG	**fRPB2-7CR:** CCCATRGCTTGYTTRCCCAT	
3.	*Bipolaris*	GAPDH	**gpd1:** CAACGGCTTCGGTCGCATTG	**gpd2:** GCCAAGCAGTTGGTTGTGC	[54]
		TEF1-α	**983F:** GCYCCYGGHCAYCGTGAYTT	**2218R:** ATGACACCRACRGCRACRGTYTGYAT	
		ITS	**ITS1:** TCCGTAGGTGAACCTGCGG	**ITS4:** TCCTCCGCTTATTGATATGC	
4.	*Botryosphaeria*	TEF1-α	**EF1-728F:** CATCGAGAAGTTCGAGAAGG	**EF1-986R:** TACTTGAAGGAACCCTTACC	[45, 67]
		ITS	**ITS5:**GGAAGTAAAAGTCGTAACAAGG	**ITS4:** TCCTCCGCTTATTGATATGC	
5.	*Botrytis*	GAPDH	**G3PDHfor:** ATTGACATCGTCGCTGTCAACGA	**G3PDHrev:** ACCCCACTCGTTGTCGTACCA	[102]
		HSP60	**HSP60for:** CAACAATTGAGATTTGCCCACAAG	**HSP60rev:** GATGGATCCAGTGGTACCGAGCAT	
		RPB2	**RPB2for:** GATGATCGTGATCATTTCGG	**RPB2rev:** CCCATAGCTTGCTTACCCAT	
6.	*Cercospora*	GAPDH	**Gpd1-LM:** ATTGGCCGCATCGTCTTCCGCAA	**Gpd2-LM:** CCCACTCGTTGTCGTACCA	[7]
		β-tubulin	**BT1-F:** GTCCWCACCGCCCCTGAT	**BT1-R:** CTTGTTRCCRGAAGCCTRTGS	

(continued)

Table 10.1 (continued)

S. N.	Genus of Fungi	Genes for molecular phylogeny	Primer name		References
			Forward primer (Primer Sequence 5′-3′)	Reverse primer (Primer Sequence 5′-3′)	
		Calmodulin	**CAL-228F:** GAGTTCAAGGAGGCCTTCTCCC	**CAL-737R:** CATCTTTCTGGCCATCATGG	
		RPB2	**fRPB2-7cF:** ATGGGYAARCAAGCYATGGG	**fRPB2-11aR:** GCRTGGATCTTRTCRTCSACC	
7.	*Colletotrichum*	ApMAT	**AM-F:** TCATTCTACGTATGTGCCCG	**AM-R:** CCAGAAATACACCGAACTTGC	[22, 84, 96]
		ITS	**ITS-1F:** CTTGGTCATTTAGAGGAAGTAA	**ITS4:** TCCTCCGCTTATTGATATGC	
		GAPDH	**GDF:** GCCGTCAACGACCCCTTCATTGA	**GDR:** GGGTGGAGTCGTACTTGAGCATGT	
		Actin	**ACT-512F:** ATGTGCAAGGCCGGTTTCGC	**ACT-783R:** TACGAGTCCTTCTGGCCCAT	
		HIS-3	**CYLH3F:** AGGTCCACTGGTGGCAAG	**CYLH3R:** AGCTGGATGTCCTTGGACTG	
		β-tubulin	**T1:** AACATGCGTGAGATTGTAAGT	**T2:** TAGTGACCCTTGGCCCAGTTG	
		CHS-1	**CHS-79F:** TGGGGCAAGGATGCTTGGAAGAAG	**CHS-345R:** TGGAAGAACCATCTGTGAGAGTTG	
8.	*Cordyceps*	SSU	**NS1:** GTAGTCATATGCTTGTCTC	**NS4:** CTTCCGTCAATTCCTTTAAG	[90]
		LSU	**LR0R:** ACCCGCTGAACTTAAGC	**LR5:** TCCTGAGGGAAACTTCG	
		TEF1-α	**983F:** GCYCCYGGHCAYCGTGAYTT	**2218R:** ATGACACCRACRGCRACRGTYTGYAT	
		RPB1	**CRPB1:** CCWGGYTTYATCAAGAARGT	**RPB1C:** CCNGCDATNTCRTTRTCCATRTA	
		RPB2	**fRPB2-5F:** GAYGAYMGWGATCAYTTYGG	**fRPB2-7cR:** CCCATRGCTTGYTTRCCCAT	
		β-tubulin	**T12:** AACAACTGGGCCAAGGGTCAC	**T22:** TCTGGATGTTGTTGGGAATCC	
		mt ATP6	**ATP6C1A:** AGAWCAATTYGAARTRAGAG	**ATPC2A:** ACAAAYACTTGWGCTTGKATWAAI GC	
9.	*Curvularia*	GAPDH	**GPD1:** CAACGGCTTCGGTCGCATTG	**GPD2:** GCCAAGCAGTTGGTTGTGC	[45]
		TEF1-α	**983F:** GCYCCYGGHCAYCGTGAYTT	**2218R:** ATGACACCRACRGCRACRGTYTGYAT	
		ITS	**ITS5:**GGAAGTAAAAGTCGTAACAAGG	**ITS4:** TCCTCCGCTTATTGATATGC	
10.	*Diaporthe*	ITS	**ITS5:**GGAAGTAAAAGTCGTAACAAGG	**ITS4:** TCCTCCGCTTATTGATATGC	[39]
		TEF1-α	**EF1-728F:** CATCGAGAAGTTCGAGAAGG	**EF1-986R:** TACTTGAAGGAACCCTTACC	
		Calmodulin	**CAL-228F:** GAGTTCAAGGAGGCCTTCTCCC	**CAL-737R:** CATCTTTCTGGCCATCATGG	
		HIS	**CYLH3F:** AGGTCCACTGGTGGCAAG	**H3-1b:** GCGGGCGAGCTGGATGTCCTT	

		β-tubulin	**Bt2a:** GGTAACCAAATCGGTGCTGCTTTC	**Bt2b:** ACCCTCAGTGTAGTGACCCTTGGC	
11.	*Fusarium*	RPB2	**5F2:** GGGGWGAYCAGAAGAAGGC **7cF:** ATGGGYAARCAAGCYATGGG	**7cR:** CCCATRGCTTGYTTRCCCAT **11aR:** GCRTGGATCTTRTCRTCSACC	[44, 52]
		RPB1	**Fa:** CAYAARGARTCYATGATGGGWC	**G2R:** GTCATYTGDGTDGCDGGYTCDCC	
		Calmodulin	**Cal228F:** GAGTTCAAGGAGGCCTTCTCCC	**CAL2Rd:** TGRTCNGCCTCDCGGATCATCTC	
		TEF1-α	**ef1:** ATGGGTAAGGA(A/G)GACAAGAC	**ef2:** GGA(G/A)GTACCAGT(G/C)ATCATGTT	
		β-tubulin	**T1:** AACATGCGTGAGATTGTAAGT	**CYLTUB1R:** AGTTGTCGGGACGGAAGAG	
12.	*Lasiodiplodia*	ITS	**ITS5:**GGAAGTAAAAGTCGTAACAAGG	**ITS4:** TCCTCCGCTTATTGATATGC	[44]
		β-tubulin	**Bt2a:** GGT AAC CAA ATC GGT GCT GCT TTC	**Bt2b:** ACCCTCAGTGTAGTGACCCTTGGC	
		TEF1-α	**EF1-728F:** CATCGAGAAGTTCGAGAAGG	**EF1-986R:** TACTTGAAGGAACCCTTACC	
13.	*Macrophomina*	β-tubulin	**T1:** AACATGCGTGAGATTGTAAGT	**CYLTUB1R:** AGTTGTCGGGACGGAAGAG	[44]
		Calmodulin	**CAL-228F:** GAGTTCAAGGAGGCCTTCTCCC	**CAL-737R:** CATCTTTCTGGCCATCATGG	
		ITS	**ITS5:**GGAAGTAAAAGTCGTAACAAGG	**ITS4:** TCCTCCGCTTATTGATATGC	
		TEF1-α	**EF1-728F:** CATCGAGAAGTTCGAGAAGG	**EF2:** GGA(G/A)GTACCAGT(G/C)ATCATGTT	
		Actin	**ACT-512F:** ATGTGCAAGGCCGGT	**ACT-783R:** TACGAGTCCTTCTGG	
14.	*Magnaporthe*	ITS	**ITS5:**GGAAGTAAAAGTCGTAACAAGG	**ITS4:** TCCTCCGCTTATTGATATGC	[103]
		LSU	**LS1:** GTACCCGCTGAACTTAAGC	**LR5:** TCCTGAGGGAAACTTCG	
		SSU	**SR1R:** TACCTGGTTGATQCTGCCAGT	**SR7:** GTTCAACTACGAGCTTTTTAA	
		MCM7	**MCM7-709:** ACIMGIGTITCVGAYGTHAARCC	**MCM7-1348:** GAYTTDGCIACICCIGGRTCWCCCAT	
		TEF1-α	**EF1-983F:** GCYCCYGGHCAYCGTGAYTT	**EF1-2218R:** ATGACACCRACRGCRACRGTYTGYAT	
		RPB1	**RPB1-Af:** GARTGYCCDGGDCAYTTYGG	**RPB1-Cr:** CCNGCDATNTCRTTRTCCATRTA	
15.	*Metarhizium*	TEF1-α	**983F:** GCYCCYGGHCAYCGTGAYTT	**2218R:** ATGACACCRACRGCRACRGTYTGYAT	[46]
		RPB1	**CRPB1:** CCWGGYTTYATCAAGAARGT	**RPB1C:** CCNGCDATNTCRTTRTCCATRTA	
		RPB2	**fRPB2-5F:** GAYGAYMGWGATCAYTTYGG	**fRPB2-7cR:** CCCATRGCTTGYTTRCCCAT	
		β-tubulin	**T12:** AACAACTGGGCCAAGGGTCAC	**T22:** TCTGGATGTTGTTGGGAATCC	
16.	*Mucor*	ITS	**ITS1:** TCCGTAGGTGAACCTGCGG	**ITS4:** TCCTCCGCTTATTGATATGC	[33]

(continued)

Table 10.1 (continued)

S. N.	Genus of Fungi	Genes for molecular phylogeny	Primer name		References
			Forward primer (Primer Sequence 5′-3′)	Reverse primer (Primer Sequence 5′-3′)	
		LSU	**LR0R:** ACCCGCTGAACTTAAGC	**LR5:** TCCTGAGGGAAACTTCG	
		MCM7	**Mcm7-709for:** ACIMGIGTITCVGAYGTHAARCC	**Mcm7-1348rev:** GAYTTDGCIACICCIGGRTCWC CCAT	
		RPB1	**RPB1-Af:** GARTGYCCDGGDCAYTTYGG	**RPB1-Cr:** CCNGCDATNTCRTTRTCCATRTA	
17.	*Mycospaerella*	LSU	**LSU1Fd:** GRATCAGGTAGGRATACCCG	**LR5:** TCCTGAGGGAAACTTCG	[93]
		ITS	**V9G:** TTACGTCCCTGCCCTTTGTA	**ITS4:** TCCTCCGCTTATTGATATGC	
		RPB2	**fRPB2-5F:** GAYGAYMGWGATCAYTTYGG	**fRPB2-7cR:** CCCATRGCTTGYTTRCCCAT	
18.	*Penicillium*	ITS	**ITS1F:** TCCGTAGGTGAACCTGCGG	**ITS4:** TCCTCCGCTTATTGATATGC	[95]
		Calmodulin	**CF1:** GCCGACTCTTGACTGAA	**CF4:** TTTYTGCATCATRAGYTGGAC	
		β-tubulin	**Bt2a:** GGTAACCAAATCGGTGCTGCTTTC	**Bt2b:** ACCCTCAGTGTAGTGACCCTTGGC	
		RPB2	**RPB2-5F_Eur:** GAYGAYCGKGAYCAYTTCGG	**RPB2-7CR_Eur:** CCCATRGCYTGYTTRCCCAT	
19.	*Phytophthora*	LSU	**LROR-O:** ACCCGCTGAACTYAAGC **LSUFint:** CKTTGACGAAATGGAGCGAT	**LR6-O:** CGCCAGACGAGCTTACC **LSURint:** TTTCCACACCCTAACACTTGC	[39]
		β-tubulin	**Btub_F1:** GCCAAGTTCTGGGAGGTCATC	**Btub_R1A:** CCTGGTACTGCTGGTAYTCMGA	
		COX II	**FM35:** CAGAACCTTGGCAATTAGG	**Phy10b:** GCAAAAGCACTAAAAATTAAATATAA	
		nad9	**Nad9-F:** TACAACAAGAATTAATGAGAAC	**Nad9-R:** GTTAAAATTTGTACTACTAACAT	
20.	*Pseudocercospora*	TEF1-α	**EF1-728F:** CATCGAGAAGTTCGAGAAGG	**EF-2:** GG(G/A)GTACCAGT(G/C)ATCATGTT	[21]
		Actin	**ACT-512F:** ATGTGCAAGGCCGGTTTCGC	**ACT-2Rd:** TACGAGTCCTTCTGGCCCAT	
		ITS	**ITS5:** GGAAGTAAAAGTCGTAACAAGG	**ITS4:** TCCTCCGCTTATTGATATGC	
		LSU	**LSU1Fd:** GRATCAGGTAGGRATACCCG	**LR5:** TCCTGAGGGAAACTTCG	
21.	*Puccinia*	LSU	**Rust28SF:** TTTTAAGACCTCAAATCAGGTG	**LR5:** TCCTGAGGGAAACTTCG	[3]
		SSU	**RustNS2-F:** GGGAGGTAGTGACMATAAATAAC AATG	**NS6:** GCATCACAGACCTGTTATTGCCTC	
		COX III	**CO3F1:** TCAGTATGTTATTTTAACGATGTAG	**CO3R1:** TCCTCATCAGTAAACACTAATA	

22.	*Pythium*	ITS	**ITS1:** TCCGTAGGTGAACCTGCGG	**ITS4:** TCCTCCGCTTATTGATATGC	[39]
		COX II	**FM58:** CCACAAATTTCACTACATTGA	**FM66:** TAGGATTTCAAGATCCTGC	
23.	*Rhizopus*	ITS	**V9G:** TTACGTCCCTGCCCTTTGTA	**LS266:** GCATTCCCAAACAACTCGACTC	[26]
		Actin	**Act-1:** TGGGACGATATGGAIAAIATCTGGCA	**Act-4ra:** TCITCGTATTCTTGCTTIGAIATCCACAT	
		TEF1-α	**MEF-4:** ATGACACCRACAGCGACGGTTTG	**MEF-10:** GTTGTCATCGGTCACGTCGATTC	
		RPB1	**RPB1-Af:** GARTGYCCDGGDCAYTTYGG	**RPB1-Cr:** CCNGCDATNTCRTTRTCCATRTA	
24.	*Sclerotinia*	ITS	**ITS1:** TCCGTAGGTGAACCTGCGC	**ITS4:** TCCTCCGCTTATTGATATGC	[83]
		β-tubulin	**β-tubulin F:** GGTAACCAAATCGGTGCTGCTTTC	**β-tubulin R:** ACCCTCAGTGTAGTGACCCTTGGC	
25.	*Sporothrix*	LSU	**LR3:** CCG TGT TTC AAG ACG GG	**LR5:** TCCTGAGGGAAACTTCG	[24]
		ITS	**ITS1F:** CTTGGTCATTTAGAGGAAGTAA	**ITS4:** TCCTCCGCTTATTGATATGC	
		β-tubulin	**T10:** ACGATAGGTTCACCTCCAGAC	**Bt2b:** ACCCTCAGTGTAGTGACCCTTGGC	
		Calmodulin	**CL1:** GA(GA)T(AT)CAAGGAGGCCTTCTC	**CL2a:** TTTTTGCATCATGAGTTGGAC	
26.	*Talaromyces*	RPB2	**fRPB2-5f:** GAYGAYMGWGATCAYTTYGG	**fRPB2-7cR:** CCCATRGCTTGYTTRCCCAT	[101]
		β-tubulin	**Bt2a:** GGTAACCAAATCGGTGCTGCTTTC	**Bt2b:** ACCCTCAGTGTAGTGACCCTTGGC	
		ITS	**V9G:** TTACGTCCCTGCCCTTTGTA	**LS266:** GCATTCCCAAACAACTCGACTC	
27.	*Trichoderma*	RPB2	**fRPB2-5f:** GAYGAYMGWGATCAYTTYGG	**fRPB2-7cR:** CCCATRGCTTGYTTRCCCAT	[42]
		TEF1-α	**EF1-728F:** CATCGAGAAGTTCGAGAAGG	**TEF1LLErev:** AACTTGCAGGCAATGTGG	
		acl1	**acl1-230up:** AGCCCGATCAGCTCATCAAG	**acl1-1220low:** CCTGGCAGCAAGATCVAGGAAG T	
28.	*Verticilium*	Actin	**VActF:** TAATTCACAATGGAGGGTAGG	**VActR:** GTAAGGATACCACGCTTGG	[40, 44]
		TEF1-α	**VEFf:** AACGTCGTCGTCATCGGCCACG	**VEFr:** CCACGCTCACGCTCGGCCTT	
		GAPDH	**VGPDf2:** GGCATCAACGGTTTCGGCC	**VGPDr:** GTAGGAGTGGACGGTGGTCATGAG	
		TS	**VTs3f:** GCGCTGCAAGGCCGAGAAC	**VTs3r:** GCGGAACGAGACGGCCTCC	
		ITS	**ITS5:** GGAAGTAAAAGTCGTAACAAGG	**ITS4:** TCCTCCGCTTATTGATATGC	

There has always been a requirement of putting all the essential information and protocols for fungal molecular taxonomy and phylogeny in one place; therefore, we assembled all the required protocols starting from fungal DNA isolation, list of recommended gene targets for fungal identification and phylogeny, their primer names, sequences and PCR cycling conditions, PCR product purification, and DNA sequencing in this chapter. Considering the vast number of fungi, we have enlisted the gene targets/markers for limited number of fungi, which are the common and important fungal pathogens. Many times, it becomes difficult to get the correct PCR cycling conditions for different gene targets/markers and for which one has to check multiple back references; therefore, we have also enlisted the primer pair names, their sequences, and their PCR cycling conditions. We have also briefed about sequence editing and analysis. Sometimes, erroneous DNA sequences in NCBI nucleotide database lead to the misidentification of fungi; therefore, to cross-check and confirm the identity of the fungi, some additional well authenticated databases are also described, which can be used for molecular identification of fungi in addition to NCBI BLASTn. An outline about the construction of phylogenetic tree using MEGA7 has been described. Though there are many advanced tools like RAxML and Mr. Bayes available for constructing the phylogenetic trees, however, MEGA7 has been given because of ease of constructing a phylogenetic tree. Especially for the beginners in the field, a chart for the entire work flow has also been given for easy understanding of all the different steps required to perform the phylogenetic analysis of fungi (Fig. 10.2). Further, the methods, materials, and protocols vary among the different laboratories; therefore, the same can be twisted or modified depending upon the availability of reagents, chemicals, and equipment.

