Development of Media for Growth and Enumeration of Fungi from Water

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Abstract

Fungi are found in water resources as natural primary inhabitants or as secondary inhabitants that enter the water source as contaminants. Many of the fungi in water resources can be directly harmful to human, animal, and plant health, or cause problems in food processing and preparation, or by producing biofilms in water-distribution systems. Hence, water fungi are of concern for consumers. The ability to detect fungi in water sources is therefore important with respect to minimizing the risk of contamination and for safety-management protocols. However, there is no one uniform method for determining fungal load in water. Various new molecular-based methods are being developed to analyze water resources, but the traditional colony-based ones are still the methods of choice for enumeration and characterization of fungal populations in water. Recent developments in those methods for water mycological quality examination, particularly with regard to media composition, are presented.

Keywords

CFU • Detection • Enumeration • Fungi • Membrane filtration • Biofilm • Treated/untreated water • Routine analyses

Introduction

An awareness of the importance of fungi in water destined for human consumption has emerged in recent years. Although still limited, the number of publications on this topic is rising, demonstrating the presence of a range of fungal species, some of which are known to be directly pathogenic to humans, cause allergic reactions, or have harmful effects due to their production and release of toxins into the water [1–3]. Fungi are also suspected of contributing to negative organoleptic qualities in drinking water [4], and to biofilm production in distribution systems [5]. Plant-pathogenic and food-spoilage fungi have also been found in treated and untreated water [2].

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In terms of human health, there is relatively little information on the role played by fungal contamination of water in illnesses and infections in the general public. However, the greater concern is related to immunosuppressed patients, who may be infected by drinking water, bath water, or recreational water bodies [6]. Hence, studies have been aimed at examining the mycological quality of hospital water systems, to assess the risk of fungal transmission to patients and possible infection. Potentially health-disruptive fungi have been detected in tap water [7–12], water-distribution systems [10, 13], bottled water [14, 15], bathing water [16], swimming pools [17, 18], surface water sources (rivers, streams, canals, lakes, and ponds) [2, 7], and groundwater [19].

Species of potentially human-pathogenic yeasts and molds recovered from drinking water include *Aspergillus fumigatus* [20], *Fusarium* [21], *Penicillium* [14], *Aureobasidium pullulans* (found in saunas) [22], *Absidia, Mucor, Candida* [23], *Trichoderma viride* [9], and *Chaetomium globosum* [4]. Various potentially pathogenic fungi, including dermatophytes, have also been isolated from swimming pools, such as *Cladosporium* spp., *Penicillium* spp., *Aspergillus* spp., *Rhizopus* spp., *Fusarium* and *Trichophyton rubrum, Mucor* spp. and *Candida albicans* [17, 18, 24], among others.

With the increasing concern about fungal contamination in consumed water, fungal examination of different water sources is on the rise, and it is recommended that water resources be monitored for fungal contamination as part of water-safety management programs. However, the field of mycological water quality, including methodologies and regulations, is far less established than the fields of bacteriological water quality and mycological food quality. In 1975, the methods for fungal detection in water and wastewater were still only tentative, as laid out in the fourteenth edition of the Standard Methods for the Examination of Water and Wastewater [25]. Even today, on a global scale, there are different regulations and methodologies in place for mycological water safety, and no uniform or standardized method for determining the mycological quality of potable water resources has been recognized.

Water is very heterogeneous with regard to the fungal diversity found within it. Fungi can be found in water resources as natural primary inhabitants or as secondary inhabitants that have entered the water source as contaminants. As summarized by Hageskal et al. [26] primary inhabitants are those that are adapted to aquatic environments, belonging to the phylum Chytridiomycota. Secondary inhabitants are all other fungi, which enter natural water bodies from the air, soil, and wastewater. The survival and proliferation of these latter fungi depend on the characteristics of the water, that is, nutrition load, temperature, pH, other microorganisms, and, in some cases, the presence of disinfectants (such as chlorine in swimming pools) [27].

The origin of the water source and its designated use also vary (e.g., tap water for drinking, bottled water, swimming pool water, recreational water, or wastewater), and these need to be considered in the establishment and application of a detection method. Each of these water bodies may support different fungal loads and diversities. It is clear that no one defined fungal indicator can give information on all of the different types of damage that can be expected from each water source. It is important to detect the individual fungus that is causing a problem and identify it, but quantifying the total fungal load may give a faster and clearer estimation of the degree of fungal contamination, so that appropriate action can be taken.

