

Chapter 2

Methods for the Isolation and Investigation of the Diversity of Cold-Adapted Yeasts and Their *Ex Situ* Preservation in Worldwide Collections

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Abstract Cold-adapted (psychrophilic and psychrotolerant) yeasts have been isolated from a variety of substrates, using a variety of cultivation methods. Yeasts able to grow at as low as 0 °C have been isolated from cold substrates such as glaciers, snow, and deep-sea sediment, but also from temperate and tropical climates. A broad diversity of media and culture conditions have been used to isolate and cultivate these yeasts. Low-temperature incubation is used to select for psychrophiles, thus depending on the strains relatively long incubation time (up to 14 weeks) may be required. Cold-adapted yeast strains belong to many species in many clades of Ascomycota and Basidiomycota. Numerous strains have been deposited in public culture collections. Online strain catalogs of some public yeast culture collections include searchable fields for growth temperatures, allowing selection of yeasts able to grow at desired temperatures. Culture-independent methods for profiling yeast diversity in mixed communities can be used to profile populations, allowing detection of yeasts whose DNA is present in a specimen but that were not cultivated.

Keywords Psychrophilic and psychrotolerant yeasts • Yeast cultivation methods • Yeast culture collections

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2.1 General Methods of Isolation of Yeasts from the Environment

Several considerations must be taken into account when designing a protocol for isolation of cold-adapted yeasts. As many of these considerations are identical for all yeasts, a brief discussion of general yeast isolation and cultivation protocols is presented. It is recommended that a combination of protocols be utilized when sampling a new substrate, to ensure that the desired results can be obtained.

Culture-based methods for isolation and cultivation of yeasts in natural substrates are desirable when living cultures are needed for subsequent studies. The major limitation of culture-based methods is that some yeast species and strains may not be culturable under the growth conditions used. Culture-independent methods (described in [Sect. 2.9](#)) have an advantage in that a broader cross section of taxa can be detected, minority species can be detected, and relative quantities may be determined. However, examination of the physiological properties of yeasts, and subsequent biotechnological developments, requires pure, living cultures. Therefore, this chapter focuses primarily on methods used for isolation of pure cultures.

Culture-based methods for enumeration and cultivation of yeasts from foods and environmental substrates have been described in detail in many recent reviews and book chapters (Martini et al. 1996; Welthagen and Viljoen 1997; Beuchat 1998; Fung 1999; Boundy-Mills 2006; Solís et al. 2009).

Three general types of culture-based methods are used for enumeration of microbes: plate count (PC) procedures (either pour plates or spread plates), membrane filtration (MF) followed by growth of colonies on the membrane placed on an agar plate, and most probable number (MPN) procedures. PC and MF methods utilize agar plates and are discussed in this chapter.

Prior to plating, a substrate is often processed to make it more amenable to growth of a statistically valid number of single colonies on or in an agar plate (Boundy-Mills 2006). Processing may include some of the following steps:

- (1) Aseptic collection of the specimen;
- (2) Storage of the specimen under appropriate conditions, such as refrigeration;
- (3) If needed, surface sterilization to avoid cultivation of microbes on the surface of the substrate;

- (4) Homogenization, disruption, or mixing of the sample to release and evenly distribute microbes;
- (5) Enrichment;
- (6) Dilution of specimens with high yeast density in an appropriate diluent;
- (7) Concentration of specimens with low yeast density, such as by filtration or precipitation.

After appropriate processing, agar plate methods can involve either spread plates or pour plates. Many researchers prefer spread plates as they are simpler to perform, and it is easier to recover a yeast from a colony on the surface of the agar, rather than embedded in the agar. Furthermore, the warm temperature of the molten agar used in pour plates may be particularly injurious to cold-adapted yeasts.

There are numerous nutrient agars available for cultivation of various types of yeasts (Beuchat 1998; Boundy-Mills 2006). Liquid or agar-based complex nutritionally rich media generally include an energy source such as glucose, a nitrogen source such as ammonium or hydrolyzed protein (peptone, tryptone, etc.), and a vitamin source such as yeast extract or malt extract. Other supplements often include compounds such as rose Bengal or dichloran to inhibit or slow growth of filamentous fungi (Beuchat 1998) and antibiotics such as tetracycline or chloramphenicol to prevent growth of bacteria. Alternatively, the pH of the medium can be reduced by addition of acid to discourage growth of bacteria. A broad range of yeasts and filamentous fungi can grow on these media. Selective and differential media containing inhibitors, selective nutrients, and indicator dyes are used to differentiate or select for growth of specific types of yeasts. For example, lysine agar is used to select for growth of non-*Saccharomyces* yeasts in brewing, as *Saccharomyces* cannot utilize lysine as a sole carbon source but unwanted spoilage yeasts can (Heard and Fleet 1986).

When selecting a media formulation, it is important to consider the composition of the substrate of origin and the types of yeast to be cultivated. For example, osmotolerant yeasts that inhabit high-sugar environments like as fruit juice, nectar, and dried fruit, such as several species of *Metschnikowia*, *Zygosaccharomyces*, and *Starmerella* species, grow well in media containing 50 % (w/v) glucose, but most other yeasts cannot. In contrast, microbes such as soil oligotrophs may fail to grow on full-strength media and can only be cultivated on diluted media such as 1/10 strength rich media (Hattori 1980).

Following enumeration of yeasts by plate counts or membrane filtration, yeasts can be selected and purified for further characterization. It is common to select one or more colonies of each morphology for purification. However, multiple species may have similar or identical colony morphologies, so some species may be overlooked. Culture-independent methods (discussed in Sect. 2.9) should be used if a more complete view of the yeast diversity is needed.

2.2 Niches that Harbor Cold-Adapted Yeasts

The term “cold-adapted” is used in this chapter to refer to yeasts able to grow at low temperatures in the laboratory. An in-depth overview of the concept of psychrophily and psychrotolerance in yeasts is reported in [Chap. 1](#).

In the food industry, the term “psychrotroph” is sometimes used to describe microbes that are able to grow at refrigeration temperatures (0–7 °C), but have optimal growth temperatures at or above 20 °C (Cousin et al. 1992). Most food-associated cold-adapted microbes are psychrotolerant (Tomkin 1963), as discussed in more detail in [Chap. 23](#).