10.2 Fungal Genomic DNA Extraction

There are various extraction methods available for fungi, and the choice of method used for DNA extraction depends on the availability of equipments and nature of fungal samples from which DNA is to be isolated. Total genomic DNA can be isolated from fungal specimen by various physical methods like crushing in liquid nitrogen, bead beating in homogenizer, and use of some cell wall degrading enzymes with detergents like sodium dodecyl sulphate (SDS) and Cetyltrimethyl ammonium bromide (CTAB). Unfortunately, sometimes few methods can lead to shearing of DNA or can be hazardous and laborious (grinding pathogenic fungi in an open mortar and pestle, or handling multiple fungal isolates). Therefore, a closed DNA isolation system is always preferred, wherein one can isolate DNA from multiple fungi simultaneously, thereby enabling high throughput DNA extraction. One such method has been outlined in the following protocol section. Researchers also use DNA extraction kits. Most popular kits are DNeasy 96 Plant Kit (Qiagen), Qiagen MOBIO Laboratories, FastDNA SPIN Kit (MP Biomedicals), and Epicentre's MasterPure™ Yeast DNA Purification Kit to isolate genomic DNA from a broad range of fungi. Specific protocols are provided with each kit for researcher.

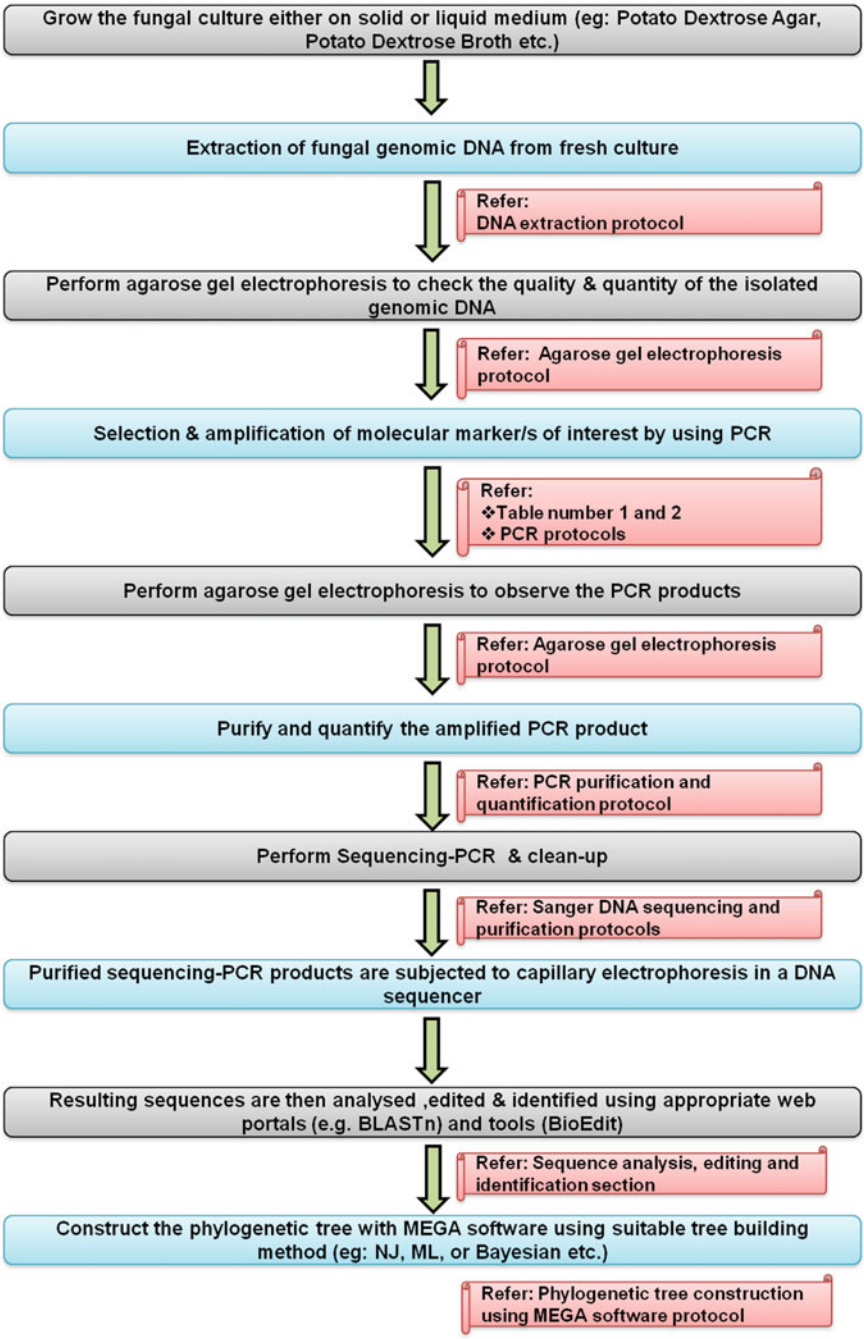

Fig. 10.2 A flow-chart of various steps towards molecular phylogenetic analysis of fungi

The basic criteria for the selection of a suitable DNA isolation method include (1) efficient DNA extraction, (2) good quantity of DNA, (3) removal of contaminants, (4) purity and quality of DNA, and (5) possibility of high throughput. Ultraviolet absorbance is mostly used to determine the purity of DNA. For a good-quality DNA, the quantitative ratio of absorbance at 260 nm and 280 nm (A260/A280) must be 1.8. If this ratio is less than 1.8, it indicates the DNA sample has protein contamination or has impurity of an organic solvent such as phenol, and if the ratio is more than 1.8, it indicates the DNA sample has RNA contamination. The quantification of dsDNA should be assessed by Qubit fluorometer analysis, while A260/A280 can be determined by NanoDrop spectrophotometer. Quality of DNA can also be assessed by visualization on agarose gel. A rapid and high throughput fungal DNA extraction protocol is mentioned below [1].

10.2.1 Materials

1. Source material – fungal culture of interest
2. DNA extraction vials containing ceramic pestle
3. Glass beads (425–600 μM, Sigma)
4. 10 ml syringe
5. Glass wool
6. Scalpel
7. Micropipettes
8. Micro centrifuge tubes/eppendorf tubes (1.5 ml)
9. Lysis buffer (100 mM Tris HCl [pH 8.0], 50 mM EDTA, 3% SDS)
10. Phenol: Chloroform: Isoamyl alcohol (25:24:1)
11. Isopropanol
12. 70% ethanol
13. 1XTE buffer (10 μl of 1 M Tris, 2 μl of 0.5 M EDTA and 988 μl dH_2O)
14. RNaseA (Sigma Aldrich, USA)
15. FastPrep®-24 tissue homogenizer (MP Biomedicals, USA)
16. Centrifuge 5415 R (Eppendorf)
17. Deep freezer (−20 °C)
18. Incubator set at 37 °C
19. NanoDrop 1000 spectrophotometer
20. Qubit fluorometer

10.2.2 Method

1. Scrape 300 mg of mycelia from agar plate or from the culture broth by filtering it through a 10–15 ml syringe containing some glass wool that will allow the fungal mass retaining in syringe and let the broth to pass through, and then

transfer the fungal mass into a screw capped tube containing ceramic pestle and some fine glass beads.
2. Add 1 ml lysis buffer and homogenize twice for 60s at 6 M/S.
3. Centrifuge the homogenized mycelia at 12,000 rpm for 15 min at room temperature (RT), and then transfer the supernatant in a fresh 1.5 ml Eppendorf tube.
4. Add equal volume of phenol-chloroform-isoamyl alcohol (PCI) and shake well (at least 30 times). Centrifuge for 10 min at 10,000 rpm at RT; separate upper aqueous layer in new 1.5 ml Eppendorf tube.
5. If any haziness or pigment persists then repeat the PCI treatment.
6. Add equal volume of Isopropanol and keep at −20 °C for 20 min.
7. Centrifuge for 10 min at 10,000 rpm at 4 °C. Remove supernatant and wash the pellet using 500 μL of 70% ethanol.
8. Again centrifuge at 10,000 rpm for 5 min at 4 °C. Remove supernatant and air dry the pellet.
9. Dissolve the pellet in 50–70 μL of 1X TE buffer.
10. Add 1 μL of RNaseA (10 mg/ml) and incubate it at 37 °C for 30 min.
11. 2 μL of genomic DNA is subjected to 0.8% agarose gel electrophoresis (refer agarose gel electrophoresis protocol).
12. Observe the gel under UV trans-illuminator gel documentation system.
13. Determination of quantity and quality of the isolated DNA can be done by NanoDrop spectrophotometer and Qubit fluorometer.

10.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis is used for separation of nucleic acid (DNA/RNA). DNA or RNA molecules are separated based on their size and molecular weight under an electrical field where, negatively charged molecules move toward positive pole known as anode. The migration flow is decided entirely according to the size where low molecular weight molecules migrate more rapidly than higher ones. The below mentioned table denotes the relationship between the Agarose gel percentage and effective DNA size separation.

Concentration of agarose gel (%)	Range of linear DNA Molecules (kb) for separation
0.6	1–20
0.8	0.5–10
1.2	0.4–6
1.5	0.2–3
2.0	0.1–2

10.3.1 Material

1. Gel electrophoretic unit including buffer tank, casting tray, gel cassette and comb
2. Voltage supplier
3. Running Buffer:
   (a) TAE (1 L – 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA of pH 8)
   (b) TBE (1 L – 242 g Tris base, 137.5 ml boric acid, 100 ml 0.5 M EDTA of pH 8)
4. Agarose
5. Conical flask
6. Ethidium bromide
7. Hot plate or microwave oven
8. Gel loading dye (6X- 1.5 g Ficoll400, 25 mg Xylene cyanol FF, 25 mg bromophenol blue – 10 ml dH_2O)
9. Gel documentation system (make Syngene, Biorad, etc.)

10.3.2 Method

1. Prepare 100 ml 0.5X Tris Borate EDTA/Tris Acetate EDTA (TBE/TAE) buffer of pH 8 in distilled water in a conical flask.
2. Weigh 0.8gm (for DNA, i.e. 0.8%)/1.2–1.5 gm (for PCR product, i.e. 1.2–1.5%) of agarose and add 100 ml of 0.5 X TBE/TAE buffer.
3. Keep it in microwave oven or on hot plate for 5–10 min to melt the gel.
4. Take the gel solution from oven or hot plate and allow it to cool (~60 °C).
5. Add 2–4 μL ethidium bromide (EtBr) of 10 mg/mL concentration and mix properly (EtBr is a well-known and widely used fluorescent dye used to visualize the DNA/RNA under UV light).
6. Assemble the gel caster with casting tray, comb and pour the gel. Let the agarose gel solidify for approx. 20–30 min.
7. Place the gel casting tray in electrophoretic chamber filled with running buffer (0.5X TBE/TAE, same as the gel preparation buffer).
8. Remove the comb and place the gel along with casting tray in the electrophoretic chamber.
9. Connect both the electrodes, switch on the current and allow the gel to run at 70–100 V for 20–30 min.
10. After 30 min, take out the gel from the electrophoretic chamber and observe it under UV transilluminator or in a gel documentation system.
    Note: If the DNA bands are bright and sharp, it indicates that the quantity and quality of DNA is good, but if a smear has been seen on gel, it indicates degradation of DNA (quantity and quality of DNA is poor).

Table 10.2 PCR conditions of different gene targets and their respective primer pairs

S. N.	Name of gene target	Primer pair/set	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles	References
1.	Internal Transcribed Spacer region of the rRNA (ITS)	**V9G/ITS4**	94 °C – 5 min	94 °C – 30 s	48 °C – 30 s	72 °C – 90 s	72 °C – 7 min	35	[37, 97]
		ITS1/ITS4	95 °C – 5 min	95 °C – 30 s	50 °C – 30 s	72 °C – 1 min	72 °C – 7 min	30	
		ITS5/ITS4	95 °C – 5 min	95 °C – 30 s	50 °C – 30 s	72 °C – 1 min	72 °C – 7 min	30	
		ITS-1F/ITS4	95 °C – 5 min	95 °C – 30 s	50 °C – 30 s	72 °C – 1 min	72 °C – 7 min	30	
2.	Large Subunit of the rRNA (LSU, 28S)	**LROR/LR6**	95 °C – 3 min	95 °C – 1 min	53 °C – 1 min	72 °C – 2 min	72 °C – 7 min	35	[94]
		LROR/LR5	94 °C – 5 min	94 °C – 1 min	52 °C – 30s	72 °C – 1 min	72 °C – 7 min	35	
		LSUFint/ LSURint	94 °C – 5 min	94 °C – 1 min	52 °C – 30s	72 °C – 1 min	72 °C – 7 min	35	[14]
		LSU1Fd/ LR5	94 °C – 3 min	94 °C – 30 s	52 °C – 30s	72 °C – 45 s	72 °C – 5 min	35	[23, 94]
		LS1/LR5	95 °C – 2 min	95 °C – 1 min	57 °C – 1 min	72 °C – 1 min	72 °C – 10 min	35	[72]
		LR3/LR5	94 °C – 5 min	94 °C – 1 min	52 °C – 30s	72 °C – 1 min	72 °C – 7 min	35	[94]
		Rust28SF/ LR5	94 °C – 5 min	94 °C – 1 min	55 °C – 30s	72 °C – 1 min	72 °C – 7 min	35	[8]
		NL1/NL4	94 °C – 5 min	94 °C – 1 min	52 °C – 30s	72 °C – 1 min	72 °C – 7 min	35	[48]
3.	Small Subunit of the rRNA (SSU, 18S)	**NS1/NS4**	94 °C – 5 min	94 °C – 1 min	51 °C – 30s	72 °C – 1 min	72 °C – 7 min	35	[89]
		RustNS2-F/ NS6	94 °C – 5 min	94 °C – 1 min	55 °C – 30s	72 °C – 1 min	72 °C – 7 min	35	[8]
		SR1R/SR7	95 °C – 2 min	95 °C – 1 min	57 °C – 1 min	72 °C – 1 min	72 °C – 10 min	35	[94]
4.	RNA polymerase II subunit 1 (*RPB1*)	**CRPB1/ RPB1C**[a]	94 °C – 5 min	94 °C – 1 min 94 °C – 35 s	37 °C – 35 s (1° every 4 s to 72 °) 45 °C – 55 s(1° every 4 s to 72°)	72 °C – 1 min 72 °C – 1 min	72 °C – 10 min	4 29	[19]

(continued)

Table 10.2 (continued)

S. N.	Name of gene target	Primer pair/set	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles	References
		Fa/G2R	94 °C – 5 min	94 °C – 1 min	45 °C – 30 s	72 °C – 1 min	72 °C – 7 min	35	[65]
		RPB1-Af/ RPB1-Cr	95 °C – 2 min	95 °C – 1 min	57 °C – 1 min	72 °C – 1 min	72 °C – 10 min	35	[15, 88]
		RBP1-F1843/ RPB1-R3096[a]	94 °C – 5 min	94 °C – 30 s 94 °C – 30 s 94 °C – 30 s	51 °C – 30 s 49°C – 30 s 47°C – 30 s	72 °C – 1 min 72 °C – 1 min 72 °C – 1 min	72 °C – 10 min	5 5 30	[76]
5.	Second largest subunit of RNA polymerase II (*RPB2*)	**RPB2-5F2/ RPB2-7cR**	94 °C – 90 s	94 °C – 30 s	55 °C – 90 s	68 °C – 90 s	68 °C – 5 min	40	[64]
		RPB2for/ RPB2rev	94 °C – 5 min	94 °C – 30 s	55 °C – 30 s	72 °C – 90 s	72 °C – 10 min	35	[86]
		RPB2-7cF/ RPB2-11aR[a]	94 °C – 5 min	94 °C – 1 min	37 °C – 35 s (1° every 5 s to 72 °)	72 °C-1 min	72 °C – 10 min	30	[51]
		bRPB2-6F/ gRPB2-6R	94 °C – 4 min	94 °C – 1 min	51 °C – 1 min	72 °C – 1 min	72 °C – 8 min	36	
6.	Beta-tubulin (β-tub)	**Bt2a/Bt2b**	94 °C – 5 min	94 °C – 1 min	58 °C-1 min	72 °C-1 min	72 °C – 7 min	32	[29]
		BT1-F/BT1-R	94 °C – 3 min	94 °C – 30 s	56 °C – 30 s	72 °C – 45 s	72 °C – 5 min	40	[7]
		T1/T2	95 °C – 4 min	95 °C – 30 s	55 °C – 30 s	72 °C – 45 s	72 °C – 7 min	40	[61]
		T12/T22	94 °C – 5 min	94 °C – 1 min	52 °C – 30 s	72 °C – 2 min	72 °C – 7 min	35	[90]
		T1/ CYLTUB1R	94 °C – 2 min	94 °C – 1 min	55 °C – 30 s	72 °C – 1 min	72 °C – 10 min	35	[22, 61]
		Btub_F1/ Btub_R1A	95 °C – 3 min	95 °C – 1 min	60 °C – 1 min	72 °C – 2 min	72 °C – 5 min	35	[14]
		T10/Bt2b	96 °C – 30s	94 °C – 1 min	58 °C – 1 min	72 °C – 1 min	72 °C – 5 min	32	[29, 61]