Traditionally, quantification of fungi in water sources has been based on culturing the colonies on general nutritional media, or culturing specific fungi on more selective media, and presenting the fungal load as CFU (colony forming units) in a certain volume of water. Although it is agreed that not all fungal species in water will grow on a specific medium under particular growth conditions, culturing and enumeration of as many members of the fungal community in the water source as possible can give a good estimation of the level of fungal contamination. Although efforts are being made today to develop rapid molecular procedures for the detection of fungal contamination (e.g., PCR-based protocols, ergosterol-content determination, the use of gene

probes, protein probes, and mass spectrometry), culturing fungal colonies on agar media is still the method of choice to enumerate and isolate fungi of interest. This chapter focuses on examinations of mycological water quality using the CFU approach, and on the recent developments in media used for this purpose.

Fungal Culturing Approach

Historically, the examination of fungi in water bodies has consisted of adopting analytical methods and media used to examine fungal loads in foods. Hence, the "pour plate" and "spread plate" techniques, used with solid products, were recommended for water examinations as well. Later, the "membrane filtration" (MF) technique was also recommended for examinations of fungi in water.

Techniques

Spread Plate Technique

This is a well-established technique for mycological examinations of food and environmental samples. This and the pour plate technique, both established and used for different types of food and soil samples, were also adopted for water examination. In this method, a water sample is spread by glass or plastic spreader on the surface of agar medium in a Petri dish. With a suitable medium and incubation conditions, this technique enables the growth of a range of fungal species, as most fungi are aerobic. The advantage of this method is that the colonies can be differentiated by appearance, and the cultured fungi of interest can be further isolated and identified separately. The disadvantage of this method is sample loss on the spreader, and the low species diversity growing on the agar surface in comparison to the pour plate technique.

Pour Plate Technique

In this technique, the water sample is placed in an empty Petri dish, to which molten medium is then added. After mixing the two, the medium is allowed to solidify, and the culture is incubated for fungal growth. This method is well suited to the enumeration of fungi since there is no loss of sample as in the spread plate technique. In addition, this technique allows the germination and growth of colonies inside the agar medium, where each colony is surrounded by a homogeneous microclimate in terms of nutrient, oxygen, and moisture levels. This allows for higher fungal diversity, as fungi can grow on the surface or at different depths in the medium. The disadvantage is that it becomes difficult to isolate the colony of interest if that colony is growing inside the medium.

Both the spread plate technique and the pour plate technique are simpler and less costly than the MF technique, but require higher quantities of medium, and more space for plates incubation.

MF Technique

Today, this is the most commonly used technique in routine microbiological quality examinations of low-turbidity water (drinking, bottled, swimming pool, and bathing water). The MF technique was initially developed as an alternative to other methods for bacteriological analyses of water samples in the late 1950s [28, 29]. In 1975, it was adopted as a standard procedure for bacterial water examination in the eleventh edition of the SMEWW [30]. It was only in 1976 that Qureshi and Dutka [31] examined the MF technique for the recovery of fungi from water, comparing different brands of MFs for this purpose. In 1978, it was used for the enumeration of *Candida* in natural water [32]. The procedure is based on filtering a volume of water through a MF, and the fungal units (conidia, chlamydospores, and other hyphal units) are trapped on the membrane surface. The filter is then transferred onto nutritional agar medium and the fungal colonies are left to develop on the filter surface. In their comprehensive work on the optimization of methodologies for fungal recovery from water, Kinsey et al. [2] showed that this technique recovers a lower number of different taxa than the direct spread plate technique. In a work conducted in our lab (S. Masaphy, unpublished data), the recovery of fungi (*Penicillium* sp., *Aspergillus* sp., *Candida albicans,* and

Saccaromyces sp.) artificially inoculated into bottled water, tap water, and swimming pool water was similar using MF and spread plate techniques in terms of fungal units. The MF technique has several advantages, especially in examining water with low microbiological loads. Moreover, this technique enables examining a large number of samples in a short period of time.

For all three of the aforementioned methods, successful fungal recovery is related mainly to selecting the right medium.

Media

Early media used for the detection of fungi in water sources were similar to those used for the detection of fungi in other products, since many of the fungi in water actually reach the water body from the surrounding environment. Today, more specific media for water examination are being developed, taking into consideration the low-nutritional conditions of the water matrix and the use of the MF technique. In general, media supporting a broad range of fungal taxa while restricting linear expansion of the fungal colonies and simultaneously inhibiting bacterial growth are preferred for the detection and enumeration of fungi in environmental samples. Different media are used, according to the type of water source and the aim of the fungal detection.