When searching for a yeast strain with specific properties, one should search in natural habitats that have those properties. For example, acid-tolerant yeasts thrive in grape juice and other acidic fruit juices. Osmotolerant yeasts can be found in dried fruit and honey. Ethanol-tolerant yeasts can be found in wine fermentations. Thus, it should not be surprising that cold-adapted yeasts are prevalent in cold habitats. However, it is important to note that yeasts able to grow as low as 4 °C have also been isolated from plant surfaces, water, soil, and insects in temperate and even tropical climates (see [Sect. 2.8](#)).

Cold-adapted yeasts have been reliably found in numerous cold habitats, including permafrost, snow, cold deserts, and glacial ice, meltwater and sediment ([Chaps. 3, 4, 5, 6](#)), the deep sea ([Chap. 7](#)), and frozen and refrigerated foods ([Chap. 23](#)). In addition to cold tolerance, yeasts able to grow in ice, permafrost, and frozen foods must tolerate low water activity, as very little liquid water is present in these substrates. Yeasts from glacial ice therefore must be osmotolerant.

It is unclear whether cold-adapted yeasts are endemic to cold habitats. With the limited number of yeast strains isolated to date, and the limited number of yeast ecologists, it is difficult to establish where a given yeast species is *not* found. For example, the yeast species *Cryptococcus victoriae*, first isolated from South Victoria Land, Antarctica (Montes et al. 1999), was subsequently found in many other habitats and geographic locations, such as cherries in California (Hamby et al. 2012). In contrast, yeast species *Cryptococcus vishniacii* is currently unknown outside of Antarctica. Yeasts belonging to the genera *Mrakia* and *Mrakiella* have been isolated exclusively from cold habitats such as Antarctica (Turchetti et al. 2008; Singh and Singh 2012), the European Alps (Margesin et al. 2002), and subglacial waters of northwest Patagonia, Argentina (Brizzio et al. 2007).

Endemic microbes, as well as exotics blown in by the wind from temperate and tropical regions, can be preserved for thousands of years deep in glacial, Arctic and Antarctic ice (Catranis and Starmer 1991; Abyzov 1993; Ma et al. 1999, 2000). Microbial species thought to be extinct, such as pathogens, could theoretically be revived re-appear as glaciers and polar ice melt due to global warming or other conditions (Ma et al. 1999).

2.3 Aseptic Collection of Specimens

For reasons of intellectual property, biosafety, and adherence to inter-institutional and international agreements and treaties such as the Convention on Biological Diversity, descriptive data should be collected including site, habitat, and other descriptive data. Projects involving isolation of large numbers of microbes, especially those performed by multi-institutional or international teams, often utilize standardized data sheets to ensure that all relevant data are collected. A very thorough data collection plan and data sheet template for collection of microbes has been recently compiled (Tadych 2008). Some types of data that are gathered in general microbiology surveys, and specific data related to cold-adapted yeasts, are listed in Table 2.1.

Some types of data are specific for the type of habitat. For example, a recent publication of yeasts isolated from a glacial lake includes descriptions of the elevation, slope, sun exposure, surface area, maximum depth, pH, dissolved solids (conductivity), Secchi disk transparency, summer air and water temperatures, and how many months per year the lake is frozen (Libkind et al. 2004). A Secchi disk is a black-and-white disk lowered into the water on a rope to determine water clarity, a property that may reflect nutrient levels in the water.

Replicate samples are recommended if possible. For example, Butinar et al. (2011) filtered and plated between four and eleven replicates per sample of melted glacial ice.

Although collection of water from shallow lakes and rivers may be relatively easy, many of the habitats that harbor cold-adapted yeasts are quite inaccessible, such as deep-sea water and sediment, and the interior of glaciers. Aseptic sampling techniques have been developed for these substrates.

Deepwater sediment: Sediment core samplers have been developed for aseptic sampling of deep-sea sediment (Ikemoto and Kyo 1993) that can be used in either a manually controlled or remote-controlled submersible.

Deepwater, lake and river water sampling: Water sampling is performed with specially designed sampling devices such as the Nansen bottle or Niskin sampler. The Nansen bottle was designed in 1910 by oceanographer Fridtjof Nansen, and later improved by Shale Niskin. It is a metal or plastic cylinder, open at both ends, that is lowered in a vertical position on a cable to the desired depth. The spring-loaded valves are activated by releasing a metal weight (“messenger”) down the cable, by a pressure switch, or by remote control, sealing the ends of the tube. The tube is then raised to the surface of the water. Similarly, the van Dorn sampler is a sample tube that is lowered into the water in a horizontal position with both ends open, and at the desired depth, the ends are sealed with spring-loaded valves by releasing a messenger down the cable. Note that in both types of sampler, the interior of the sampling tube is exposed to surface water as it is lowered. Microbes from the higher zones are diluted but not excluded. A variant, the Niskin bio-sampler, allows aseptic sampling. Another sampling apparatus, the J-Z sampler, has been modified to reduce contamination (Kimball et al. 1963).

Table 2.1 Categories and types of data to be collected in general microbial surveys, and specific data related to cold-adapted yeasts

Category	Data collected in general microbial surveys	Data collected for cold-adapted yeasts
Collector documentation	Names and institutions of primary and secondary personnel Date specimen was collected Date specimen was processed and plated Photograph filenames, name of photographer, type of camera Collecting permit documentation	Name and institution of experiment station facility, including cold storage facility
Locality	Site description: public or private land, national park, city, province, state, country GPS coordinates Elevation Forest, soil, or water type Slope (such as north-facing mountain); sun exposure Type of ecosystem	Name of mountain, glacier, fjord, bay, river, or deepwater trench Depth of river, lake, or ocean Surface area of lake Average salinity and pH of river, lake, or ocean Average summer air and water temperatures How many months per year a lake is frozen Average ice thickness of a glacier
Ambient conditions	Water and air temperatures, humidity Relevant weather conditions such as recent rain, flood, drought, heat wave Sun exposure Season	Depth of ice core specimen Secchi disk transparency of water Underground temperature at various depths after collecting ice or permafrost core samples Temperature of sediment, water, and ice
Substrate	Plant or insect host, and name and institution of person who identified it	Depth of ice core specimen Whether sample was from glacier superficial meltwater, supraglacial sediments, deep piping sediments, subglacial sediments, etc. Whether ice core or meltwater specimens are sediment rich or clear Physicochemical parameters including dry mass, pH, salinity, Na ⁺ , Mg ²⁺ , K ⁺ , total C, N, P; organic C, N, P