7.	Translation elongation factor 1-alpha (*TEF1*-α)	**EF1-728F/ EF1-986R**	94 °C – 5 min	94 °C – 30s	52 °C – 30 s	72 °C – 45 s	72 °C – 7 min	40	[18]
		EF1-983F/ EF1-2218R[a]	94 °C – 4 min	94 °C – 1 min	56–62 °C – 1 min (1° increase in every cycle till 7 cycle)	72 °C – 1 min	72 °C – 8 min	40	[71]
		EF1/EF2[a]	94 °C – 4 min	94 °C – 1 min	62–52 °C –30 s (1° decrease in every cycle till 9 cycle)	72 °C – 1 min	72 °C – 8 min	30	[61]
		EF1-728F/ EF2	94 °C – 2 min	94 °C – 1 min	52 °C – 30 s	72 °C – 1 min	72 °C –10 min	35	[18, 61]
		EF1-1577F/ EF1-1567R[a]	94 °C – 4 min	94 °C – 1 min	56–62 °C –1 min (1° increase in every cycle till 7 cycle)	72 °C – 1 min	72 °C – 8 min	40	[71]
		EF1-728F/ TEF1LLErev	94 °C – 2 min	94 °C – 1 min	52 °C – 30s	72 °C – 1 min	72 °C – 10 min	35	[18, 41]
		MEF-4/MEF-10	94 °C – 2 min	94 °C –15 s	55 °C – 30s	72 °C – 1 min	72 °C – 5 min	35	[62]
		VEFf/VEFr	94 °C – 2 min	94 °C – 10s	60 °C – 20s	72 °C – 1 min	72 °C –7 min	32	[40]
8.	Actin	**ACT-512F/ ACT-783R**	94 °C – 5 min	94 °C – 30s	58 °C – 30s	72 °C – 45 s	72 °C – 7 min	35	[18]
		Act1/Act4a	95 °C – 5 min	95 °C –1 min	58 °C-1 min	72 °C – 2 min	72 °C – 7 min	35	[5]
		VActF/ VActR	94 °C – 2 min	94 °C – 10s	48 °C – 20s	72 °C – 1 min	72 °C – 7 min	32	[40]
9.	Sixth subunit of ATP synthase (*ATP6*)	**ATP6C1A/ ATP6C2A**[a]	94 °C – 5 min	94 °C – 1 min 94 °C – 35 s	37 °C – 35 s (1° every 4 s to 72 °) 45 °C – 55 s(1° every 4 s to 72 °)	72 °C – 1 min 72 °C – 1 min	72 °C – 10 min	4 29	[19]

(continued)

Table 10.2 (continued)

S. N.	Name of gene target	Primer pair/set	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles	References
10.	HSP60	**HSP60for/ HSP60rev**	94 °C – 5 min	94 °C – 30s	55 °C – 30 s	72 °C – 90 s	72 °C – 10 min	35	[86]
11.	GAPDH	**GPD1/GPD2**	94 °C – 5 min	94 °C – 30s	48 °C – 30s	72 °C – 90s	72 °C – 7 min	35	[9]
		G3PDHfor/ G3PDHrev	94 °C – 5 min	94 °C – 30s	64 °C – 30s	72 °C – 90s	72 °C – 10 min	35	[86]
		Gpd1-LM/ Gpd2-LM	94 °C – 5 min	94 °C – 45 s	53 °C – 45 s	72 °C – 90s	72 °C – 5 min	40	[7]
		GDF/GDR	95 °C – 4 min	95 °C – 30 s	60 °C – 30 s	72 °C – 45 s	72 °C – 7 min	35	[91]
		VGPDf2/ VGPDr	94 °C – 2 min	94 °C – 10 s	56 °C – 20 s	72 °C – 1 min	72 °C – 7 min	32	[40]
12.	Calmodulin (*CaM*)	**CF1/CF4**	94 °C – 5 min	94 °C – 45 s	55 °C – 45 s	72 °C-1 min	72 °C – 7 min	35	[66]
		CF1L/CF4	96 °C – 2 min	96 °C – 30 s	51 °C – 30 s	72 °C – 90s	72 °C – 5 min	42	
		CF1M/CF4	96 °C – 2 min	96 °C – 30 s	51 °C – 30 s	72 °C – 90s	72 °C – 5 min	42	
		Cal228F/ CAL2Rd	95 °C – 8 min	95 °C – 15 s	55 °C – 20 s	72 °C-1 min	72 °C – 5 min	35	[18]
		cmd5/cmd6	96 °C – 2 min	96 °C – 30 s	51 °C – 30 s	72 °C – 90s	72 °C – 5 min	42	[35]
		Cal228F/ Cal737R	94 °C – 2 min	94 °C – 1 min	55 °C – 30 s	72 °C-1 min	72 °C – 10 min	35	[18]
		CL1/CL2a	95 °C – 5 min	95 °C – 30 s	55 °C – 30 s	72 °C-1 min	72 °C – 8 min	35	[63]
13.	Mini chromosome maintenance protein(*MCM7*)	**MCM7–709/ MCM7–1348**	94 °C – 10 min	94 °C – 45 s	56 °C – 50 s	72 °C-1 min	72 °C – 5 min	38	[78]

14.	CHS-1	**CHS-79F/ CHS-345R**	95 °C – 8 min	95 °C – 15 s	55 °C – 20 s	72 °C-1 min	72 °C – 7 min	32	[18]
15.	Histone-3 (HIS3)	**CYLH3F/ CYLH3R**	96 °C – 5 min	96 °C – 30s	52 °C – 30 s	72 °C – 1 min	72 °C – 5 min	30	[22, 29]
		CYLH3F/H3-1b	94 °C – 5 min	94 °C – 30s	58 °C – 30 s	72 °C – 1 min	72 °C – 5 min	40	
16.	Cox2	**FM35/ Phy10b**	94 °C – 3 min	94 °C – 1 min	54 °C – 1 min	72 °C – 2 min	72 °C – 5 min	35	[56]
		FM58/ FM66	94 °C – 5 min	94 °C – 1 min	52 °C – 1 min	72 °C – 2 min	72 °C – 7 min	32	[55]
17.	Cox3	**CO3F1/ CO3R1**	94 °C – 5 min	94 °C – 1 min	50 °C – 1 min	72 °C – 1 min	72 °C – 7 min	35	[8]
18.	Nad9	**Nad9-F/ Nad9-R**	94 °C – 3 min	94 °C – 1 min	61 °C – 1 min	72 °C – 2 min	72 °C – 5 min	35	[56]
19.	Acl1	**acl1-230up/ acl1-1220low**[a]	95 °C – 3 min	95 °C – 45 s 95 °C – 45 s 95 °C – 45 s	64 °C – 45 s 62 °C – 45 s 56 °C – 45 s	72 °C – 2 min 72 °C – 2 min 72 °C – 2 min	72 °C – 8 min	5 5 30	[31]
20.	TS	**VTs3f/VTs3r**	94 °C – 2 min	94 °C – 10s	59 °C – 20s	72 °C – 1 min	72 °C – 7 min	32	[40]
21.	ApMAT	**AM-F/AM-R**	94 °C – 3 min	94 °C – 45 s	62 °C – 45 s	72 °C – 1 min	72 °C – 7 min	30	[84]
22.	Cytochrome B (CYTB)	**E1M4/E2mr3**	94 °C – 4 min	94 °C – 1 min	46 °C – 1 min	72 °C – 1 min	72 °C – 4 min	36	[12]
23.	Alt a1	**Alt-for/Alt-rev**	94 °C – 4 min	94 °C – 30s	57 °C – 30 s	72 °C – 1 min	72 °C – 7 min	35	[36]
24.	OPA1–10	**OPA10-2L/ OPA10-2R**	94 °C – 5 min	94 °C – 30s	62 °C – 30 s	72 °C – 45 s	72 °C – 7 min	36	[6]
25.	Endo PG	**PG3/PG2b**	94 °C – 5 min	94 °C – 30s	50 °C – 30 s	72 °C – 30 s	72 °C – 7 min	40	

[a] Touchdown PCR conditions

10.4 Selection of Gene Target and Their Amplification

Selection of gene target is very crucial step in the fungal molecular taxonomy and phylogeny. Most common DNA markers proposed for molecular identification and phylogeny of fungi are the nuclear ITS region of the ribosomal RNA gene. We have enlisted the suitable gene targets/markers which can be used for specific fungal genera and/or species level identification, their respective primer pair, primer sequences, and PCR cycling conditions (Tables 10.1 and 10.2). We have provided this information for few selected fungi, and for the non-listed fungi, the researchers are advised to find out the recommended gene targets/markers by following the recent literature on the same.

10.4.1 Materials

1. Taq DNA polymerase and 10X DNA polymerase buffer (Sigma)
2. Forward and reverse primers
3. dNTPs mix (Sigma)
4. DNA template
5. Sterile distilled water
6. 0.2 ml PCR tubes
7. Micropipettes
8. PCR tube stand
9. Thermal cycler

10.4.2 Method

The steps for PCR amplification are as described below. The total volume can either be 25 or 50 μL.

1. Thaw all the PCR reagents completely on ice before use. (Make sure the reagents were kept at −20 °C).
2. Prepare PCR reaction mix in a thin-walled 0.2 mL PCR tubes for 25/50 μL reaction volume.
3. All the PCR reagents can be added in the following order: water, buffer, dNTPs, forward and reverse primers, Taq DNA polymerase and DNA template, or if one has PCR master-mix, then the order should be water, master-mix (e.g. EmeraldAmp GT PCR Master Mix, Takara), both primers and DNA template.

Reagents	Volume		Final concentration
PCR buffer (10X)*	2.5 µl	5 µl	1X
dNTPs mixture	2 µl	4 µl	200 mm
Forward primer	0.5 µl	1 µl	10pM/µl
Reverse primer	0.5 µl	1 µl	10pM/ µl
Taq DNA polymerase	0.25 µl	0.25 µl	1unit/µl
Template	1 µl	1 µl	10 ng/µl
dH_2O	18.25 µl	37.75 µl	
Total	**25 µl**	**50 µl**	

*10x PCR buffer can be used either (Mg^{2+} free), or $MgCl_2$ solution shall be used with 10x Buffer, if necessary

4. Gently pipette the reaction mixture to allow good mixing and give short spin to settle down the reagents.
5. Prepare positive and negative control reactions with and without template DNA of known size, respectively along with test sample. (**Note:** positive control confirms that all PCR reagents are working properly, whereas negative control confirms that there is contamination in reagents which will result into false band).

   Note: If one wants to perform a greater number of PCR reactions, then it is recommended to first prepare a PCR master mix, except the reagents which are to be varied. (e.g., DNA template). For all the PCR related information (Primer name and sequences, PCR conditions), refer Tables 10.1 and 10.2.
6. 2 µL of PCR product is subjected to 1.2% agarose gel electrophoresis (refer agarose gel electrophoresis protocol).
7. Observe the gel under UV transilluminator gel documentation system.

10.5 PCR Product Purification and Quantification

Purification of PCR product is essential before proceeding with DNA sequencing, which aids the removal of remaining enzymes, primers, nucleotides, and buffer components. Traditionally, purification was done by organic extraction methods like phenol chloroform treatment, followed by ethanol precipitation. Nowadays, there are various rapid and less hazardous PCR clean-up kits are available. These kits utilize spin columns having a matrix of silica on which DNA is bound selectively. Finally, elution of the DNA occurs from the column with elution buffer or milli-Q water and is ready for DNA sequencing. The DNA fragment of interest can be purified by gel extraction method (gel extraction kits can be used) if the PCR product is contaminated with any non-specific amplification product and primer dimers. The most commonly used kits are GenElute PCR Clean-Up Kit Sigma-Aldrich, FavorPrep™ GEL/PCR Purification Kit – Favorgen, QIAquick PCR Purification Kit – Qiagen, etc. (each kit has its own protocol; one can follow the manufacturer's guidelines provided with the kits).

After PCR product purification, quality and quantity of amplicons must be checked before proceeding with DNA sequencing. To check the quality and size of PCR product, they are analyzed on agarose gel electrophoresis along with appropriate DNA marker/ladder (see agarose gel electrophoresis protocol). For quantity and quality check, NanoDrop spectrophotometer and Qubit fluorometer can be used. Details and basic principle of quantification of DNA/PCR product is given in section High throughput DNA extraction protocol.

10.6 DNA Sequencing (Sanger Method)

DNA sequencing is the process to determine the order of nucleotides in a given DNA molecule. The first-generation sequencing approach emerged in the 1970s, which included the Maxam-Gilbert method and the Sanger method (or dideoxy method), discovered by English biochemist Frederick Sanger. Eventually, Sanger method became the more commonly employed technique among these two approaches because it is a rapid and easy method for DNA sequencing. This conventional chain-termination approach requires a single-stranded DNA template, a primer, DNA polymerase, dNTPs, and modified di-deoxynucleotide triphosphates (ddNTPs), the latter of which plays a key role in terminating DNA strand elongation. These chain-terminating ddNTPs lack a 3'-OH group, which is required for phosphodiester bond formation between two consecutive nucleotides; hence, the moment it gets incorporated into newly synthesised strand it halts the extension of DNA [77]. The ddNTPs are fluorescently labelled for detection in automated sequencing machines. The protocol mentioned here is of Applied Biosystems – BigDye™ Terminator v3.1 cycle sequencing kit.

The direct approach of cycle sequencing of amplified and purified PCR products depends on two factors, i.e. source material (type and amount) and contamination like proteins, RNA or chromosomal DNA, excess primers, dNTPs, enzyme, residual salts along with some organic chemicals like phenol and chloroform. Before proceeding with sequencing, the critical requirement is to quantify the purified DNA and calculate A260/280 ratios (A260/280 = 1.8–2.0). The optimum concentrations of DNA template are mentioned below:

Recommended DNA Quantities

DNA template (purified PCR product)	Quantity
100–200 bp	1–3 ng
200–500 bp	3–10 ng
500–1000 bp	5–20 ng
1000–2000 bp	10–40 ng
>2000 bp	20–50 ng

10.6.1 Materials

1. BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific)
2. Distilled water
3. Hi-Di formamide (Thermo Fisher Scientific)
   Note: Not required for BigDye XTerminator purification kit (Thermo Fisher Scientific)
4. Primers of interest
5. MicroAmp clear adhesive film (Thermofisher)
6. MicroAmp optical 96-well reaction plate (Thermo Fisher Scientific)
7. Vortex machine
8. Centrifuge with swinging bucket (with PCR plate adapter)
9. BigDye XTerminator purification kit (Thermo Fisher Scientific)
10. EDTA (125 mM)
11. 100% and 70% ethanol
12. Micropipettes
13. 0.2 ml PCR tubes
14. PCR tube stand

10.6.2 Method

1. Thaw all the reagents of the BigDye Terminator v3.1 cycle sequencing kit and primers completely (place on ice).
   Note: Prevent dye terminators to light exposure.
2. Vortex and centrifuge briefly the tubes for 3–4 s to collect all tube contents at bottom.
3. Add components in 0.2 ml thin-walled tube to prepare reaction mix as indicated below:

S. N.	Reagents	Volume
1.	5X sequencing buffer	2 μL
2.	Primer (primer of interest)	0.6 μL
3.	BigDye terminator 3.1	1.2 μL
4.	Template-purified PCR product (10-50 ng depend on length of template, refer recommended DNA quantities)	1 μL
5.	Sterile dH_2O	1 μL
6.	Total	10 μL

Note: One can use BDT 3.1 ready mix (3–4 μL) that has sequencing buffer also, so no need to add sequencing buffer separately

10.7 Perform Sequencing PCR in Thermal Cycler Using Following Conditions

Parameter	Temperature	Time	Cycle
Initial denaturation	96 °C	1 min	1
Denaturation	96 °C	30 s	25
Annealing	55 °C	15 s	
Extension	60 °C	4 min	
Final extension	60 °C	2 min	1
Hold	4 °C	Hold	

10.7.1 Purification of Sequencing PCR Product

1. Add 2 μL of freshly prepared EDTA (125 mM) to sequencing PCR product.
2. Add 50 μL of 95–100% ethanol to the tube, mix well by pipetting.
3. Incubate it for 15 min at RT and centrifuge at 12,000 rpm for 20 min at 4 °C.
4. Decant the supernatant and add 200 μL of 70% ethanol, and centrifuge at 12,000 rpm for 10 min at 4 °C.
5. Repeat 70% ethanol wash and air dry the pellet.
6. Add 10–12 μL of Hi-Di formamide.
   Note: One can use BigDye XTerminator™ purification kit (catalog number: 4376486) for the purification of sequencing PCR product using manufacturers' instruction and protocol.
7. Place the purified sequencing PCR product in MicroAmp optical 96-well reaction plate.
8. Seal the plate with MicroAmp clear adhesive film.
9. Vortex it for 3–4 s, then give short spin in a swinging bucket centrifuge, to collect all the plate contents at bottom of the wells for 8–10 s.
10. Put the plate in a thermal cycler and denature the DNA at 96 °C for 5 min.
11. The plate containing DNA samples is then subjected to appropriate Genetic Analyser or DNA Sequencer (e.g. ABI PRISM ® 3100-Avant Genetic Analyzer).