Nutritional characteristics are the most influential factor in the suitability of a particular medium for the recovery of water fungi. To detect a wide range of fungal propagules present in the water source, a nutrition-rich medium, with the addition of an antibacterial agent, is used, such as Sabourand dextrose agar (SDA) [33]. However, low-nutrition media have also been recommended for fungal recovery from water [2]. Comparing poor and rich media, half-strength corn meal agar (CMA/2) was recommended by Kinsey et al. [2] for routine fungal examination since it provided good results, with recovery of higher fungal diversity, and is inexpensive. The rich SDA medium supported higher fungal counts, but mostly from common hypomycete (Fungi Imperfecti) genera such as *Penicillium* and *Aspergillus,* whereas CMA/2 supported other genera [2].

Medium pH is also important. Generally, fungi tend to grow in more acidic media than bacteria; hence, many of the mycological media are adjusted to be more acidic, thereby supporting fungal growth while inhibiting bacterial growth. This is even more relevant when fungi are being detected in food products that may themselves be acidic. Thus, due to adoption of media from the food discipline, some officially recommended media are already acidic, such as modified aureomycin-rose bengal-glucose-peptone agar (MARGPA), which has a pH of 5.4 [34]. Other recommended media, such as SDA, corn meal medium, and dichloran-18% glycerol (DG18), have pH values between 5.6 and 6 [35]. In 1962, Mossel et al. [36] showed better recovery of molds and yeasts from foods using media with more neutral pH containing an antibacterial agent than with media based on acid pH alone. In a recent work in our lab [37], we also showed that for recovery of fungi from a range of fungus-inoculated water sources, rose bengal-chloramphenicol (RBC) medium with pH 7.2 was superior to MARGPA.

Another important consideration in fungal recovery is their rapid growth. Unlike most bacterial colonies, filamentous fungi tend to form expansive colonies, which may cover small colonies of slower germinating or growing fungi and yeast. Therefore, chemicals that inhibit hyphal growth need to be added to limit overgrowth of the fast-growing colonies. Dichloran and rose bengal were shown to perform this function and were incorporated into a nutrition-rich medium to restrict the linear expansion of hyphal growth [38–41]. In 1973, Jarvis [42] developed and used rose bengal-chlortetracycline medium, and in 1979, King et al. [43] showed that introducing dichloran and rose bengal together (with reduced rose bengal concentration) allowed greater recovery of molds. More recently, DG18 has been recommended in water examinations and widely used by Hageskal et al. [9, 10]. This medium was developed for xerophilic fungi from foods [44],

and was recommended by Samson et al. [35] as a general medium for the isolation and enumeration of fungi in food with water activity (a_w) >90. Askun et al. [45] compared DG18 and RBC medium for fungal examination in raisins and obtained higher fungal species diversity with RBC, although both media gave similar results for total fungal counts. We compared RBC medium with MARGPA for the recovery of fungi from different water sources and found RBC to be superior.

As to the antibacterial agent, Korburger and Rodgers [46] showed the positive effect of adding antibiotic to the medium on enumeration of yeasts and molds, and today, as mentioned above, media for detection of fungi in water samples include a wide spectrum of different antibiotics, such as chlortetracycline (auromycin) and streptomycin [34, 47], in addition to the hyphal-restricting agent. We found that the antibiotic chloramphenicol is simplest to use as it is autoclavable.

Assessing and Counting

The observed fungal colonies are counted and referred to as CFU. There are two important issues to consider. First, filamentous fungi tend to spread over the medium, overlapping other slow-growing fungi. As mentioned, to overcome this problem, rose bengal or dichloran are incorporated into the medium. However, the concentration of the added compounds is important, as it can limit fungal growth too severely. The second issue involves observation of the fungal colonies on the medium surface. Some of the fungi are colored due to colored spores, whereas others appear pale and are difficult to observe. Upon using rose bengal to limit the overgrowth of fungi, we found that it also strongly improves the colony count: the filamentous fungi and yeast colonies tend to absorb the rose bengal, giving them a sharper color and reducing the need for optical magnification [37]. This is especially true when MF technique is used, as it is difficult to observe light-colored colonies on the white filter.

Procedures

Media

Some of the more common media used for water fungal detection and enumeration are presented here.