(continued)

Table 2.1 (continued)

Category	Data collected in general microbial surveys	Data collected for cold-adapted yeasts
Sampling regimen	Number of sites samples and number of replicate specimens collected per site Aseptic sampling method Size of samples How samples were stored after collection and before microbial plating Amount of time between collecting specimen and plating Processing methods: surface sterilization, homogenization, concentration, or dilution Volume plated	Temperature and length of storage of ice core samples Type of sampling device used, such as Nansen bottle or Niskin sampler, spatula, rotary drill

Glacial ice cores: Viable yeasts have been cultivated from ice samples up to 3250 years old (Abyzov 1993). Ice cores are collected using a hollow drill which cuts in a cylindrical pathway. The tube surrounds the core as it proceeds. The length of the core is limited by the length of the drill barrel, such as the 6-m drill at Vostok Station in Antarctica (Abyzov 1993). Deep cores are collected by cutting to the length of the drill assembly, raising it to the surface, emptying the barrel, and repeating. Deep cores more than 300 m may require use of a fluid to prevent the hole from deforming and closing due to pressure. Cores are sealed in plastic bags, stored frozen, and analyzed in clean rooms. Precautions such as storing the core below $-15\text{ }^{\circ}\text{C}$ and transporting in foam shippers are taken to avoid introduction of microfractures that could allow microbes to enter the interior of the ice core. Ice cores are examined visually on a light table to detect fractures (Ma et al. 1999). More extensive measures to detect microbial contamination into the interior of ice core samples have been developed, including fluorescent microspheres, a chemical tracer, dissolved organic carbon (DOC) signatures, and comparison of bacterial 16S clone libraries from drilling mud to bacterial species recovered from core samples (Gronstal et al. 2009). When collecting core samples, prevention of contamination from the borehole can be difficult. Gilichinsky et al. (2005) devised a method to investigate the contribution of contaminating bacteria from surface and borehole microbes in cryopeg samples, by measuring incorporation of ^{14}C from glucose to determine relative metabolic activity under hypersaline conditions. They determined that metabolic activity in sterilized brine inoculated with surface contaminants was low, but high in native brine; thus, the contaminants were relatively metabolically inactive in brine.

Permafrost: The microbial diversity of permafrost was recently reviewed (Wagner 2008; Margesin 2009). It has been suggested that microbes, especially bacteria, may survive in a hypometabolic state deep in permafrost for millions of

years (Vorobyova et al. 1997). Although less prevalent than bacteria, yeasts are present in deep permafrost and were the dominant culturable aerobic eukaryotes in samples of ancient Oleorian permafrost, 2–3 million years old (Vorobyova et al. 1997). Yeasts were more frequently found in young permafrost with high plant residue content such as peat.

2.4 Surface Sterilization of Specimens

Ice and permafrost cores have been collected from depths up to 4 km. Several researchers have examined these ice cores for viable microbes or microbial DNA, as described by Rogers et al. (2004). It can be difficult to confirm that viable microbes were indeed isolated from the interior of the ice cores, as contamination from external sources such as drilling equipment is hard to exclude. Ma et al. (2000) surface sterilized the ends of Greenland glacier ice cores by UV irradiation, then used a sterile drill and saw to remove a subcore from the center of the ice sample. Rogers et al. (2004) compared a variety of protocols for decontaminating ice core samples, including exposure to bleach, ethanol, UV radiation, acid and base, and found that bleach was the most effective method in killing microbial contaminants on the surface of the ice. They confirmed that microorganisms that they spread on the surface of the ice core were not recovered in the melted material. Abyzov (1993) devised a method to aseptically sample the interior of Antarctic ice cores, using a circular knife blade to crack off the end of an ice core sample, followed by melting and collecting water from an interior section using a cone-shaped heater applied to the freshly revealed interior surface.

Ice samples collected from surface environments such as glacier surfaces and sea ice do not require such extensive surface sterilization. For example, Butinar et al. (2011) simply melted and discarded the surface layer from ice samples and then melted the remaining ice sample.

2.5 Homogenization, Dilution, or Concentration of Specimens

Homogenization: Some samples must be homogenized, disrupted, or mixed to release and disperse microbes. Depending on the composition and texture of the substrate, a sample may be aseptically ground, shaken, pounded, pummeled, stirred, swirled, or pureed (Boundy-Mills 2006). For example, samples of giant white clams and tube worms collected at over 1,000 m depth in Sagami Bay, Japan, were surface sterilized with ethanol and then pulverized in artificial seawater (Nagahama et al. 2001, 2003). After surface sterilization, ice core and subcore samples are simply melted to release microbes from the ice matrix and

Table 2.2 Yeast abundance in select substrates known to harbor cold-adapted yeasts