10.8 Sequence Editing, Analysis, and Identification

Once the DNA sequences are generated, the most important task is to ensure the quality of the DNA sequences. Different tools like Finch TV and Chromas Lite can be used to visualize the DNA sequence in the form of electropherogram. Both these tools are freely available to download. Another important point is that one should try

to get full length DNA sequence of any given gene target/marker. If the sequence length is short, one should sequence both the strands of the DNA and make a consensus sequence to get the full-length sequence of the gene. Usually the quality scores of the initial and last few bases (20–30) in a given DNA sequence are not always up to the mark; therefore, while performing similarity searches, these few bases should not be considered.

10.8.1 Basic Local Alignment Search Tool (BLAST)

BLAST is an algorithm for comparing primary biological sequence information, such as the amino acid or nucleotide sequences of proteins and DNA/RNA, respectively. It has been the most widely used portal for comparison of query sequences with a library or database of sequences, to identify the resembling sequences above a certain threshold value. There are different tools which can be employed according to ones' need for example BLAST (blastn) program (DNA/RNA), in which given DNA query is compared with the most similar DNA sequences from the DNA database as per user specifications. Pairwise sequence alignment allows you to compare the sequence of interest with all known sequences in a database so that all homologous sequences are attained. Basically, they show most closely related sequences first, followed by sequences with diminishing similarity. These matches are generally reported to assess the statistical significance like an Expectation-value.

The important point to be considered while working with fungal query sequences using BLAST search is its results against GenBank database because approximately 27% of GenBank fungal ITS sequences were deposited with inadequate taxonomic identification [59] and 20% of fungal sequences are incorrectly annotated, but this varies according to taxonomic group [16, 69].

10.8.2 MycoBank Database Search

MycoBank is a recognized nomenclature repository for all published fungal novelties since 2004, which provides an open access database to mycologists [22]. It also allows online pairwise sequence alignment and identification (www.mycobank.org/biolomicssequences.aspx) against other well-curated databases such as CBS collections websites, Q-bank, EUBOLD system, ITS Database for Human and Animal Pathogenic Fungi (ISHAM), Fungal Barcoding, or UNITE. The unknown DNA sequences are compared against all the required reference databases either at the same time or against individual database, on the basis of which the results are shown centrally, as a unique matching list of fungi. This particular database identifies new species in reference with information based on morphological, physiological, and molecular data.

10.8.3 UNITE Database Search

UNITE (https://unite.ut.ee/) is an online database managing large number of sequences which is useful for the fungal molecular identification. It targets the nuclear rDNA-ITS region and offers 10,00,000 public fungal ITS reference sequences. There are 4,59,000 species hypothesized cluster to which digital object identifiers (DOIs) are assigned to avoid ambiguous referencing across studies. In the past 15 years, the curation and annotation of sequence by a third party have made approximately 2,75,000 improvements, both in-house and web-based. UNITE in collaboration with other community resources and fungal sequence database provides a large amount of data for a number of meta-barcoding software pipelines [58].

10.8.4 Method

1. Obtain sequences from sequencer in the form of an electropherogram and edit either manually using Chromas Lite software or by BioEdit software for inconsistencies.
2. Align with sequences in the NCBI BLASTn tool for identification.
3. Cases where no substantial similarity is found with sequences in the databases confirm the identity with the help of sequences from other loci of same strain or sequences from additional strains.

Note: A large part of the known fungal diversity is not sequenced at all; hence, one will be unable to identify an unknown fungus, as the sequence information will not be available in the database. Moreover, few of the sequences deposited in the databases might be having errors; therefore, one has to be cautious in identification and drawing any phylogenetic conclusion based purely on NCBI database comparisons. In such situations, one can also try using other databases, e.g., MycoBank and UNITE.

10.9 Molecular Phylogeny and Phylogenetic Tree

Phylogenetics is the branch of Science that estimates and evaluates evolutionary relationships among individuals/organisms for their identification. In order to determine phylogenetic relationship, alignment and comparison of different fungal sequences can be done and on the basis of their physical and genetic features a number of differences can be determined. This relationship is represented in the form of “phylogenetic tree” in which the branch’s position and length represent the relation between different fungi. Closely related fungi will exhibit lesser sequence differences than distantly related fungi.

10.9.1 Phylogenetic Tree Construction

Phylogenetic trees can be constructed using DNA sequences of closely related fungi and various computational phylogenetic methods. The basic and simple method of tree construction is distance-based method including neighbour joining and UPGMA method, which performs multiple sequence alignment using ClustalW method and calculate genetic distances. The most common tree construction method is character-based: include maximum parsimony that involves an inherent model of evolution (i.e. parsimony), and maximum likelihood which is an advanced method that uses Bayesian scheme and applies a precise open model of evolution. Identification of a most favourable phylogenetic tree is non-parametric; therefore, the optimization and combination of these methods are required to identify a best possible and optimal phylogenetic tree, while building trees bootstrapping is followed. A bootstrap is a statistical method for assessing phylogenetic tree where the bootstrap value represents that out of 100 how many times the same specific branch is observed when repeating the phylogenetic tree reconstruction from the same set of data.

10.9.2 Phylogenetic Tree Construction Using MEGA Software

1. A number of taxonomical target DNA sequences are retrieved from BLAST searches and GenBank.
2. The DNA sequence of test strain and all other relevant DNA sequences are saved in FASTA file format.
3. The FASTA file format is converted to .fas file format.
4. The **.**fas file is opened in MEGA 7 or X software and subjected to multiple sequence alignment (MSA) using ClustalW or MUSCLE.
5. The alignment file can be saved for further analysis as MAS file.
6. The MAS file is opened in MEGA 7/X software.
7. The MSA data is subjected to phylogenetic tree construction using suitable method like maximum parsimony, maximum likelihood, NJ, and UPGMA.
8. Different parameter needs to be instructed to the program to construct a phylogenetic tree.
9. An example is shown in below mentioned table.

Analysis	Phylogenetic tree construction
Statistical method	Maximum parsimony
Test of phylogeny	Bootstrap method
Number of bootstraps	1000
Model	Kimura-2 parameter model
Branch swap filter	Very strong
Tree inference option	Neighbour joining

10. Once the tree is constructed the session of tree can be saved as MTS file.
11. Post-construction, various modifications can be done in the tree.
12. The tree can be obtained as JPEG image/PDF file along with the caption.

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Chapter 11
Fluorochrome-Based Methods for Fungal Sample Examination

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Contents

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11.1 Introduction

Fungi are eukaryotic, usually filamentous, spore-producing organisms and can be obligate parasites, nonobligate parasites, or biotrophs, developing several interactions with plants, animals, or the environment and can be used to produce food and enzymes for industrial processes [27]. Most fungi species have microscopic structures and studies on this organism group depend on various microscopy techniques types.

Stokes related the fluorescence phenomenon in 1852, describing a photon molecular absorption generating the emission of another photon with greater wavelength, the principle from which it was possible to develop techniques of fluorescence microscopy [20]. Therefore, natural or induced fluorescence characteristics have been explored for organisms and macromolecules localization explaining several types of biological phenomena, mainly by techniques of epi-fluorescence or laser confocal microscopy.

These techniques enable many morphological and physiological analyzes in cells and tissues, locating cellular components, interaction with plants, nuclear dynamics, reactive oxygen species accumulation, and cellular death.

Fluorescence microscopy studies may include analyzes of fluorescence or autofluorescent samples. Some fungi like Basidiomycota are autofluorescent, and

others such as *Cercospora* spp. produce fluorescent phytotoxins. Other studies types are conducted by inducing fluorescence in the samples. This may occur through the use of fluorochromes, immunofluorescence techniques, nucleic acids hybridization, and molecular markers. Fluorochromes are molecules capable of specifically binding to cellular components by inducing their fluorescence under the excitation of certain wavelengths. These components can be the fungal cell wall, nuclei, chromosomes, mitochondria, and others. Other fluorochromes may indicate physiological aspects, such as cell death, accumulation of reactive oxygen species, or evidence of defense reactions in plant tissues colonized by fungi. Immunofluorescence, also called immunostaining, is a technique where fluorescent molecules are attached to antibodies corresponding to antigens to which they will be located. Nucleic acid hybridization, called FISH (fluorescent in situ hybridization), allows the use of nucleic acid probes attached to fluorescent molecules. These probes are complementary to target sequences, which can identify specific regions on chromosomes, the expression of certain genes by mRNAs, or specific groups of organisms using regions of phylogenetically important DNA. One of the most commonly used molecular markers is fluorescent proteins such as GFP (green fluorescent protein). By genetic transformation, the fluorescent markers genes are associated with the genes whose products will be localized. Thus, the expressed proteins are localized through the fluorescent protein anchored.

In this chapter, protocols for fluorescence microscopy will be discussed in studies on fungi using only fluorochromes or autofluorescence of structures for localization techniques.

All following procedures were done at the Electron Microscopy and Ultrastructural Analysis Lab at Federal University of Lavras, using an inverted Epi-Fluorescence Zeiss Axio Z.1 and an inverted Laser Confocal Zeiss LSM780 Observer Z.1 and Zen 2012 software.

11.2 Sample Preparation to Observe Fungi Nuclei and Chromosome

Ascomycota fungi have the characteristic of maintaining monocariotic state but may present some dicariotic cells throughout the intermediary processes of their reproduction, such as plasmogamy and karyogamy. In the case of Basidiomycota, they may have different phases throughout their life cycle, monocariotic or even dicariotic (Fig. 11.1e). Basidiomycota may present multinucleate cells, as basidia before nuclei migration to the basidiospores, and the in certain *Rhizoctonia* hyphae (Fig. 11.1f). These nuclear dynamics processes can be studied using fluorescence markers, such as DAPI, one of the most used. Often, DAPI is used associated with immunofluorescence and hybridization techniques, for proteins or DNA sequences specific localization in nuclei. Other fluorescent nuclei dyes can be used, such as SyBrGreen and Propidium Iodide (in the case of living cells).

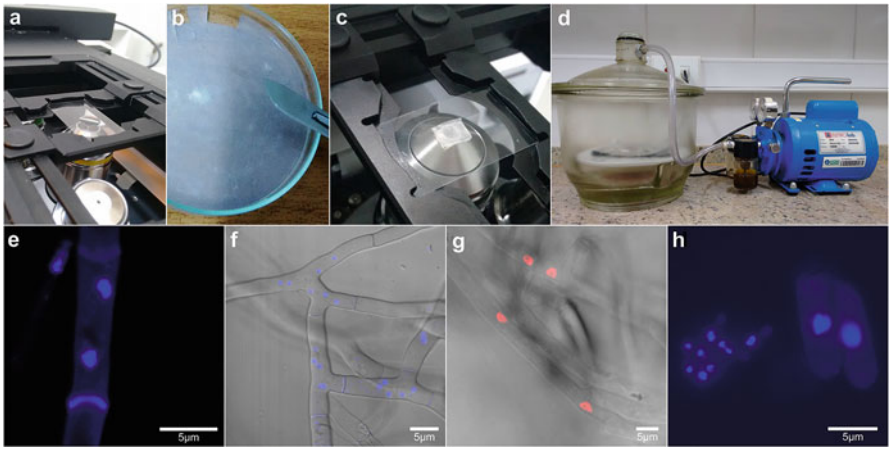

Fig. 11.1 Fungi nuclei and chromosome observations by fluorochrome-based methods. (**a**) Positioning of DAPI drops above coverslip coupled in an inverted microscope. (**b**) Sampling of *Rhizoctonia solani* colony growth in SNA media. (**c**) Transferring of colony fragment to the dye. (**d**) Vacuum pump linked to a hermetically sealed recipient. Laser scanning confocal micrographs of *Rhizoctonia solani* from binucleated (**e**) and multinucleated lineages (**f**) marked with DAPI; *Rhizoctonia* sp. nuclei marked with Propidium Iodide (**g**); and *Colletotrichum* chromosome at metaphase observation by germ tube burst method using DAPI (**h**)

11.3 DAPI Methods

Materials to prepare fungi for EFM or LSM observation. The necessary materials can vary depending on the protocol used.

11.3.1 Materials

1. Nucleus marker DAPI (4′6-Diamidine-2′-phenylindole dihydrochloride) (VectaShield® H-1200 Vector®) mounting medium.
2. Calcofluor White (Fluorescent brightener 28, Sigma, CAS-4404-43-7) 0.01% (p/v) prepared in 0.01 M potassium phosphate buffer solution (PBS) pH 7.2.
3. Fungi colony growth within a clear media, such as Synthetic Nutrient-poor Agar (SNA) [4], with living mycelia, or fixed fungi fragments.
4. Kolle handle with a needle
5. Scalpel.
6. Tweezer.
7. Bunsen burner.
8. Alcohol.
9. Automatic pipette.
10. Slides and regular coverslips (e.g. 20 × 20 mm).

11. Large coverslips (e.g. 24 × 40 mm).
12. Nail polish or other coverslip sealants.
13. Vacuum pump linked to a hermetically sealed container.
14. Stereomicroscope.
15. EFM or LSM, vertical or inverted.
16. For EFM, a filter cube that works near 405 ηm excitation and 450 ηm emission (ex. Filter cube Zeiss #49).
17. For LSM, excitation with Diode 405 ηm laser line and emission filter with 420–480 ηm range. An EC Plan-Neofluar 40×/1.30 Oil DIC M27 objective was used, with 1024 × 1024 resolution, and around fourfold zoom. The bright-field image was acquired using the TPM-T detector.

11.3.2 Methods

The methods presented below are for marking filamentous fungi nuclei, and variations will be presented and discussed.

11.3.3 Culture Block on Inverted Microscope

Firstly, we will show a quick preparation to observe nuclei in fungi (Fig. 11.1e, f) using an inverted microscope (EFM or LSM) as made by Melo et al. [17].

1. Put a previously cleaned large coverslip (e.g. 24 × 40 mm) on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
2. Put 10 μL of DAPI-VectaShield mounting media above the coverslip (Fig. 11.1a). In case of many samples working, use a marker pen to delimitate and identify spots with around 0.5 × 0.5 cm where each sample will be placed. Avoid the coverslip corners, where the objective lens does not reach. Develop it in a room with diffuse light, or as dark as possible.
3. Take colony fragments with around 0.5 × 0.5 cm with a scalpel (Fig. 11.1b), choosing the region of interest using a stereomicroscope, if needed. The use of clear media like SNA allows better light transmission. Besides, it favors the fungi sporulation and reduces the aerial mycelia growth rate. The colony fragments may be fresh or fixed.
4. Transfer the upside-down colony fragment above the DAPI drop. The mycelia should be in contact with the dye (Fig. 11.1c).

5. Leave it in the dark for 5–20 min. The incubation time will depend on the fungus species. The thinner cell wall species as well as the younger mycelia usually need less time to mark the nuclei. Some thicker cell wall species demand incubation with vacuum for dye infiltration. This can be done using a vacuum pump linked to a hermetically sealed container (Fig. 11.1d). In these cases, it is recommended to use younger cultures and colony border regions.
6. Observe in inverted EFM or LSM.

If the LSM is equipped with a detector like T-PMT, the bright-field images acquired overlaid with fluorescent nuclei may dispense the need of other fluorochromes for fungi cell walls marking, such as Calcofluor White. Sometimes, DAPI colors well the cell walls also. Once the LSM is not equipped for bright field images, the Calcofluor White use is recommended. For this, the DAPI needs to be applied before and directly in the culture fragment. After this, 10 μL of the 0.01% (p/v) Calcofluor White (Fluorescent brightener 28, Sigma, CAS-4404-43-7) prepared in 0.01 M potassium phosphate buffer solution (PBS) pH 7.2 are positioned above coverslip, as described in step 2. Follow, the colony fragment DAPI-treated should be positioned upside-down above the Calcofluor drop and incubate for around 20 min in darkness before observation. The excitation/emission parameters are the same used for DAPI in EFM or LSM.

11.3.4 Slide and Coverslip Preparation

Below is a procedure to observe fungi nuclei using slide and coverslip (vertical or inverted microscope, EFM or LSM):

1. Put 10 μL of DAPI- VectaShield mounting media above a previously cleaned slide.
2. Sample fungi structures from fresh or fixed cultures using a needle previously cleaned with alcohol and flamed. Choose the region of interest using a stereoscopic microscope, if needed. In this case, the DAPI fluorescence signal in slides prepared with fixed structures may persist for weeks, if slides kept in the refrigerator, in darkness.
3. Transfer the specimens to DAPI drop.
4. Cover the drop with specimen with coverslip and seal with nail polish or other coverslip sealants.
5. Leave it in the dark for 5–20 min. The time may vary, as discussed above. A vacuum step may be required.
6. Observe in a vertical or inverted microscope, EFM or LSM.

11.4 Culture Block on Inverted Microscope and Propidium Iodide in Nuclei Marking

Propidium Iodide can be used as a fungi nuclei marker in the case of living cells (Fig. 11.1g). After the cell death, the fluorochrome passes through the nuclear envelope and occupies the cytosol, marking the entire cell. Thus, this marker is important in cellular death studies, as will be discussed later.

11.4.1 Materials

1. Propidium Iodide (Sigma-Aldrich, CAS-11348639001) work solution 1.0 μg. mL^{-1} prepared in 0.01 M PBS pH 7.2.
2. Fungi colony growth within a clear media, such as SNA, with living mycelia.
3. Scalpel.
4. Tweezer.
5. Automatic pipette.
6. Large coverslips (ex. 24 × 40 mm).
7. Stereomicroscope.
8. Inverted EFM or LSM.
9. For EFM, a filter cube that works near 500 ηm excitation and 630 ηm emission (e.g. Filter cube Chroma TxRed#39004).
10. For LSM, excitation with Argon 514 ηm laser line and emission filter with 620–660 ηm range. An EC Plan-Neofluar 40×/1.30 Oil DIC M27 objective was used, with 1024 × 1024 resolution, and around fourfold zoom. The bright-field image was acquired using the TPM-T detector.

11.4.2 Method

1. Put a previously cleaned large coverslip (e.g. 24 × 40 mm) on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
2. Put 10 μL of Propidium Iodide work solution (PIWS) above the coverslip. In case of many samples working, use a marker pen to delimitate and identify spots with around 0.5 × 0.5 cm where each sample will be placed. Avoid the coverslip corners, where the objective lens does not reach. Develop it in a room with diffuse light, or as dark as possible. It is recommended to test PIWS dilutions, as it is possible that concentrations as 0.1 and 0.01 $\mu g.mL^{-1}$ may provide good results depending on the case.

3. Take colony fragments with around 0.5 × 0.5 cm with a scalpel, choosing the region of interest using a stereoscopic microscope, if needed. The colony fragments should be fresh.
4. Transfer the upside-down colony fragment above the PIWS drop. The mycelia should be in contact with the dye.
5. Leave it in the dark for 15–20 min.
6. Observe in inverted EFM or LSM.

11.5 Sample Preparation to Observe Fungi Chromosome

Karyotyping involves characterize the number, morphology, and size of chromosomes of a species, and the main methods used for fungi are pulsed-field gel electrophoresis (PFGE) and germ tube burst method (GTBM) [28]. The techniques are important to determine the polymorphisms in the size and number of chromosomes between strains from the same species, or even extra chromosome presence. The study of these chromosomes is very important, since they may contain pathogenicity-essential genes of fungi to cause plant diseases [9]. Following, the GTBM method for *Colletotrichum* chromosome observation (Fig. 11.1h).

11.5.1 Materials

1. Petri dishes with PDA culture media.
2. PD liquid culture media.
3. Sterilized 40 μm miracloth filter.
4. Autoclaved 1.5 mL microtubes.
5. Centrifuge for microtubes.
6. Neubauer chamber.
7. Bunsen burner.
8. Tweezers.
9. Mili-Q water.
10. Sterilized distilled water.
11. Autoclaved slides.
12. Coverslips.
13. Poly-L-lysine.
14. Rubber glue.
15. Automatic pipette.
16. Wet chamber.
17. Incubator adjusted for 22 °C.
18. Bright field microscope.
19. Thiabendazole (TBZ) solution 50 $\mu g.mL^{-1}$.
20. Methanol.

21. Glacial acetic acid.
22. DAPI 1 μm.mL^{-1} and VectaShield mounting medium.
23. Propidium Iodide 1 μm.mL^{-1}.
24. Nail polish.
25. EFM or LSM.
26. For EFM, a filter cube that works near 405 ηm excitation and 450 ηm emission (e.g. Filter cube Zeiss #49). (A 100× objective was used on an inverted EFM).
27. For LSM, excitation with Diode 405 ηm laser line and emission filter with 420–480 ηm range.

11.5.2 Method

The following method was refined by Gonçalves [11], adapted from Taga et al. [24], to observe chromosomes from *Colletotrichum* species.

1. Growth of fungi in PDA at 22 °C in darkness. After sporulation, the conidia suspension is acquired with mili-Q water and scraping and collected with an automatic pipette.
2. The suspension is filtered with miracloth 40 μm filter and centrifuged within microtubes at 3000–3500 *g* for 5 min.
3. After centrifugation, the supernatant should be discarded and the pellet washed with sterile water twice. Resuspend the pellet in a nutrient medium and adjust the concentration to 2 to 3 × 10^6 conidia.mL^{-1} using a Neubauer chamber.
4. The previously autoclaved slides are treated with poly-L-lysine solution and marking a rectangle on the slide with the rubber glue.
5. Pipetting 450–600 μL conidia solution into the rectangle marked with the rubber glue on the face of the poly-L-lysine slide.
6. Place the slides in a humid camera and incubate them in the dark at 22 °C. Monitor the germination after 6 h of incubation using a light microscope, prolong the incubation time if necessary.
7. Remove the liquid excess from the slide surface without drying out completely.
8. Add 400–600 μL of the nutrient medium containing thiabendazole (TBZ) at the final concentration of 50 μg.mL^{-1} to stop mitosis during metaphase.
9. Incubate the slide at 22 °C in the dark for 2–3 h, then remove the TBZ solution using an automatic pipette and remove the rubber glue using tweezers.
10. Slowly immerse the slide in mili-Q water to wash off excess TBZ that may still be present on the blade and remove excess water using a filter paper, but still leaving the slide moist.
11. Dry the slide by passing it over a flame quickly, without letting it dry completely or overheating the slide.
12. Add 20–25 μL of the propidium iodide 1 μg.mL^{-1} on the slide surface and wait 15 min. After, immerse the slide in sterile water to remove the dye excess.

13. Add 15–20 μL of the VectaShield mounting medium with DAPI and incubate in the dark for 10–15 min and then seal the slide with coverslip and nail polish.
14. Observe in EFM or LSM.

The DAPI-VectaShield mounting media may be used instead of the DAPI and VectaShield mounting media separated.

11.6 Sample Preparation to Observe Fungi Cell-Wall

The fungi cell wall staining with fluorochromes is very important in many studies, such as during infection processes in plant tissues, delimitating the septa during the nuclei dynamic studies, and associated with other methods, such as immunolabeling or hybridization. Calcofluor White is a commonly used dye for glucans, as the fungi chitin and the plant cellulose. On the other hand, AlexaFluor 488® WGA Conjugate (Alexa488-WGA) marks exclusively fungi. Thus, fungi-plant interaction can be studied with both used together, Alexa488-WGA to dye fungi and Calcofluor marking plant tissues, as will be discussed later.

11.7 Calcofluor White Staining

This fluorochrome dyes the fungi cell wall (Fig. 11.2a) usually with a short time incubation, around 10–30 min, depending on species and cell type. The procedure below was performed with a 7-days-old *Pyricularia oryzae* colony.

11.7.1 Materials

The necessary materials can vary depending on the protocol used. The samples can be mounted in slide-coverslip with fungal structures in 0.01 mg.mL^{-1} CalcoFluor in a vertical microscope or with culture block facing-dawn above 10 μL of 0.01 mg. mL^{-1} CalcoFluor in large coverslip on an inverted microscope, as discussed in DAPI methods. The excitation and emission conditions are also similar.

11.8 AlexaFluor 488® WGA Conjugate Staining

Alexa488-WGA labels the fungi cell wall (Fig. 11.2b) and the time incubation may vary depending on species and cell type. Another very important point is that some fungi with thick conidia cell walls such as *Pyricularia* and *Alternaria* require a

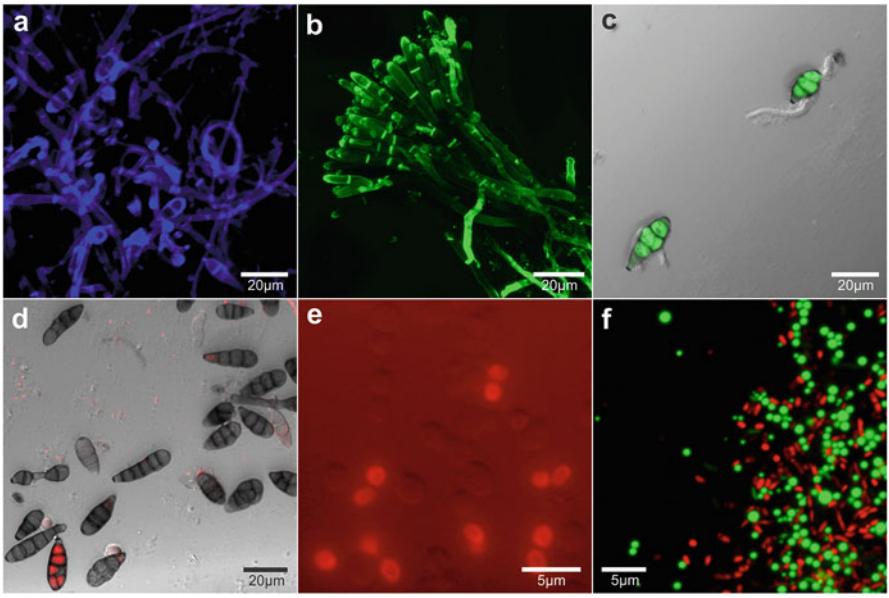

Fig. 11.2 Fungi structures were fluorochrome-stained. (**a**–**b**) Fungi cell wall dyed with CalcoFluor White (**a**) and AlexaFluor488–WGA (**b**). (**c**) Reactive oxygen species labeled with DCF-DA. (**d**–**e**) Cellular-death observation using Iodide Propidium for conidia of filamentous fungi (**d**) and yeasts (**e**). (**f**) Live-dead test for yeasts and bacteria using Iodide Propidium for dead (red) and Syto9 for living cells (green)

vacuum-infiltration step for 20 min to efficient dyeing [13]. Fungus with thin cell walls such as *Fusarium* can be marked without vacuum step. The method shown was made with common bean leaves infected with fungus.

11.8.1 Materials

1. Wheat Germ Agglutinin (WGA) AlexaFluor 488® Conjugate (Alexa488-WGA) (ThermoFischer, CAT-W11261) 10 µg.mL^{-1} work solution prepared in 0.01 M PBS pH 7.2.
2. 0.01 M PBS pH 7.2.
3. Scalpel, tweezers, and scissors.
4. Sterilized 96-well ELISA plates.
5. Vacuum pump linked to a hermetically sealed container.
6. Clean coverslips.
7. Glass piece (2 × 2 × 1 cm).
8. Aluminum paper.
9. Automatic pipette.
10. Clean tips.

11. Large coverslips (e.g. 24 × 40 mm).
12. Inverted EFM or LSM.
13. For EFM, a filter cube that works near 488 ηm excitation and 520 emission (ex. Filter cube Zeiss #38HE).
14. For LSM, excitation with Argon 488 ηm laser line and emission filter with 500–550 ηm range, for Alexa 488. A Plan-Apochromat 63×/1.40 Oil DIC M27 objective was used, with 1024 × 1024 resolution.

11.8.2 Method

1. Sampling of common bean leaves infected with fungus and cutting in 4 × 4 mm pieces.
2. After, the leaves fragments are place within Elisa plates with 10 μg.mL^{-1} Alexa488-WGA.
3. Wrap the Elisa plate with aluminum foil and keep it at vacuum (Fig. 11.1d) for 1 h.
4. Wash with PBS.
5. Put a leaf fragment above the previously cleaned large coverslip on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
6. Put the leaf fragment above the coverslip with the region of interest facing down.
7. Place a glass piece above the sample (to minimize the irregular topography of the sample).
8. Observe in inverted EFM or LSM.

11.9 Reactive Oxygen Species (ROS) in Fungi

Reactive oxygen species (ROS) are ubiquitous in fungi living cells, with high damaging potential but are also essential for gene expression signaling and development in several biological processes [14] such as the programmed cellular death [10]. One way to study the ROS dynamics is using the 2′,7′-Dichlorofluorescein diacetate (DCF-DA) fluorochrome. DCF-DA dyes several ROS, as hydrogen peroxide (H_2O_2), peroxyl (ROO•), hydroxyl (HO•), peroxynitrite anion (ONOO-) and nitric oxide (•NO), and it's useful for assessing various stress types in plants, such as osmotic, thermal, and by pathogen infection [2, 13, 19, 29]. Another fluorochrome variant, H_2DCFDA, was important to understanding the ROS role during rice blast pathogenesis [6]. The strobilurin resistance in *Alternaria alternata* is caused by a non-synonymous mutation that changes the cytochrome b product, replacing a glycine by an alanine at the condon 143 (G143A mutation) [5]. Resistant strains remain with low DCF-DA signal when cultivated in PDA media plus high concentration fungicide (Fig. 11.2e), while the wild type presents a high signal (not shown).

11.9.1 Materials

1. 2′,7′-Dichlorofluorescein diacetate (DCF-DA) (Sigma, CAS-4091-99-0) 10 μM in filtered dimethyl sulfoxide (DMSO).
2. Conidia suspension without fixation.
3. Water-Agar 1.5% in thin layer on a Petri dish.
4. Kolle handle with a needle.
5. Scalpel.
6. Tweezer.
7. Automatic pipette.
8. Slides and regular coverslips (e.g. 20 × 20 mm).
9. Large coverslips (e.g. 24 × 40 mm).
10. Nail polish or other coverslip sealant.
11. EFM or LSM, vertical or inverted.
12. For EFM, a filter cube that works near 488 ηm excitation and 525 ηm emission (ex. Filter cube Zeiss #38HE).
13. For LSM, excitation with Argon 488 ηm laser line and emission filter with 500–550 ηm range. An EC Plan-Neofluar 40×/1.30 Oil DIC M27 objective was used, with 1024 × 1024 resolution. The bright-field image was acquired using the TPM-T detector.

11.9.2 Methods

11.9.2.1 Conidia Suspension

1. Cut water-agar (WA) in 0.5 × 0.5 cm block.
2. Carry 10 μL of conidia suspension above the WA block.
3. Wait around 10 min for decanting.
4. Apply 10 μL of DCF-DA 10 μM above the WA block, and leave for 5 min in darkness.
5. Put a previously cleaned large coverslip (e.g. 24 × 40 mm) on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
6. In case of many samples working, use a marker pen to delimitate and identify spots with around 0.5 × 0.5 cm where each sample will be placed in the coverslip. Avoid the coverslip corners, where the objective lens does not reach.
7. Take the WA block with a dyed sample using a scalpel and transfer the upside-down block above the coverslip.
8. Observe in inverted EFM or LSM.

This procedure may be done for observation in a vertical microscope, using slide-coverslip preparing, mixing 5 μL sample suspension and 5 μL DCF-DA. In this case,

the fluorochrome concentration will be diluted, and maybe a higher concentration may be necessary.

11.10 Fungi Cellular Death Studies with Propidium Iodide

Experiments with cellular death marking of fungi can help to characterize phenotypes in many contexts, such as confirming the fungicide activity of a control product, instead fungistaticity. Another possible subject is the the programmed cell death (PCD), which may occur during the sexual and asexual reproduction, in some infection processes for phytopathogenic fungi, and in the non-self-recognition mechanism heterokaryon incompatibility (HI) [10]. The strobilurin resistance of *Alternaria alternata* is caused by the G143A change in cytochrome b [5], and low mortality of conidia when cultivated in PDA media plus high fungicide concentration (Fig. 11.2d). Other assay was for yeast mortality observation during a fermentation process (Fig. 11.2f). The cell-death marker used was Propidium iodide [8].

11.10.1 Materials

1. Propidium iodide (Sigma, CAS-25535-16-4) 1.0 μg.mL^{-1} prepared in 0.01 M potassium phosphate buffer solution (PBS) pH 7.2.
2. Fungi conidia suspension without fixation.
3. Yeast suspension from a fermentation process.
4. Water-Agar 1.5% in a thin layer on a Petri dish.
5. Kolle handle with a needle.
6. Scalpel.
7. Tweezer.
8. Automatic pipette.
9. Slides and regular coverslips (e.g. 20 × 20 mm).
10. Large coverslips (e.g. 24 × 40 mm).
11. Nail polish or other coverslip sealants.
12. EFM or LSM, vertical or inverted.
13. For EFM, a filter cube that works near 543 ηm excitation and 645 ηm emission (ex. Filter cube Zeiss #45). A 100× objective was used on an inverted EFM – yeast suspension.
14. For LSM, excitation with HeNe 543 ηm laser line and emission filter with 625–665 ηm range. An EC Plan-Neofluar 40×/1.30 Oil DIC M27 objective was used, with 1024 × 1024 resolution – *Alternaria alternata* conidia suspension. The bright-field image was acquired using the TPM-T detector.

11.10.2 Methods

The methods presented below are for filamentous fungi or yeast cell-death marking, and variations will be considered.

11.10.3 Conidia or Yeast Suspension

1. Cut water-agar (WA) in 0.5 × 0.5 cm block.
2. Carry 10 μL of conidia or yeast suspension above the WA block.
3. Wait around 10 min for decanting.
4. Apply 10 μL of Propidium iodide 1.0 $\mu g.mL^{-1}$ above the WA block, and leave for 5 min in darkness.
5. Put a previously cleaned large coverslip (e.g. 24 × 40 mm) on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
6. In case of many samples working, use a marker pen to delimitate and identify spots with around 0.5 × 0.5 cm where each sample will be placed in the coverslip. Avoid the coverslip corners, where the objective lens does not reach.
7. Take the WA block with dyed sample using a scalpel and transfer the upside-down block above the coverslip.
8. Observe in inverted EFM or LSM.

This procedure may be done for observation in vertical microscope, using slide-coverslip preparing, mixing 5 μL sample suspension and 5 μL Propidium iodide. In this case, the fluorochrome concentration will be diluted, and maybe higher concentration may be necessary.