Substrate	Yeast abundance	Reference
Cryoconite	7×10^3 to 1.4×10^4 CFU g ⁻¹ dry mass	Singh and Singh (2012)
Cryoconite	500– 1.5×10^5 CFU g ⁻¹ dry mass	Margesin et al. (2002)
Antarctic soil	$5-1 \times 10^5$ cells g ⁻¹ soil	Di Menna (1966)
Surface layers of glacier ice	Up to 25 CFU ml ⁻¹	Butinar et al. (2007)
Glacial ice cores and meltwater	0.11–0.56 CFU ml ⁻¹	Turchetti et al. (2008)
Supra- and subglacial sediment	12–21 CFU g ⁻¹ dry mass	Turchetti et al. (2008)
Cryokarst formations in glacier	Up to 400 CFU ml ⁻¹	Butinar et al. (2007)
Basal glacier ice	Up to 4,000 CFU ml ⁻¹	Butinar et al. (2007)
Permafrost	200–2,000 CFU g ⁻¹ dry mass	Vorobyova et al. (1997)
Cryopegs (hypersaline underground water surrounded by permafrost)	3–400 CFU ml ⁻¹	Gilichinsky et al. (2005)
Arctic subglacial environments	Up to 4×10^3 CFU ml ⁻¹	Butinar et al. (2007)
Supraglacial samples	25 CFU ml ⁻¹	Butinar et al. (2005)
Subglacial ice	Up to 10 CFU ml ⁻¹ (ascomycetes)	Butinar et al. (2011)
Sea ice	84 CFU L ⁻¹ (ascomycetes) and 2×10^3 CFU L ⁻¹ (basidiomycetes)	Butinar et al. (2011)
Seawater in a fjord	Up to 500 CFU L ⁻¹ (ascomycetes) and 400 CFU L ⁻¹ (basidiomycetes)	Butinar et al. (2011)
Snow/ice in the tidal zone bordering the fjord	6.7×10^3 CFU L ⁻¹ ascomycetes	Butinar et al. (2011)

evenly distribute the microbes (Abyzov 1993; Rogers et al. 2004). Yeast abundance varies significantly in different substrates, or in the same type of substrate sampled in different locations and seasons. Examples of yeast abundance reported for several different types of substrates that harbor cold-adapted yeasts are listed in Table 2.2. For example, yeast populations were reported to be denser in freshwater than marine water in Brazil (Hagler and Ahearn 1987).

Dilution: Some substrates such as cryoconite (organic and inorganic dust containing microbes found on the surface of glaciers), soil or glacial or marine sediment require suspension or washing to release suspended microbes. They may also contain sufficiently high concentrations of viable yeasts to require dilution prior to plating. Diluents used include liquid media, saline, water, or various phosphate buffers (Boundy-Mills 2006). A surfactant such as 0.01–0.05 % (v/v) Tween 80 can be added to aid in separation of cells from soil particles, cell clumps, and filamentous structures (Deak 2003). For example, cryoconite was shaken with phosphate buffer and Tween 80 at 170 rpm, and then, appropriate dilutions in saline were surface plated onto agar plates (Margesin et al. 2002). The water activity of the diluent should resemble that of the substrate, or yeast viability may be affected (Hocking et al. 1992; Hernandez and Beuchat 1995). Because frozen substrates such as ice and permafrost have very little available liquid water, media with quite low water activity have been successfully used to isolate yeasts from melted snow and ice (Butinar et al. 2011). Yeasts in diluent must be plated promptly, particularly in saline solutions, which may have adverse effects on yeast viability (Andrews et al. 1997).

Concentration: Specimens with low yeast density can be concentrated using filtration or centrifugation. Filters such as nitrocellulose membranes, 0.2- or 0.45- μm pore size, are used in a sterile filtration device to concentrate the microbes in the water. To exclude particles larger than yeasts, the material can be pre-filtered with a larger pore size membrane such as 5 μm (López-García et al. 2001). The filter is then aseptically removed from the filtration device and placed filtrate side up on the surface of an agar plate. The volume of water to be filtered depends on the concentration of resident microbes. Table 2.2 lists yeast abundance reported in selected substrates from which cold-adapted yeasts have been isolated. As it may be difficult to predict the yeast density in advance, multiple concentrations should be plated in order to obtain a statistically significant number of yeasts.

Organic contents including yeast cells in dilute samples such as lake water have been concentrated using a coagulant (Sláviková et al. 1992). Water samples can also be concentrated using a Foerst-type continuous flow centrifuge (Kimball et al. 1963).

2.6 Cultivation Methods for Cold-Adapted Yeasts

Many different media and growth conditions have been used for isolation and cultivation of cold-adapted yeasts. Table 2.3 describes a variety of media, incubation temperatures, and incubation times used in selected published descriptions of isolation and cultivation of cold-adapted yeasts from many of the cold substrates discussed in this chapter.

Media composition: The composition of media including nutrient concentration and water activity can dramatically affect recovery of viable microbes. Recovery of microbes from oligotrophic environments such as water may be more successful

Table 2.3 Media and growth conditions used to isolate and cultivate cold-adapted yeasts from various cold substrates in selected recent publications

Substrate, location	Isolation medium and growth conditions	Reference
Glacier, Patagonia, Argentina	Meltwater filtered on 0.45- μ m nitrocellulose filter placed on MYP agar with chloramphenicol. Incubated at 4–15 °C for up to 1 month	de Garcia et al. (2007)
Glacier, Ny-Ålesund, Norway	Cryoconite diluted in unnamed diluent, plated on six media by pour plate and spread plate: YPD, MYP, MEA, PDA, SDA, PCA, rose Bengal plus tetracycline. Incubated at 5, 10, 15, 20, and 25 °C for 2–4 weeks	Singh and Singh (2012)
Glacier, Kongsfjorden, Svalbard, Norway	Basal ice, subglacial meltwater, subsurface ice from cryokarst formations, snow/ice mixtures, seasonal meltwaters, seawater, and sea ice. Up to 100 mL of liquid filtered onto 0.22- and 0.45- μ m membranes, and filters placed on DRBCA, DG18, MY10-12, MY20G, MY35G, MY50G, MEA, MEA5NaCl, MEA10NaCl, MEA15NaCl, MEA17NaCl, MEA24NaCl, MEA30NaCl (all with chloramphenicol, 50 mg L ⁻¹). Incubated 4, 10, and 24 °C for up to 14 weeks	Butinar et al. (2011)
Glacier, Tyrolean Alps, Austria	Cryoconite suspended in diluent and then plated on MP plus chloramphenicol and lactic acid. Incubated at four temperatures: 2 °C for 10 days, 20 °C for 3–5 days, 37 °C for 2 days or 55 °C for 2–4 days	Margesin et al. (2002)
Glacial cores and meltwater, Forni and Szforzellina glaciers, Italy	50–100 ml of melted glacial cores or meltwater filtered through 0.22- μ m filters, and filters placed on RB + tetracycline, DG18, and DRB + chloramphenicol. Incubated at 4 °C for 12 weeks and 20 °C for 3 weeks	Turchetti et al. (2008)
Supra- and subglacial sediment, Forni and Szforzellina glaciers, Italy	Solid samples diluted with 0.1 % sodium pyrophosphate, serial dilutions plated in triplicate on RB + tetracycline, DG18, and DRB + chloramphenicol. Incubated at 4 °C for 12 weeks and 20 °C for 3 weeks	Turchetti et al. (2008)
Ancient ice cores, Greenland	200 uL of melted subcores plated on eight media: SAB, YMA, acidified YMA, NA, MEA, PDA, MA, OMA. Incubated at 8 °C for 6 weeks, then 15 °C for 2 weeks	Ma et al. (1999, 2000)