11.11 Live-Dead Test for Fungi Cells

The methods presented below are for yeasts and bacteria, and variations will be considered. In Fig. 11.2f, observation of yeasts and bacteria from a probiotic product was analyzed. The following protocol was developed based on Stiefel et al. [23] and Batista et al. [1].

11.11.1 Materials

1. Propidium iodide (Sigma, CAS-25535-16-4) 1.0 $\mu g.mL^{-1}$ in 0.01 M potassium phosphate buffer solution (PBS) pH 7.2.
2. Syto9 (Thermo Fischer-S34854) 20 μM in filtered DMSO.

3. Probiotic yeasts and bacteria suspension.
4. Water-Agar 1.5% in a thin layer on a Petri dish.
5. Kolle handle with a needle.
6. Scalpel.
7. Tweezer.
8. Automatic pipette.
9. Slides and regular coverslips (e.g. 20 × 20 mm).
10. Large coverslips (e.g. 24 × 40 mm).
11. Nail polish or other coverslip sealants.
12. EFM or LSM, vertical or inverted.
13. For EFM, a filter cube that works near 543 ηm excitation and 645 ηm emission (ex. Filter cube Zeiss #45) in Propidium iodide localization; filter cube to works near 488 ηm excitation and 535 emission (ex. Filter cube Zeiss #38HE) in Syto9 working.
14. For LSM, excitation with HeNe 543 ηm laser line and emission filter with 625–665 ηm range in Propidium iodide localization; excitation with Argon 488 ηm laser line and emission filter with 520–550 ηm range in Syto9 observation. (Plan-Apochromat 63×/1.40 Oil DIC M27 objective was used, with 1024 × 1024 resolution, and around twofold zoom).

11.11.2 Method

1. Cut water-agar (WA) in 0.5 × 0.5 cm block.
2. Carry 5 μL of yeast suspension above the WA block.
3. Wait around 10 min for decanting.
4. Above the same surface apply 5 μL of Syto9 20 μM and leave for 40 min in darkness.
5. After, apply 10 μL of Propidium iodide 1.0 $\mu g.mL^{-1}$ above the WA block, and leave for 5 min in darkness.
6. Put a previously cleaned large coverslip (e.g. 24 × 40 mm) on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
7. In case of many samples working, use a marker pen to delimitate and identify spots with around 0.5 × 0.5 cm where each sample will be placed in the coverslip. Avoid the coverslip corners, where the objective lens does not reach.
8. Take the WA block with a dyed sample using a scalpel and transfer the upside-down block above the coverslip.
9. Observe in inverted EFM or LSM.

This method may be adapted also for filamentous fungi and other organisms, adjusting the concentration and the time incubation. The Syto9 fluorochrome dye all cells and the Propidium iodide the dead cells only. Thus, the merged green and red channels will show the living cells green and the dead cells in red color

(Fig. 11.2f). If the Syto9 is not available, it may be done using only the Propidium iodide, merging the red channel with the bright field image (Fig. 11.2d).

11.12 Sample Preparation to Observe Fungi-Plant Interactions

Below, a protocol for staining plant tissues with Calcofluor White and fungi with Alexa488 WGA. Before the staining steps, a clarification protocol was develop based on Warner et al. [26] and Minker et al. [18]. Common bean leaves infected with phytopathogenic fungus were used (Fig. 11.3).

11.12.1 Materials

1. CalcoFluor White (Fluorescent brightener 28, Sigma, CAS-4404-43-7) 0.01 mg. mL^{-1} work solution prepared in 0.01 M PBS pH 7.2.
2. Wheat Germ Agglutinin (WGA) AlexaFluor 488® Conjugate (Alexa488-WGA) (Thermo Fischer, CAT-W11261) 10 µg.mL^{-1} work solution prepared in 0.01 M PBS pH 7.2.
3. Clarifying solution (6 M Urea, 30% Glicerol, 0.01% Tween 20).
4. 0.01 M potassium phosphate buffer solution (PBS) pH 7.2.
5. Potassium hydroxide (KOH) solution 10% (p/v).
6. Scalpel, tweezers and scissor.
7. Sterilized 96-well ELISA plates.
8. Vacuum pump linked to a hermetically sealed container.
9. Clean coverslips.
10. Glass piece (2 × 2 × 1 cm).
11. Aluminum paper.
12. Automatic pipette and clean tips.
13. Large coverslips (e.g. 24 × 40 mm).
14. Incubator.
15. Inverted fluorescence microscope.
16. For EFM, a filter cube that works near 405 ηm excitation and 450 ηm emission (ex. Filter cube Zeiss #49) to CalcoFluor, and filter cube to works near 488 ηm excitation and 520 emission (ex. Filter cube Zeiss #38HE) to Alexa 488.
17. For LSM, excitation with Diode 405 ηm laser line and emission filter with 420–480 ηm range, for CalcoFluor; excitation with Argon 488 ηm laser line and emission filter with 500–550 ηm range, for Alexa 488. A Plan-Apochromat 63x/1.40 Oil DIC M27 objective was used, with 1024 × 1024 resolution.

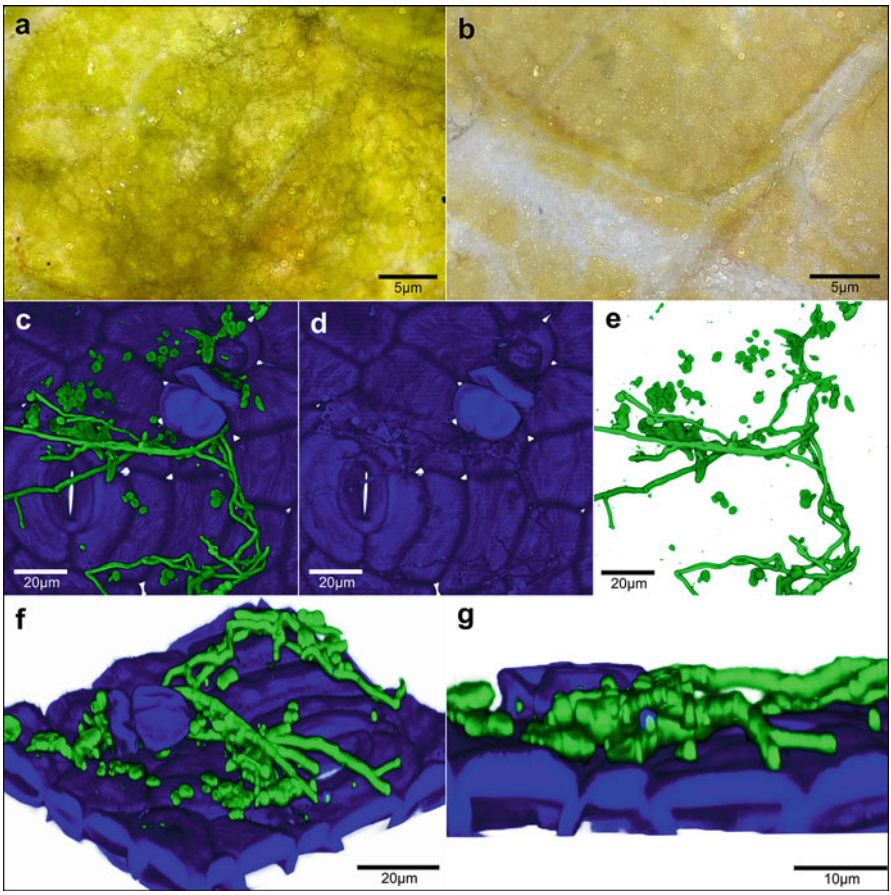

Fig. 11.3 Fungi-Plant interactions. Common bean leaf surface before (**a**) and after (**b**) clarifying treatment. (**c**–**g**) Common bean colonized superficially by fungi. Plant tissue dyed with CalcoFluor and fungi marked with AlexaFluor 488 – WGA. (**c**) 2D overlay of blue and green channels from 98 focal plans within 30 μm range in Z-axis using the maximum intensity projection method. (**d**–**e**) 2D of separated channels. (**f**–**g**) 3D software reconstruction from focal plans acquired

11.12.2 Methods

1. Sampling of common bean leaves infected with phytopathogenic fungus and cutting in 4 × 4 mm pieces.
2. After, the leaves fragments are placing within Elisa plates well containing KOH 10%, following incubation at 10 °C for 4 days in darkness.
3. Wash with PBS and replace for a new KOH 10% solution.
4. Other incubation steps at the same conditions.
5. PBS wash and transfer fragments to Elisa wells with clarifying mix [Urea 6 M, Glycerol 30% (v/v) and Tween 20 0.05% (v/v)].

6. After 4 days in clarifying mix at 10 °C and darkness, wash with PBS, and incubate in a new clarifying mix for 4 days.
7. Wash with PBS, and add 100 μL Alexa488–WGA 10 $\mu g.mL^{-1}$.
8. Wrap the Elisa plate with aluminum foil and keep it at vacuum (Fig. 11.1d) for 1 h.
9. Add 80 μL of CalcoFluor 0.01 $mg.mL^{-1}$ and incubate for 10 min at the darkness.
10. Put a previously cleaned large coverslip on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
11. Put the leaf fragment above the coverslip with the region of interesting facing down.
12. Place a glass piece above the sample (to minimize the irregular topography of the sample).
13. Observe in inverted LSM.
14. In this assay, the z-stack method was used to capture 98 focal plans within 30 μm ranges in Z-axis, to obtain the 2D with the maximum intensity method and the 3D reconstruction, using the Zen software (Carl Zeiss).

The clarifying protocol showed was for leaf tissues. Adjustments are needed depending on the tissue type and the plant species, changing the incubation time. For example, the common bean pods can be clarified with 20 days in KOH 10% and 15 days in clarifying mix, changing the solutions every 5 days of incubation.

The importance of the clarification step is the high amount of autofluorescent compounds such as cell wall components, phenols, and alkaloids [21]. Thus, many protocols have been developing to minimize these fluorescence signals and optimize the specific fluorescent label [18, 25, 26]. The transparency increasing in plant tissues improves the three-dimensional structures imaging quality, especially valuable during the investigation of plant–fungi interactions, once it provides better fluorochrome infiltration, photon and or laser penetration, and so high quality of fluorescence signal [18]. Other clarification protocol with very satisfactory results was developed by Ursage et al. [25] which uses the *ClearSee* solution [xylitol 10% (w/v); sodium deoxycholate 15% (w/v); urea 25% (w/v)] in the clarification and for fluorochromes preparing. The *ClearSee* method also has the advantage of allowing the fluorescent proteins such as GFP and m-Cherry observation in plant tissues even after treatment. In cases where the sample preparation evolves alcohol gradients, for embedding and microtome cutting, clarification is not necessary. And, for sure, for non-autofluorescent samples.

Fungi-plant interactions fluorochrome-based studies can be made using other dyes combinations. Ha et al. [13] observed the wheat tissues colonization by *Pyricularia graminis-tritici* using Alexa488-WGA and *Fusarium graminearum* genetically transformed for GFP expression. In both cases, the fixed plant tissues were dyed with Propidium iodide. The plant cell wall marker, pontamine fast scarlet (S4B), is specific for cellulose and may be used combined with other fluorochromes, such as aniline blue, which label callose [7].

11.13 Important Plant Structures Defence Against Phytopathogenic Fungi

The plant resistance factors against phytopathogens are divided into biochemical and structural, preformed or postformed. Through histochemical analysis, some structural resistance components such as callose, lignin, and cuticle, and biochemical, as ROS, can be evaluated by microscopy techniques. Then, callose and lignin protocols are shown.

11.14 Callose Deposition

The callose polymer (β-1,3-glucan) is a plant cell wall component and may plant defense responses, and their deposition may increase in response to infections or resistance-inducing agents [7, 16]. Aniline blue is the fluorochrome used in callose deposition studies, such as in papillae observation [7]. Figure 11.4a shown callose deposition marked with aniline blue in tangerine leaf.

11.14.1 Materials

1. Aniline blue diammonium (Sigma, CAS-415049) 0.1 mg.mL^{-1} in PBS 0.01 M pH 7.2.
2. Tangerine leaves fragments with around 0.5 × 0.5 cm were previously fixed in 4% paraformaldehyde and clarified as seen before for common bean leaves.

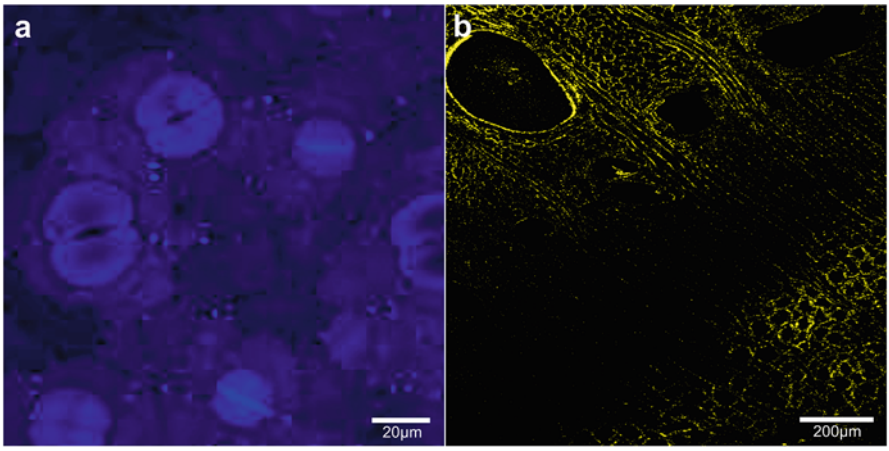

Fig. 11.4 (**a**) Callose deposition in tangerine leaf observed with Aniline blue fluorochrome. (**b**) Lignin localization in *Eucalyptus* cambial region using Auramine-O

3. Scalpel, tweezers, and scissors.
4. Sterilized 96-well ELISA plates.
5. Vacuum pump linked to a hermetically sealed container.
6. Clean coverslips.
7. Glass piece (2 × 2 × 1 cm).
8. Aluminum paper.
9. Automatic pipette and clean tips.
10. Large coverslips (e.g. 24 × 40 mm).
11. Inverted fluorescence microscope.
12. For EFM, a filter cube that works near 405 ηm excitation and 450 ηm emission (ex. Filter cube Zeiss #49).
13. For LSM, excitation with Diode 405 ηm laser line and emission filter with 420–480 ηm range. The 40× objective was used with zoom resource.

11.14.2 Method

1. Take the clarified leaf fragment and incubate samples within Elisa plate wells in 80 µL Aniline blue 0.1 $mg.mL^{-1}$.
2. Wrap the Elisa plate with aluminum foil and keep it at vacuum for 1 h.
3. Incubate overnight at around 25 °C.
4. Wash with PBS.
5. Transfer the abaxial side to a large coverslip supported in the microscope stage.
6. Place the glass piece on a sample.
7. Observe with EFM or LSM the leaf surface (In the case of LSM, paradermic examination is possible).

11.15 Lignin Localization in Plant-Fungi Interactions

Lignification has an important role in plant defense, in resistance to fungi mechanical penetration, and participating in gene expression signaling and metabolic pathways of the plant resistance [3]. The usual fluorescent markers in lignin labeling are Auramine-O [22], which dyes cutin also, and Basic fucsin [25], which dyes lignin only. In Fig. 11.4b, a cross section of *Eucalyptus* cambial region with lignin localization using the Auramine-O.

11.15.1 Materials

1. Auramine-O (Merck, CAS-2465-27-2) 1.0 mg.mL^{-1} work solution.
2. *Eucalyptus* cross sections of 7 μm thickness prepared in a microtome, in pith to bark direction.
3. Slides and coverslips.
4. Nail polish or other coverslip sealants.
5. Automatic pipette and tips.
6. EFM or LSM.
7. For EFM, a filter cube that works near 488 ηm excitation and 520 emission (ex. Filter cube Zeiss #38HE).
8. For LSM, excitation with Argon 488 ηm laser line and emission filter with 500–550 ηm range. An EC Plan-Neofluar 10x/0.30 M27 was used.

11.15.2 Method

1. Take the slides with sections and apply around 5 μL of Auramine-O 1.0 mg.mL^{-1} to cover all sample areas.
2. Incubate in darkness for 20–40 min.
3. Wash gentlly with PBS.
4. Cover with coverslip and seal.
5. Observe in an inverted or vertical microscope, EFM or LSM.

11.16 Sample Preparation to Observe Autofluorescent Fungi and Specific Structures

11.17 Autofluorescent Rust Fungi

Several Basidiomycota presents autofluorescence such as some mushroom producers and rust fungi [30, 31]. Its feature permits localization in the environment and plants. For *Hemileia vastatrix* and many other rust fungi, autofluorescence can be observed at the green or blue emission spectra. Here, we observed the autofluorescence of *H. vastatrix* pustules in coffee leaves (Fig. 11.5a).