(continued)

Table 2.3 (continued)

Substrate, location	Isolation medium and growth conditions	Reference
Polythermal glacier ice from edge of glacier, Kongsfjorden, Norway	Ice melted, then either diluted or filtered and filter plated on agar; plated on MEA, MEA5NaCl, and MEA20G. Incubated at 4, 10, and 24 °C, number of days not stated	Butinar et al. (2007)
Cryopegs, East Siberian Sea coast	Cryopeg brine plated directly, or filtered and filter placed on agar, on MA with or without sodium purryvate, and on Czapek media, with added sucrose or NaCl. Incubated at 4 or 26 °C for 1 month	Gilichinsky et al. (2005)
Permafrost, Dry Valley, Antarctica	Core sample surface sterilized, 1 ml of melted microcore spread plated on RB with tetracycline. Incubated at 4 °C for 12 weeks or 20 °C for 3 weeks	Zucconi et al. (2012)
Deep-sea sediments, Suruga Bay, Japan	Sediment plated on YMA, 1/5 YMA, PDA, and CMA, all made with artificial seawater and supplemented with 0.01 % chloramphenicol. Incubated 5–10 °C for 2 weeks then 20 °C for 1 month	Nagahama et al. (2003)
Deep-sea sediments, giant white clams and tubeworm from Suruga Bay, Iheya Ridge and Mariana Trench	Sediments plated directly on agar; animals surface sterilize and then pulverized in artificial water. Specimens plated on YMA, PDA, NA with 0.5 % glucose, YNB without amino acids containing 0.5 % glucose and 2 % agar; all dissolved in artificial seawater with 0.01 % chloramphenicol, 0.002 % streptomycin. Plates incubated 5–10 °C for 2 weeks, then 20 °C for one month	Nagahama et al. (2001)

YPD agar yeast extract peptone dextrose agar; *MYP agar* malt extract yeast extract soytone agar (Bandoni 1972); *MP agar* malt extract mycological peptone agar (3 % malt extract, 0.5 % mycological peptone, 1.5 % agar, pH 5.4) (Margesin et al. 2002); *SAB* Sabouraud's agar; *YMA* yeast–malt extract agar; *NA* nutrient agar; *MEA* malt extract agar; *MEA5NaCl* malt extract agar containing 5 % (w/v) NaCl; *MEA20G* malt extract agar containing 20 % glucose; *PDA* potato dextrose agar; *MA* mycobiotic agar; *OMA* oatmeal agar; *SDA* Sabouraud dextrose agar; *PCA* potato carrot agar; *RB* rose Bengal agar; *CMA* corn meal agar; *DRB* dichloran rose Bengal agar (Difco); *DRBCA* dichloran rose Bengal chloramphenicol agar (King et al. 1979); *DG18* dichloran 18 % glycerol agar (Hocking and Pitt 1980); *MY* 10–12 malt yeast 10 % glucose and 12 % NaCl agar (Samson et al. 2004); *MY20G* malt yeast 20 % glucose agar (Gunde-Cimerman et al. 2003); *MY35G* malt yeast 35 % glucose agar (Gunde-Cimerman et al. 2003); *MY50G*, malt yeast 50 % glucose agar (Samson et al. 2004); *MEA5NaCl* malt extract 5 % NaCl (Gunde-Cimerman et al. 2003); *MEA10NaCl* malt extract 10 % NaCl (Gunde-Cimerman et al. 2003); *MEA15NaCl* malt extract 15 % NaCl (Gunde-Cimerman et al. 2003); *MEA17NaCl* malt extract 17 % NaCl (Gunde-Cimerman et al. 2003); *MEA24NaC* malt extract 24 % NaCl (Gunde-Cimerman et al. 2003); *MEA30NaCl* malt extract 30 % NaCl (Gunde-Cimerman et al. 2003); *YNB*, yeast nitrogen base

with low nutrient media. Vishnivetskaya et al. (2000) observed that recovery of viable bacteria from permafrost samples up to 2 million years old was more successful using 1/10 strength TSA (tryptone soy agar) than with full-strength TSA, but recovery of bacteria from surface tundra soil was higher on full-strength TSA. Water activity is also an important consideration. Although frozen substrates such as ice and permafrost have very little dissolved solutes, they also have very little available liquid water and thus have low water activity. Media with low water activity have been successfully used to isolate yeasts from melted snow and ice (Butinar et al. 2011).

Temperature: Cultivation temperatures must be carefully selected for cold-adapted yeasts. These yeasts can be either psychrophilic or psychrotolerant. For example, three to ten times more yeasts were isolated from cryoconite on the surface of a glacier at 2 °C than at 20 °C, and none could be isolated at 30 °C (Margesin et al. 2002). Kutty and Philip (2008) recommend that after inoculation onto agar plates, yeasts from polar and deep-sea habitats should be incubated at 5 °C. However, the growth temperature range of yeasts isolated from cold substrates can be surprisingly high: Butinar et al. (2011) found that most ascomycetous yeasts they isolated from glacier ice and meltwater could grow up to 25–30 °C, in addition to growing at 4 °C.