11.17.1 Materials

1. Coffee leaf with rust fungi *Hemileia vastatrix* pustules.
2. Incubator at 20 °C.

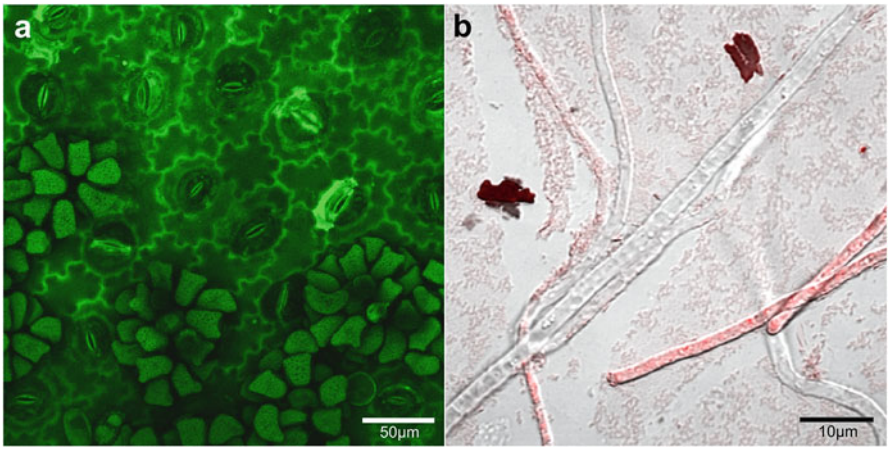

Fig. 11.5 (**a**) Autofluorescence of *Hemileia vastatrix* pustules in coffee leaves. (**b**) Autofluorescence of cercosporin crystals produced by *Cercoscopa coffeicola* in MEA

3. Dissecting scissor.
4. Tweezer.
5. Large coverslips (e.g. 24 × 40 mm).
6. Inverted EFM or LSM.
7. For EFM, a filter cube that works near 488 ηm excitation and 520 ηm emission (ex. Filter cube Zeiss #38 HE).
8. For LSM, excitation with 488 ηm laser line and emission filter with 490–560 ηm range. An LCI Plan-Neofluar 25×/0.8 Imm Korr DIC M27 objective was used, with 512 × 512 resolution.

11.17.2 Method

1. Put a previously cleaned large coverslip (ex. 24 × 40 mm) on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
2. Positioning a leaf fragment with pustules directed to an objective lens (upside-down). Small fragments, as 1 × 1 cm are desirable to minimize the irregular topography of the sample, as well as avoid the midrib.
3. Use a piece of glass as a weight to minimize the irregular topography of the sample.
4. Observe in inverted EFM or LSM.

11.18 Autofluorescent Cercosporin

The autofluorescence may be useful information to detect certain fungi metabolites and other endogenous fluorophores [15]. The cercosporin crystals produced by *Pseudocercosporella capsellae* are detectable at 561 nm excitation condition using a laser confocal microscope [12]. In the method described below will be shown the location of cercosporin crystals produced by *Cercoscopa coffeicola* in vitro (Fig. 11.5b).

11.18.1 Materials

1. *Cercoscopa coffeicola* colony growth in malt extract agar (MEA) 3 weeks at 20 °C.
2. Inverted EFM or LSM.
3. Scalpel.
4. Tweezer.
5. For EFM, a filter cube that works near 561 ηm excitation and 650 ηm emission (ex. Filter cube Zeiss #45).
6. For LSM, excitation with 543 ηm laser line and emission filter with 600–710 ηm range An EC Plan-Neofluar 40×/1.30 Oil DIC M27 objective was used, with 1024 × 1024 resolution, and around three-fold zoom. The bright-field image was acquired using the TPM-T detector.

11.18.2 Method

1. Put a previously cleaned large coverslip (e.g. 24 × 40 mm) on an inverted microscope stage. In case of immersion objective using, drop firstly the appropriated immersion liquid on the objective lens before that.
2. Take colony fragments with around 0.5 × 0.5 cm with a scalpel, choosing the region of interest using a stereoscopic microscope, if needed.
3. Transfer the upside-down colony fragment above the coverslip.
4. Observe in inverted EFM or LSM.

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Chapter 12
Yeast Isolation Methods from Specialized Habitats

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Contents

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12.1 Introduction

Yeasts are a unicellular heterogeneous group of fungi which were first observed under a microscope by Antonie Van Leeuwenhoek in 1680 and successfully isolated for the first time by Louis Pasteur in the late 1860s. Since 1865, yeast research has undergone significant progress with respect to their distribution, isolation and characterization. Currently, more than 1500 yeast species have been reported from all over the world, which comprise of only 1% of the actual diversity while the remaining still needs to be explored [18]. Yeasts are ubiquitous in nature and have been isolated from aquatic, atmospheric and terrestrial habitats. While some species can be found in great numbers in different habitats, others may be restricted geographically to only a few specialized habitats; suggesting that the overall yeast distribution is not uniform.

It would seem that the distribution of yeasts would not be affected by geographical barriers since they can be dispersed by air currents [34], organismal vectors, plant material as epiphytes or endophytes. But actually, distinct yeast species occur in different regions and seasons. Studies on yeasts associated with beetles, drosophilids, bees, and ephemeral flowers disprove the fact that yeasts are ubiquitous [13, 19, 34]. The yeast diversity of common habitats like soil, plants and other terrestrial habitats has been thoroughly explored since the isolation techniques are easily available and well described [29, 34]. In contrast, the isolation techniques for the less exploited niches (Fig. 12.1) like rumen, insect gut, hot spring, rotten wood, flowers, nectar and other anthropogenic habitats like compost, molasses, distillery wastes etc. are rather cumbersome and not elaborately outlined, since the procedures vary greatly depending on the yeast density, the volume and shape of the source [25]. The correlation between yeasts and the habitats that they occupy is determined by the overall intrinsic factors (chemical, physical and physiological), availability of nutrients, beneficial interactions with other organisms, and the presence of competitors.

In the past decade, several novel yeasts have been isolated from natural habitats which are not fully exploited; one such niche is insect gut [1, 36, 37]. The insect gut has become increasingly recognized as an important source for the isolation of new ascomycetous and basidiomycetous yeast taxa. The major purpose for the yeast-insect association is that the yeasts provide essential amino acids, vitamins, sterols and allelochemicals to attract the insect dispersers for targeted dispersal to a fresh environment [4]. Such associations are believed to have promoted fungal diversity and the expansion of insects into nutrient-poor substrates [37]. Several novel yeasts have been isolated from the gut of beetles. Likewise, the gut of termites has also been explored for novel yeasts, which produce valuable enzymes and can degrade xylan and/or ferment xylose [1] to ethanol. These properties can be exploited in industries for the sustainable production of ethanol or valuable enzymes. The gut of termites and other wood-feeding insects can be considered as natural mini bioreactors with the understudied microbiome, therefore, we have provided a detailed protocol for isolation of such industrially important yeasts from the termite gut.

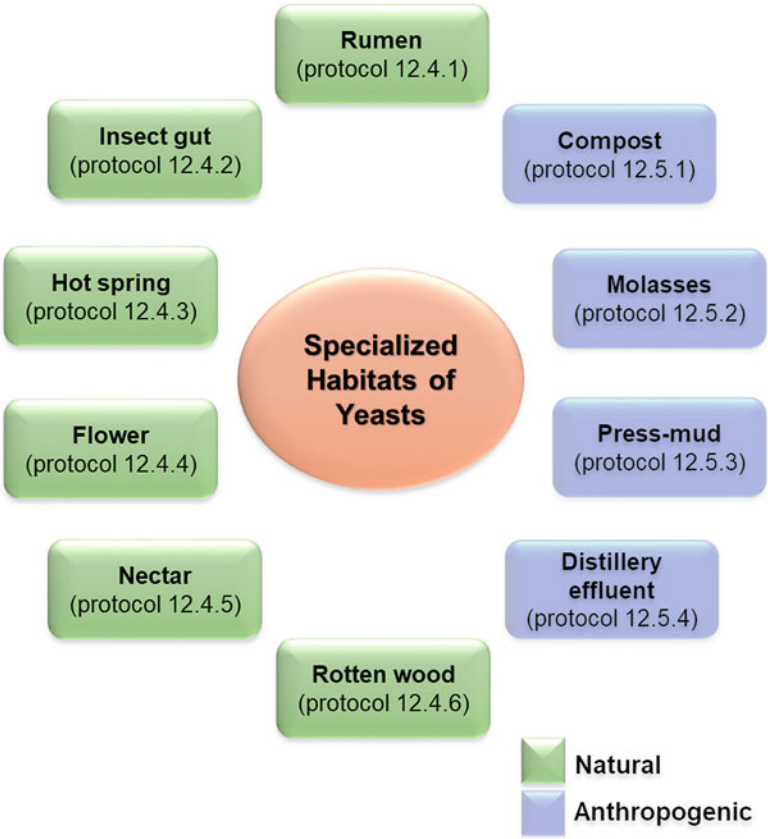

Fig. 12.1 Specialized habitats for isolation of yeasts

In a similar manner, yeasts play a mutualistic role in the rumen of cattle and other ruminants. The rumen is an anaerobic, cellulose and hemicellulose-rich habitat, which harbours many microbes including bacteria, protozoa, filamentous fungi and yeasts [10]. Yeasts isolated from rumen digesta can grow at elevated temperatures since the temperature of rumen itself is 39.6 ± 1 °C. There are few reports of yeasts isolated from the rumen of sheep, bovine, cattle and musk oxen [8, 21, 22]. The exact role of yeasts in the rumen is unclear but it is proposed that they act as probiotics to prevent various diseases in ruminants and stimulate the growth and activity of various fibrolytic ruminal bacteria by providing proteins, vitamins and other growth factors [24, 33]. Since the rumen is also a less explored niche, a protocol for rumen yeast isolation is given in this chapter.

There are some 'wood-feeding yeasts' found on rotting wood, which is a nutrient-rich substrate for yeast populations. This niche has recently received attention after isolation of xylose-fermenting yeasts from rotting wood in Brazilian forests and a novel yeast capable of arabitol production was reported [6, 17]. The hemicellulosic

fraction of wood is broken down to simpler monomers by the yeasts' enzymes. The hydrolytic abilities of the enzymes secreted by these yeasts are of great significance to biotechnological industries, therefore. a brief protocol for yeast isolation from rotting wood has also been given.

Another interesting niche for obtaining novel or rare yeasts is the flower or floral nectar. There are many publications on the properties and characteristics of nectar, but very little is known regarding its role as a natural habitat for micro-organisms, especially yeasts. The presence of yeasts in the nectar of flowers is well known but very little effort has been taken for the isolation of yeasts and studying their role in compositional changes of nectar fluid [16, 43]. It is possible that the yeasts inhabiting nectars of angiosperms play a significant role as intermediate agents in plant-pollinator signalling by the production of volatile compounds by fermentation of available sugars [16, 43]. It is suggested that some osmophilic species can be found in nectar since it contains high levels of sugars, and this trait can be beneficial from a biotechnological point of view.

Some extreme environments have been established as niches for various species of yeasts capable of enduring harsh conditions [34]. One such naturally occurring habitat is the hot spring which harbours thermotolerant and thermophilic yeasts. Novel thermotolerant yeasts have been reported from hot springs [38] which can be used for high-temperature fermentations to produce ethanol. Enzymes like β-galactosidase, cellulase and lipases produced by thermotolerant/thermophilic yeasts are expected to have thermal tolerance, which is one of the most desirable properties of an enzyme to be used for industrial applications [12]. A detailed protocol for the isolation of yeasts from hot springs has been provided in this chapter.

Besides the naturally existing habitats, there are a number of anthropogenic sources from which yeasts can be recovered. Urbanization and human activities have created multiple ecological or functional niches for specific yeast populations. One such artificial habitat is compost. Composting is an aerobic process which involves the microbial conversion of complex organic compounds into their simpler forms [31]. As a habitat rich in carbohydrates and phenolics, composts harbour a variety of microbes including yeasts that have the ability to utilize C5 and C6 sugars and are tolerant to high temperatures and low pH [9, 14]. In the past decade, most research on composts has been focused on their ecological and functional biodiversity. Very few studies have been undertaken to explore yeast diversity from composts despite being one of the rarest and unexplored habitats [9]. Yeasts isolated from composts might be possible candidates for fermentation in biofuel or bioethanol industries.

The yeast biota of carbon-rich substrates like residual juice, molasses and press mud from sugar cane/beet in sugar industries has not been delved into completely. Press mud is also a type of waste which is rich in lignocellulosic material, obtained after ethanol production from molasses and is usually burnt or discarded by most distilleries. Few yeasts of the genera *Saccharomyces*, *Schizosaccharomyces* and *Torulaspora* have been reported from this niche [5, 39, 40]. Molasses from sugar cane and agave are used as the starting material for the production of ethanol or other

distilled spirits [11, 20] and the waste generated (residual molasses and press mud) can possibly harbour high sugar-tolerant or osmophilic microbes, especially yeasts [15, 26, 40]. Isolation of such indigenous yeasts and their employment in fermentation industries can increase the efficiency of ethanol production from molasses [7]. Protocols for yeast isolation from press mud and molasses have been provided.

Much like molasses, distillery waste also constitutes a very rich ecosystem in which varied yeast species can flourish and carry out fermentation spontaneously. The yeast community thrives on residual sugars and other by-products generated after the distillation process to produce ethanol from grape pomace. Very few surveys have been carried out to study the yeast diversity from distillery waste [41, 42]. More recently, distillery waste has also been found to be a potential reserve for novel thermotolerant yeasts [3]. Such yeasts once successfully isolated and characterised can be of interest in the field of biotechnology for efficient production of ethanol from lignocellulosic biomass [27].

The above mentioned natural or anthropogenic ecosystems are all reservoirs of rare and novel yeasts that can significantly contribute to the sustainable advancement of biotechnological research across the globe. Yeast diversity of such specialised habitats has not been completely exploited as a consequence of insufficient knowledge or unavailability of detailed step-by-step isolation protocols. The current chapter presents a comprehensive compilation of available methods that have been suitably modified to successfully isolate yeasts from specialized and less explored habitats like rumen, rotting wood, insect gut, flower/floral nectar, hot spring, compost, molasses/press mud and distillery waste (Fig. 12.1). We aim to provide a complete set of methods as a reference for researchers who are interested in exploring yeast biota from rare ecosystems.

12.2 Common Materials

1. 0.9% saline solution
2. Autoclave
3. Chloramphenicol (HiMedia)
4. Cover-slips
5. Cryo-Vials (2 ml)
6. Deep freezer (−20 °C & −80 °C)
7. Differential Interference Contrast (DIC) Microscope
8. Distilled water
9. Erlenmeyer flasks (250 ml).
10. Ethanol (70%)
11. Falcon tubes (50 ml)
12. Glass slides
13. Glass spreader
14. Glycerol
15. Hand gloves

16. Incubator
17. Laminar air flow cabinet
18. Measuring cylinders (500 ml
19. Micropipettes (P20, P200 & P1000)
20. Milli-Q water
21. Penicillin (HiMedia)
22. Petri-plates (90 mm)
23. pH meter,
24. Plastic bags (6″ × 10″ or 10″ × 16″ size)
25. Plastic containers (500 and 1000 ml)
26. Refrigerated centrifuge
27. Refrigerator (4 °C)
28. Screw-cap glass bottles (500 ml & 1000 ml)
29. Shaker Incubator
30. Streptomycin (HiMedia)
31. Weighing balance
32. Yeast Extract Peptone Dextrose (YEPD) agar plates, (10 g l^{-1} yeast extract, 20 g l^{-1} mycological peptone, 20 g l^{-1} dextrose, 20 g l^{-1} agar; pH 5.0)

12.3 Methods

12.4 Yeast Isolation Protocols from Natural Habitats

12.4.1 Rumen Fluid/Digesta

12.4.1.1 Materials

(a) Common materials
(b) Specific materials:

(i) Whirl-pak® bags
(ii) BagMixer® CC
(iii) Vortex
(iv) Muslin cloth
(v) Manifold machine (anaerobic media preparation)
(vi) Nitrogen cylinder
(vii) Carbon dioxide cylinder
(viii) Serum bottles (120 ml)
(ix) Resazurin (0.1%)
(x) L-Cysteine-hydrochloride (L-Cys-HCl)
(xi) Dispenser (5–50 ml)
(xii) Sealer and de-sealer machines

(xiii) Sterile syringes (1 ml and 10 ml)
(xiv) Syringe needles (gauge 16)
(xv) Potato Dextrose (PD) agar (200 g l^{-1} potato infusion, 20 g l^{-1} dextrose, 25 g l^{-1} agar; pH 5.0±0.5)

12.4.1.2 Protocol

1. Collect approximately 100 g of rumen digesta from buffalo/goat/sheep in sterile whirl-pak® bags (triplicates) from a slaughterhouse or any other source and transfer them to the laboratory immediately.
2. Store all rumen samples at 39.6 ± 1 °C in an incubator until further processing. (Note: It is recommended to process the sample as soon as possible to obtain maximum diversity of yeasts. Samples can be stored at 4 °C temporarily).
3. Pool the samples and equilibrate with CO_2 gas using a manifold machine. Take 10 g of this rumen digesta and suspend it into 100 ml of 0.9% saline solution.
4. Homogenize the above solution using BagMixer® CC for 30 seconds or filter through a muslin cloth to obtain a uniform suspension of rumen digesta before it is used for yeast enrichment and isolation processes.
5. Aerobic isolation on solid media:
   (a) Spread 100 µl of appropriately diluted suspension (10^{-3}, 10^{-4} and 10^{-5}) on YEPD and PD agar plates containing antibiotics (200 µg ml^{-1} streptomycin, 200 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol).
   (b) Incubate the plates at 39.6 ± 1 °C to obtain thermotolerant yeasts, and 30 ± 1 °C for 48–72 h to obtain the complete diversity of yeasts.
6. Aerobic isolation in liquid media (enrichment):
   (a) Inoculate 10 ml of homogenized rumen suspension into 100 ml of YEPD and Potato Dextrose broth containing antibiotics.
   (b) Incubate the flasks at 39.6 °C and 30 °C for 12–24 h at 150 rpm to enrich the indigenous yeast flora.
   (c) After observing considerable growth, spread 100 µl of the above culture broth on YEPD plates and incubate at 39.6 ± 1 °C and 30 ± 1 °C for 24–72 h to obtain yeast colonies.
7. Anaerobic isolation on solid media: serum roll bottle method [23].
   (a) Add 0.5 ml of appropriately diluted rumen suspension (usually 10^{-3} or 10^{-4}) in 10 ml YEPD and PDA media prepared anaerobically in 120 ml glass serum bottles containing 0.1 ml of resazurin (0.1%), 1 g l^{-1} of L-Cys-HCl and antibiotics (200 µg ml^{-1} streptomycin, 200 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol).
   (b) Incubate all bottles at 39.6 ± 1 °C and 30 ± 1 °C separately for 24–72 h and inspect regularly for the development of yeast colonies.