2.7 Cold-Adapted Yeast Species

Cold-adapted yeasts belong to multiple clades of Ascomycota and Basidiomycota. While ascomycetous yeasts are the predominant agent of spoilage of chilled or frozen foods (Davenport 1980; Schmidt-Lorenz 1983), they are less abundant than basidiomycetous yeasts in polar soil (Vishniac and Onofri 2003), cold seawater, and subglacial ice (Butinar et al. 2011). The medium used for isolation of yeasts can impact the species recovered: Butinar et al. (2011) found that ascomycetous yeasts dominated on media containing elevated NaCl, but basidiomycetous yeasts dominated on other media with lower NaCl concentrations. In samples from the northwest Pacific Ocean, Nagahama et al. (2001, 2003) found that ascomycetes dominated in deep-sea sediments sampled from less than 2,000 m depth, but basidiomycetes dominated in deeper sediments.

Several recent publications detail the range of yeast species isolated from numerous cold habitats and locations. A review on yeasts isolated from Antarctica recently published by Buzzini et al. (2012) includes a list of species found in various Antarctic habitats. Shivaji and Prasad (2009) reviewed the biodiversity of yeasts from Antarctica, including a list of strains available from the Centraalbureau voor Schimmelcultures, CBS). Recent reports listing numerous yeast species include yeasts from four glaciers in Argentina (de García et al. 2007) and Italy (Turchetti et al. 2008), deep-sea sediment in Japan (Nagahama et al. 2001, 2003), and permafrost (Zucconi et al. 2012).

Some of these species are ubiquitous, with strains found in cold as well as warm habitats, such as *Cryptococcus laurentii*, *Cryptococcus macerans* (anamorph of *Cystofilobasidium macerans*), *Rhodotorula mucilaginosa* (de García et al. 2007). These species are also able to grow at temperatures of 20 °C and higher. However, some species isolated from Antarctica are psychrophiles. Species in the genus *Mrakia* and its anamorphic genus *Mrakiella*, by definition of the genera, have a maximum growth temperature of 20 °C or lower. Some species names reflect the cold-adapted nature of this genus, including *Mrakia frigida*, *Mrakia gelida*, and *Mrakia psychrophila*. Many *Mrakia* strains were isolated from cold climate soil and glaciers, such as the many *M. gelida* and *M. frigida* strains isolated from glaciers in Italy (Turchetti et al. 2008).

2.8 Preservation of Cold-Tolerant Yeasts in Public Culture Collections

Many scientific journals require that microbial strains cited in scientific publications be made available to the scientific public for research. Some are maintained in research collections at the institution of the researcher that performed the study, and others are deposited in public collections to allow broader availability and long-term preservation. For example, yeasts from glaciers and seawater described in a recent publication (Butinar et al. 2011) are maintained in the EX-F Culture Collection of Extremophilic Fungi of the Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia.

The mission of public microbial culture collections is to preserve and distribute pure, authenticated microbial strains. Culture collections acquire yeasts through deposit by researchers from other institutions, through in-house research, or a combination of these (Boundy-Mills 2012). Many of these collections have online strain catalogs that can be searched by genus, species, geographic origin, and habitat of origin such as Antarctica, Norway, or Alaska, or cold habitats such as glaciers, deep-sea sediment, frozen foods, or permafrost. Researchers searching for a specific strain or species can search the species or strain fields in the online catalogs of these collections to locate strains. Representative culture collections, their websites, and examples of habitats sampled to obtain cold-adapted yeasts are listed in Table 2.4.

In addition to this basic descriptive data, some public culture collections have made physiological data including growth temperatures available in a searchable format in their online catalogs. For instance, the Industrial Yeasts Collection (DBVPG) at the University of Perugia, Italy, has a strong emphasis on environmental isolates. Historically, this collection focused on wine yeasts and has expanded in recent decades to include yeasts useful for other biotechnological applications such as cold-active enzymes. To fill this need, DBVPG personnel have built a sizeable collection of yeasts isolated from glaciers and snow. The

Table 2.4 Cold-adapted yeast species and strains available from public yeast culture collections

Yeast culture collection	Website	Website searchable for growth temperatures	Examples of cold substrates and geographic origins	Genera of yeasts able to grow at 4 °C
Industrial yeasts collection, University of Perugia, Italy (DBVPG)	http://www.dbvpg.unipg.it	Yes	Glacial ice, glacial meltwater, subglacial sediment	<i>Aureobasidium</i> , <i>Barnettozyma</i> , <i>Bensingtonia</i> , <i>Bullera</i> , <i>Bulleromyces</i> , <i>Candida</i> , <i>Cryptococcus</i> , <i>Cuniculitrema</i> , <i>Curvibasidium</i> , <i>Cystoflobasidium</i> , <i>Debaryomyces</i> , <i>Dioszegia</i> , <i>Glaciocyba</i> , <i>Holtermanniella</i> , <i>Kazachstanii</i> , <i>Kluyveromyces</i> , <i>Hannaella</i> , <i>Leucosporidiella</i> , <i>Leucosporidium</i> , <i>Metschnikovia</i> , <i>Mrakia</i> , <i>Mrakietta</i> , <i>Ogataea</i> , <i>Pichia</i> , <i>Rhodospiridium</i> , <i>Rhodotorula</i> , <i>Saccharomyces</i> , <i>Sporidiobolus</i> , <i>Sporobolomyces</i> , <i>Tortillaspora</i> , <i>Trichosporon</i> , <i>Udeniomyces</i> , <i>Wickerhamomyces</i> , <i>Willtipsis</i> , and <i>Xanthophylomyces</i>
Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS)	http://www.cbs.knaw.nl/Collections/DefaultInfo.aspx?Page=Home	Yes (12 temperatures between 4 and 45 °C)	Snow, sea ice, glacial ice, glacial meltwater, subglacial sediment, glacier cryoconite, deep-sea sediment, refrigerated and frozen foods	<i>Barnettozyma</i> , <i>Bensingtonia</i> , <i>Bortryozyma</i> , <i>Bullera</i> , 36 species of <i>Candida</i> , 20 species of <i>Cryptococcus</i> , <i>Cuniculitrema</i> , <i>Curvibasidium</i> , <i>Cystoflobasidium</i> , <i>Debaryomyces</i> , <i>Dioszegia</i> , <i>Guehomyces</i> , <i>Hannaella</i> , <i>Hanseniaspora</i> , <i>Holtermanniella</i> , <i>Kazachstanii</i> , <i>Kluyveromyces</i> , <i>Lachancea</i> , <i>Leucosporidiella</i> , <i>Metschnikovia</i> , <i>Mrakia</i> , <i>Ogataea</i> , <i>Phytobolhora</i> , <i>Pichia</i> , <i>Rhodospiridium</i> , <i>Rhodotorula</i> , <i>Saccharomyces</i> , <i>Sporidiobolus</i> , <i>Tortillaspora</i> , <i>Trichosporon</i> , <i>Udeniomyces</i> , <i>Wickerhamomyces</i> , <i>Willtipsis</i> , and <i>Xanthophylomyces</i>
National collection of yeast cultures (NCYC)	http://www.nycy.co.uk	Yes (opt., max and min temperatures)	Frozen foods, frozen soil, Antarctica	<i>Cystoflobasidium</i> , <i>Cryptococcus</i>
Phaff yeast culture collection, University of California Davis, USA (UCDFST)	http://www.phaffcollection.org	No	Glaciers, Antarctic sea, Alaska, frozen foods	n/a
Colección Española de Cultivos Tipo (CECT), Universidad de Valencia, Spain	http://www.cect.org	No	Antarctic seawater, soil, and lake sediment, ice cream	n/a
Belgian coordinated collections of microorganisms (BCCM/MUCL), Belgium	http://bccm.belpo.be/db/muel_search_form.php	No	Snow, Antarctica, fjord	n/a

(continued)

Table 2.4 (continued)

Yeast culture collection	Website	Website searchable for growth temperatures	Examples of cold substrates and geographic origins	Genera of yeasts able to grow at 4 °C
Culture collection of extremophilic fungi (EX-F), University of Ljubljana, Slovenia	http://www.ex-genebank.com	No	Sea ice, seawater, subglacial ice, surface ice, surface glacier ice, glacier meltwater	<i>Cryptococcus</i> , <i>Rhodotorula</i> , <i>Mrakia</i>
NITE biological resource center (NBRC), Tokyo, Japan	http://www.nbrc.nite.go.jp/NBRC2/NBRCDispSearchServlet	No	Antarctica, snow, frozen foods	<i>M. frigida</i>
USDA-ARS culture collection (US department of agriculture–agricultural research service), USA (NRRL), Peoria, Illinois, USA	http://nrl.lcaur.usda.gov	No	n/a	n/a
Culture collection of the National Institute of Chemistry, Slovenia (MZKI)	http://www.nbc.ki.si/slo_katalog.html	No	Glacial basal ice, surface ice	<i>Bulleromyces</i> , <i>C. Rhodotorula</i> , <i>Rhodospiridium</i> , <i>Trichosporon</i>

n/a not available



Fig. 2.1 Geographic origin of yeast strains in the Centraalbureau voor Schimmelcultures (CBS) collection (Utrecht, The Netherlands) that tested positive for growth at 4 °C. The number of CBS strains from each region is indicated. Data taken from the CBS online strain database (www.cbs.knaw.nl/Collections/Biolomics.aspx)

online DBVPG strain database is searchable for many fields including growth at low temperatures and “enzymatic and industrial surveys.” A search for “growth at 4 °C” in this field generated a list of over 500 strains, most of which were isolated from snow and glacial ice, meltwater, and sediments from Italy and Antarctica.

The Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands, has posted quite extensive physiological data for the strains in the public CBS catalog. Data are posted for growth of CBS yeasts across a broad range of growth temperatures ranging from 4 to 45 °C. Over 300 strains able to grow at 4 °C were found in a search of the CBS online strain database (www.cbs.knaw.nl/Collections/Biolomics.aspx). Some interesting trends appear in this set of strains.

The geographic origin of CBS psychrotolerant strains is depicted in Fig. 2.1. Psychrotolerant yeasts able to grow at 4 °C were isolated from chilly geographic locations such as Antarctica (1 strain) and Iceland (39 strains), but also from unexpectedly warm temperate and tropical climates such as Egypt (2 strains) and the Bahamas (4 strains). For example, *Lachancea meyersii* CBS 9925 was isolated in 1999 by J. W. Fell and colleagues from seawater in a mangrove creek on Andros Island in the Bahamas (Fell et al. 2004) and can grow at 4 °C through 30 °C, but not at 35 °C or higher (CBS online database for growth temperatures). The bias toward temperate and tropical origins indicated in Fig. 2.1 most likely reflects the travel preferences of the field microbiologists who collect yeasts, rather than the actual biogeography of psychrotolerant yeasts.

Psychrotolerant yeasts in the CBS catalog were isolated from cold habitats such as glaciers, snow, and chilled beef, but also from various mid-temperature habitats such as fruits, flowers, trees, seawater, soil, and insects. These yeasts could be useful for many applications as discussed in Part IV of this book. A broad variety

of psychrotolerant yeast genera and species are represented in the CBS catalog, as listed in Table 2.4. Both basidiomycetous and ascomycetous yeasts are represented, though basidiomycetes are more prevalent, as has been seen in an evaluation of yeasts isolated from Antarctica (Buzzini et al. 2012). Basidiomycetes made up 85 % of yeasts isolated from subglacial ice, glacial meltwater, seawater, sea ice, and melted snow in Norway (Butinar et al. 2011).

The National Collection of Yeast Cultures (NCYC), located at the Institute of Food Research, Norwich, UK, has a historic emphasis on brewing strains and has expanded in recent decades to embrace yeasts with biotechnology or food value. Like CBS, NCYC has an online strain database (www.ncyc.co.uk) that can be searched for strains with specific growth temperatures. Eighty-six strains had a reported minimum growth temperature of 4 °C. Notable strains include *Phaffia rhodozyma* type strain NCYC 874 (UCDFST 67-210), a species used for industrial production of the carotenoid pigment astaxanthin, and multiple wine, brewing and baking strains of *Saccharomyces cerevisiae*. Use of low-temperature-adapted yeasts for wine making is addressed further in Chap. 19.