(c) Select different yeast colonies after observation of morphology under a DIC microscope and pick them anaerobically in the presence of N_2 gas.
(d) Subculture these colonies on aerobic (YEPD plate) and anaerobic media (serum roll bottles).

8. Anaerobic isolation on liquid media (enrichment):

(a) Inoculate 3 ml of homogenized rumen suspension in a 120 ml glass serum bottle containing 30 ml of YEPD and PD broth with 0.1 ml resazurin (0.1%), 1 g l^{-1} L-Cys-HCl and antibiotics as mentioned above.
(b) Incubate all serum bottles at 39.6 ± 1 °C and 30 ± 1 °C separately for 12–24 h at 150 rpm or until considerable growth has been obtained.
(c) Inoculate 100 µl of the above culture broth into serum bottles containing YEPD media with antibiotics.
(d) Incubate all serum bottles at 39.6 ± 1 °C and 30 ± 1 °C separately for 24–72 h.
(e) Select morphologically distinct yeasts by observing under microscope and subculture on aerobic (YEPD plate) and anaerobic media (roll bottles).

9. Preserve purified yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.4.2 Insect Gut (Termite/Beetle)

12.4.2.1 Materials

(a) Common materials
(b) Special materials:

(i) Forceps
(ii) Xylose
(iii) Stereomicroscope
(iv) Ethanol (90%)
(v) Sterile syringe (5 ml)
(vi) Yeast Nitrogen Base (YNB) + 1% xylose (pH 5.0)

12.4.2.2 Protocol

([35] with few modifications)

1. Collect 15–30 live termites/beetles from rotting wood logs, soil, bark of trees and transfer them to the laboratory at ambient conditions.
(Note: Dead termites/beetles are difficult to dissect and affect the gut micro-flora, therefore, it is important to keep the insects alive until dissection)

2. Surface-disinfect the insect body using 95% ethanol for 2 min and then rinse with sterile distilled water.
3. Dissect and remove the gut from the insect under a stereomicroscope using dissection needles/forceps and transfer the gut into a separate tube for crushing in 0.9% sterile saline solution.
4. Pass the gut suspension through a 2 or 5 ml syringe twice to macerate the gut contents.
5. Direct isolation on solid media:
   (a) Spread 100 µl of the crushed suspension onto YEPD and YNB + xylose plate containing antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (Note: Do not autoclave YNB+ xylose medium; sterilize by filtration)
   (b) Incubate all plates at 25–27 °C for 24–96 h and observe frequently for growth of yeast colonies.
6. Enrichment in liquid media:
   (a) Inoculate 1 ml of gut suspension into 10 ml of YEPD and YNB + xylose media in 50 ml flasks, each containing antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (b) Incubate all flasks at 25–30 °C for 24–48 h at 150 rpm.
   (c) After observing considerable growth, spread 100 µl of the culture broth on YEPD and YNB + xylose plates containing antibiotics and incubate at 25–30 °C for 24–72 h.
7. Select well-separated, morphologically different yeast colonies and streak on agar plates (YEPD and YNB + xylose) to obtain purified cultures.
8. Preserve purified yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.4.3 Hot Spring

12.4.3.1 Materials

(a) Common materials.
(b) Special materials:
   (i) Hand shovel
   (ii) Ice bucket
   (iii) Membrane filter (0.45 µm)
   (iv) PD medium (pH 5.0)

12.4.3.2 Protocol

([2] with few modifications)

1. Collect approximately 1000 ml of water and 500 ml of wet sediments from hot springs in sterile plastic containers.
2. Store the samples at 4 °C while transferring to the laboratory and process immediately.
3. Concentrate the hot spring water sample by passing through a membrane filter and resuspend the membrane filter into 10 ml of 0.9% saline solution to obtain a uniform suspension.
4. Mix wet sediment samples thoroughly before isolation of yeasts.
5. Direct isolation on solid media:
   (a) Spread 100 and 200 µl from the concentrated hot spring water suspension / wet sediments onto YEPD and PD agar plates with antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (b) Incubate all plates at 30, 35, 40 and 45 °C separately for 24–96 h or until yeast growth is observed.
   (Note: High incubation temperatures are employed for isolation of thermotolerant/thermophilic yeasts)
6. Enrichment in liquid media:
   (a) Inoculate 2 ml of concentrated hot spring water suspension and wet sediment separately into 20 ml of YEPD and PD media with antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (b) Incubate all flasks at 30, 35, 40 and 45 °C separately for 12–96 h at 150 rpm.
   (c) Take 5 ml culture broth from the above flask and inoculate into 100 ml YEPD medium with antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (d) Incubate each flask at 30, 35, 40 and 45 °C separately for 12–18 h at 150 rpm.
   (e) After observing considerable growth, spread 100 µl of culture broth on YEPD plates containing antibiotics and incubate at 30, 35, 40 and 45 °C separately for 24–72 h.
7. Examine yeast morphology microscopically; select different yeasts and streak on YEPD/PD agar plates to obtain purified cultures.
8. Preserve all yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.4.4 Flower

12.4.4.1 Materials

(a) Common materials.
(b) Special materials:

(i) Mortar and pestle
(ii) Muslin cloth
(iii) PD medium (pH 5.0)

12.4.4.2 Protocol

([32] with slight modifications)

1. Collect fresh flowers and transfer them to the laboratory for processing.
2. Rinse the flower with sterile distilled water for 10 min to remove adhered dust particles and other contaminants.
3. Dry the flower and surface-sterilize by transferring it into 70% ethanol for 2 min.
4. Rinse the flower again in sterile distilled water for 5 min and air dry maintaining sterile conditions.
5. Take 1 g of surface-sterilized flower in mortar and pestle and crush thoroughly in 3 ml sterile water.
6. Add 7 ml sterile water to this slurry, mix well and filter through muslin cloth to obtain a uniform suspension.
7. Spread 100 µl of the appropriately diluted (10^{-4} and 10^{-5}) suspension on YEPD and PD agar plates containing antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol).
8. Incubate the plates at 25 to 30 °C for 24–96 h and observe frequently for yeast colonies.
9. Select well-separated yeast colonies and observe under a microscope for morphological differences; subculture on YEPD/PD agar plates to obtain purified colonies.
10. Preserve purified yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.4.5 Nectar

12.4.5.1 Materials

(a) Common materials.
(b) Special materials:

(i) Eppendorf tube (1.5 ml and 2 ml)
(ii) Sterile syringe (1 ml)
(iii) Yeast Maintenance (YMA) media (3 g l^{-1} yeast extract, 3 g l^{-1} malt extract, 5 g l^{-1} mycological peptone, 10 g l^{-1} glucose, 25 g l^{-1} agar; pH 5.5)

12.4.5.2 Protocol

([43] with apt modifications)

1. Collect sufficient amount of nectar (~1 ml) from inflorescences with a sterile syringe into a 2 ml tube, transfer to the laboratory as soon as possible and store at 4 °C until further processing. (Note: The volume of nectar collected will vary from flower to flowerFlowers; some flowers may have as little as 5 µl of nectar fluid)
2. Dilute 100 µl of nectar with 900 µl of sterile distilled water and spread 100 µl of diluted nectar on YMA and YEPD plates containing antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol). (Note: In case of overgrowth of yeast colonies, prepare further dilutions to obtain well-separated yeast colonies)
3. Incubate all plates at 25 to 30 °C for 5 days and observe intermittently for yeast colonies.
4. Select well-separated yeast colonies and streak on agar plates (YMA and YEPD) to obtain purified cultures.
5. Microscopically examine yeast cells to determine their morphology and select different yeasts.
6. Preserve purified yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.4.6 Rotten Wood

12.4.6.1 Materials

(a) Common materials
(b) Special materials:

(i) Membrane filter (0.45 µm)
(ii) Sterile syringe (50 ml)
(iii) Yeast Nitrogen Base (YNB) + 1% xylose (pH 5.0)
(iv) YMA medium (pH 5.0)

12.4.6.2 Protocol

([6] with modifications)

1. Collect 50 g of rotten wood in a sterile plastic bag, transfer immediately to the laboratory and process the sample for double enrichment. (Note: Xylanolytic and xylose utilizing yeasts can be isolated through double enrichment as they are present in less numbers)
2. Add 1 g of rotten wood separately into 20 ml of YMA, YEPD and YNB + xylose media each containing antibiotics (100 μg ml^{-1} streptomycin, 100 μg ml^{-1} ampicillin and 25 μg ml^{-1}chloramphenicol).
3. Incubate all flasks at 25 °C for 3–10 days at 150 rpm on a reciprocal shaker.
4. Inoculate 5 ml of the above culture broth into 100 ml of YMA, YEPD and YNB + xylose media containing antibiotics.
5. Incubate all flasks at 25 °C for 12–24 h at 150 rpm on a rotary shaker.
6. Spread 100 μl of culture broth on YMA, YEPD and YNB + xylose plates containing antibiotics.
7. Incubate all the plates at 25 °C for 5 days or until observation of yeast colonies.
8. Select well-separated yeast colonies and streak them on agar plates (YEPD and YNB + xylose) to obtain purified cultures.
9. Select morphologically different yeasts by microscopic observation and streak on YEPD/YNB + xylose agar plates.
10. Preserve all yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) for long-term maintenance.

12.5 Yeast Isolation Protocols from Anthropogenic Habitats

12.5.1 Compost

12.5.1.1 Materials

(a) Common materials
(b) Special materials:

(i) Hand shovel
(ii) Acidified YEPD medium (pH 3.5)
(iii) YEPD 5 (5% dextrose) medium (pH 3.5)
(iv) YEPD 10 (10% dextrose) medium (pH 3.5)
(v) Glass wool
(vi) Glass funnel

12.5.1.2 Protocol

([9] with modifications)

1. Collect approximately 100 g of compost from composting heaps (at least 90 days old) after excavating at a depth of 1 m from the surface and transfer immediately to the laboratory under aseptic conditions.
   (Note: Compost samples can also be stored at 4 °C until further processing, but not for more than 2 weeks)
2. Inoculate 10 g of compost into 100 ml of 0.9% saline solution, to obtain a uniform suspension prior to enrichment and isolation experiments.
   [Note: In order to avoid mould growth, enrich the samples in an acidified liquid broth (pH 3.5) using sulphuric acid, as recommended by [28]. Filter the enriched culture broth with sterile glass wool and use this filtrate for further isolation experiments]
3. Direct isolation on solid media:
   (a) Spread 100 µl of appropriately diluted suspension on acidified YEPD, YEPD 5 and YEPD 10 agar plates containing antibiotics (200 µg ml^{-1}streptomycin, 200 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol).
      [Note: Acid hydrolysis of agar at low pH while autoclaving can be avoided by adding the acid to sterilized molten agar (45–50 °C) and mixing gently to avoid air bubbles]
   (b) Incubate the plates at 30, 35, 40 and 45 °C separately for 24–96 h to obtain yeast colonies.
      (Note: High incubation temperatures are employed for isolation of thermotolerant/thermophilic yeasts)
4. Enrichment in liquid media:
   (a) Inoculate 10 ml of suspension into 100 ml of acidified YEPD, YEPD 5 and YPD 10 media with antibiotics (200 µg ml^{-1} streptomycin, 200 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (b) Incubate each flask at 30, 35, 40 and 45 °C separately for 12–24 h at 150 rpm.
   (c) After considerable growth has been observed, take 5 ml culture broth from the above flasks and inoculate into 100 ml YEPD, YEPD 5 and YPD 10 media with antibiotics.
   (d) Incubate all flasks at 30, 35, 40 and 45 °C separately for 12–18 h at 150 rpm.
   (e) After observing considerable growth in the flasks, spread 100 µl of culture broth on YEPD and YEPD 10 agar plates containing antibiotics (200 µg ml^{-1} streptomycin, 200 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol).
      (Note: Dilute the culture broth before inoculation in case there is excessive yeast growth)
5. Select well-separated yeast colonies and subculture on YEPD media plates to obtain pure yeast cultures.

6. Preserve purified yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.5.2 Molasses

12.5.2.1 Materials

(a) Common materials
(b) Special materials:

(i) YMA medium (pH 5.5)
(ii) Malt Extract Peptone Dextrose (MEA) agar plates, (20 g l^{-1} malt extract, 6 g l^{-1} mycological peptone, 20 g l^{-1} dextrose, 20 g l^{-1} agar; pH 5.0)
(iii) Sugarcane Blackstrap Molasses (SCBM) medium (2.6% molasses, 0.3% yeast extract, 0.2% KH_2PO_4, 0.1% $(NH4)_2SO_4$, 0.1% $MgSO_4.7H_2O$, 2% agar; pH 5.0)

12.5.2.2 Protocol

([15] with appropriate modifications)

1. Collect 100 ml of molasses from sugar industries in 500 ml plastic containers in triplicates; transfer to the laboratory immediately and store at 4 °C until further processing.
2. Dilute the molasses sample ten times with 0.9% saline solution prior to enrichment and isolation experiments.
3. Direct isolation on solid media:

   (a) Spread 100 µl of the appropriately diluted suspension on YMA, YEPD and SCBMM agar plates containing antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol). (Note: Dilute further if there is excessive yeast growth, e.g. 10−3 or 10−4)
   (b) Incubate the plates at 30 °C for 24–96 h to obtain yeast colonies.

4. Enrichment in liquid media:

   (a) Inoculate 10 ml of appropriately diluted molasses suspension into 100 ml of YMA, YEPD and SCBMM broth, each containing antibiotics.
   (b) Incubate the flasks at 30 °C for 12–18 h at 150 rpm.
   (c) After observing considerable growth, spread 100 µl of culture broth on YMA, YEPD and SCBMM plates and incubate at 30 °C for 24–72 h to obtain yeast colonies.
   (Note: Dilute the culture broth suitably before plating in case of over-growth of yeasts)

5. Select well-separated yeast colonies; observe under a DIC microscope to select morphologically distinct yeasts and streak on YMA, YEPD, and SCBMM agar plates to obtain purified cultures.
6. Preserve all yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.5.3 Press Mud

12.5.3.1 Materials

(a) Common materials
(b) Special materials:

(i) Hand shovel
(ii) YMA medium (pH 4.0)
(iii) SCBM medium (pH 4.0)

12.5.3.2 Protocol

([30] with suitable modifications)

1. Collect 100 g to 1 kg press mud from sugar-cane composting heaps in sterile plastic containers and transfer to the laboratory immediately or store at 4 °C until further processing.
2. Suspend 100 g of press mud in 1000 ml of 0.9% saline solution to obtain a uniform suspension prior to enrichment and isolation experiments.
3. Direct isolation on solid media:

(a) Spread 100 µl of the appropriately diluted suspension on YMA, YEPD and SCBMM acidified agar plates (pH 3.5) containing antibiotics (200 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
(b) Incubate the plates at 30 °C for 24–96 h to obtain yeast colonies.
(Note: Prepare serial dilutions (e.g. 10^{-4}, 10^{-5}) in case of overgrowth of yeasts and repeat direct isolation)

4. Enrichment in liquid media:

(a) Inoculate 10 ml of suspension into 100 ml of YMA, YEPD and SCBMM media supplemented with antibiotics.
(b) Incubate the flasks at 30 °C for 12–18 h at 150 rpm.
(c) After considerable growth has been observed, spread 100 µl of the above culture broth on YMA, YEPD and SCBMM plates and incubate at 30 °C for 24–72 h to obtain yeast colonies.

7. Examine well-separated yeasts under the microscope and subculture them on agar plates (YMA, YEPD, and SCBMM) to obtain purified cultures.
8. Preserve purified yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.5.4 Distillery Effluent/Spent Wash

12.5.4.1 Materials

(a) Common materials
(b) Special materials:

(i) YMA medium (pH 4.5)
(ii) SCBM medium (pH 4.5)

12.5.4.2 Protocol

[3]

1. Collect approximately 500 ml of effluent from distillery units associated with sugar industries and transfer it to the laboratory immediately under aseptic conditions.
2. Dilute the distillery effluent ten times with 0.9% saline solution prior to enrichment and isolation experiments.
3. Direct isolation on solid media:
   (a) Spread 100 µl of the diluted suspension on YMA and YEPD agar plates containing antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (b) Incubate the plates at 30 °C for 24–96 h or until observation of yeast colonies. (Note: Prepare serial dilutions (e.g. 10^{-4}, 10^{-5}) in case of overgrowth of yeasts and repeat direct isolation)
4. Enrichment in liquid media:
   (a) Inoculate 10 ml of diluted distillery effluent suspension into 100 ml of YMA and YEPD media containing antibiotics (200 µg ml^{-1} streptomycin, 200 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (b) Incubate the flasks at 30 °C for 12–18 h at 150 rpm.
   (c) Spread 100 µl of the above culture broth on YEPD plates and incubate at 30 °C for 24–72 h.
5. Pick well-separated yeast colonies and subculture on YMA and YEPD media plates to obtain pure yeast cultures.

6. Select morphologically different yeasts and streak them on YMA and YEPD agar plates.
7. Preserve purified yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

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