2.9 Methods for Detecting Uncultured Yeasts

As discussed in Sect. 2.6, cultivation methods can bias recovery of different types of yeasts. Many yeast species may not be cultivable under the conditions selected. Molecular methods of profiling yeast biodiversity do not generate living cultures for further analysis, but can provide more detailed profiles of mixed populations. Molecular methods for the analysis of mixed communities of bacteria or yeasts were reviewed recently (Bokulich 2012).

Targeted methods are aimed at specific taxonomically defined groups of microbes, such as all bacteria, or a specific genus, species, or strain. These methods include fluorescence *in situ* hybridization (FISH) and quantitative PCR (qPCR). Broader profiling methods utilize universal primers aimed at larger taxonomic groups, such as kingdom or phylum level. The PCR products can be analyzed and profiled using several methods.

Amplified fragments can be ligated into a cloning vector, transformed into *E. coli*, and sequenced. This method is rather low-throughput, as each clone must be individually sequenced.

Denaturing gradient gel electrophoresis (DGGE) and its close relative temperature gradient gel electrophoresis (TGGE) have been used for the past 20 years to profile mixed microbial communities. These methods separate short PCR products along a chemical or temperature gradient. DGGE was used by Cocolin et al. (2000) to profile the succession of yeasts in wine fermentations. Yeasts are identified by comparison of band lengths to those of standards, or by extracting from the gel, re-amplifying, and sequencing. This method is limited by the low-throughput, and difficulty in performing the procedure. More information about DGGE can be found in a review by Ercolini (2004).

Terminal restriction fragment length polymorphism (TRFLP) differentiates microbial members of a community based on differences in length of a fluorescently labeled PCR fragment, cut with restriction enzymes, and separated by capillary electrophoresis on a DNA sequence analyzer. The fragment lengths are compared to those in databases, such as that compiled by Bokulich et al. (2012) for analysis of yeasts associated with wine fermentations. This method can be used in high-throughput, but is limited to fragment sizes for the species found in relevant databases.

Next-generation sequencing (NGS) methods include several recently developed technologies for massively parallel sequencing of a mixture of diverse DNA sequences. PCR is used to amplify marker genes, primarily 16S rRNA for prokaryotes and ITS genes for fungi. The two methods currently used for profiling microbial communities are 454 Life Sciences pyrosequencing (Margulies et al. 2005) and Illumina sequencing platforms (Bennett 2004). These two methods both generate large numbers of short segments of DNA sequence, with the fragments being physically partitioned and the growing DNA strand detected using different technologies. They differ in error rates, sequence lengths, and cost. Pyrosequencing generates longer reads (600 bp) than Illumina (150 bp), but Illumina generates greater sequence coverage (10^9 reads versus 10^6).

Users of these methods are cautioned to carefully select the reference database for identification of species from the raw DNA sequence data. Public DNA sequence databases contain sequences that are too short, misannotated, or contain too many ambiguous base calls to be useful. Fungal databases that are curated include the UNITE database of hand-curated fungal ITS sequences, primarily ectomycorrhizal fungi, and the Silva reference database of fungal large subunit (LSU) rDNA sequences. Different reference databases will yield different taxonomic assignments (Bokulich 2012). Illumina sequencing was recently used to profile both bacterial and yeast communities in a winery (Bokulich et al. 2013).

Some molecular methods have been used to profile fungal diversity in cold habitats. Bass et al. (2007) amplified an 18S rDNA segment from genomic DNA isolated from filtered seawater collected at several marine sites between 250 and 4,000 m depth, cloned the PCR fragments into the TOPO TA vector, and sequenced the inserts; 115 sequences were fungal, and included 19 fungal 18S sequence types, with 3–11 sequence types per site. Eleven of these sequences have only been detected in deep-sea samples. Yeasts predominated, particularly basidiomycetous yeasts. DNA sequences were similar to those of known cold-adapted yeast species including *C. vishniacii*, *Rhodospidium diobovatum*, *Filobasidium globisporum*, *R. mucilaginosa*, and other basidiomycetous and ascomycetous yeast species.

Bellemain et al. (2013) used next-generation sequencing of the ITS ribosomal region to analyze the fungal diversity in 26 samples of Siberian permafrost aged 16,000–32,000 years. Seventy-five fungal operational taxonomic units (OTUs) belonging to 21 orders in three phyla were detected, 75 % of which were ascomycetous. Species detected from this study include many filamentous fungi, as well as yeasts that have been cultivated from other cold substrates including

Mrakiella aquatica (99.5 % identical), *Rhodotorula minuta* (100 % identical), and *Cryptococcus albidus* (100 % identical).

These two examples demonstrate that molecular methods, including next-generation sequencing methods, can be useful to detect, identify, and estimate the relative proportions of yeasts in substrates such as deep-sea water and may be used more extensively in coming years.

2.10 Conclusions

General methods used to isolate and cultivate yeasts have been modified to allow cultivation of cold-adapted yeasts. These modifications include selection of appropriate cold incubation temperature, osmolarity, and nutrient levels. Cold-adapted yeasts can be isolated from rather inaccessible habitats, such as deep-sea sediment and glacial ice cores, which requires particular aseptic sampling and processing methods. Some species isolated from these cold habitats only grow at cold temperatures, while others can tolerate mesophilic temperatures as well. Because these yeasts are particularly difficult to sample, isolate, cultivate, and preserve, it is important to archive them in properly equipped institutions with personnel knowledgeable about their cultivation, such as public culture collections. As culture-independent methods of microbe community profiling gain prominence, it may be important to archive the hard-won environmental samples and associated environmental DNA samples in public archives as well. Culture-independent methods, although powerful, still rely on data acquired using representative pure cultures to identify the species of microbes present. Isolation of cold-adapted yeasts is helping to expand our knowledge of the ecology of cold habitats and is leading to taxonomic discoveries and biotechnology advances.

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<http://www.springer.com/978-3-642-39680-9>

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Biotechnological Significance

Buzzini, P.; Margesin, R. (Eds.)

2014, XI, 549 p. 61 illus., 28 illus. in color., Hardcover

ISBN: 978-3-642-39680-9