1. Rose bengal-chloramphenicol (RBC) agar (commercially available). Add 5 g peptone, 10 g glucose, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.05 g rose bengal, and 15.5 g agar to 1 L distilled water. Heat to dissolve with stirring, and autoclave. Adjust final pH to 7.2 ± 0.2.
2. Dichloran-RBC (DRBC) agar. Add 5 g peptone, 10 g glucose, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.002 g dichloran, 0.025 g rose bengal, 0.1 g chloramphenicol, and 15 g agar to 1 L distilled water. Adjust pH to 5.4–5.8.
3. Aureomycin-rose bengal-glucose-peptone agar (ARGPA). Add 5 g peptone, 10 g glucose, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.035 g rose bengal, and 20 g agar to 800 mL distilled water. Prepare separately: dissolve 70 mg chlorotetracycline (aureomycin hydrochloride) in 200 mL distilled water, filter-sterilize. Add to the cooled (42–45 °C) melted agar medium. Adjust final pH to 5.4.
4. Modified ARGPA (MARGPA). Add 5 g peptone, 10 g glucose, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.035 g rose bengal, and 20 g agar to 800 mL distilled water. Prepare separately: dissolve 200 mg chlorotetracycline in 200 mL distilled water and filter-sterilize. Add to cooled (42–45 °C) melted agar medium. Adjust final pH to 5.4.
5. Dichloran-18% glycerol agar (DG18). Add 5 g peptone, 10 g glucose, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 mL dichloran (0.2% in ethanol), 220 g glycerol, 0.1 g chloramphenicol, and 15 g agar to 1 L distilled water. Adjust to pH 5.4–5.8.
6. Neopeptone-glucose-rose bengal-auromycin. Add 5 g neopeptone, 10 g glucose, 3.5 mL rose bengal solution (1 g/100 mL), and 20 g agar to 1 L distilled water. Separately filter-sterilize chlorotetracycline or tetracycline

(1 g/150 mL water). Add 5 mL of this solution to 1 L agar solution immediately before use. Adjust to pH 6.5.

7. Czapek Dox (CZ) agar. This medium is recommended for *Aspergillus, Penicillium,* and similar fungi, among others, but not for total fungal recovery. Add 30 g saccharose, 3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4$, 0.5 g KCl, and 15 g agar to 1 L distilled water. Adjust to pH 7.3.
8. SDA. Add 10 g mycological peptone, 40 g glucose, and 15 g agar to 1 L distilled water. Adjust to pH 5.4–5.8.

If dehydrated commercial medium is being used, preparation should be as per the manufacturer's recommendations. Otherwise, weigh each of the medium components into 1 L or 800 mL sterile distilled water, heat while stirring on a hotplate to near boiling until the agar is dissolved and the medium is homogeneous. Autoclave (121 °C for 15 min). Cool medium to 45 °C, and then pour into the plates. If autoclave-sensitive antibiotic is to be used, add 200 mL of the filter-sterilized antibiotic at relevant concentration to 800 mL cooled medium, mix by stirring, and then pour into the plates. Keep at 45 °C, and adjust pH as required with HCl or NaOH.

Materials

1. Sterile distilled water
2. Ethanol or methanol in wide-mouth container for flame-sterilization of the forceps
3. 0.1 M NaOH and 0.1 M HCl
4. Dilution buffer composed of:
   (a) Potassium dihydrogen phosphate (KH_2PO_4) solution: Weigh 17 g KH_2PO_4 into 250 mL sterile water. Mix to complete dissolution. Adjust to pH 7.2 with 1 N NaOH, bring to 500 mL with sterile water.
   (b) $MgCl_2$ solution: Weigh 40.55 g of $MgCl_2 \cdot 6H_2O$ into 500 mL sterile water. Mix to complete dissolution.
   (c) Working dilution buffer: Transfer 1.25 mL from solution (a) and 5 mL from solution (b) into 1 L sterile distilled water. Sterilize solution before use.
5. Screw-cap bottles for dilutions, 100–500 mL volume
6. Borosilicate glass flasks, 250–1,000 mL volume
7. Sterile pipettes, glass or plastic, of appropriate volumes
8. Graduated cylinder, 100–1,000 mL
9. Sterile L-shaped glass rod or plastic disposable spreader rod
10. Petri dishes (either 50 or 90 mm), sterile, plastic
11. Forceps, with smooth tips, to handle filters without damaging them
12. Membrane filtration unit: filter funnel manifolds and filter manifolds (47 mm)
13. Membrane filter: 0.45-μm pore size white hydrophobic mixed cellulose acetate membrane filter, grid marked, 47 mm, preferably presterilized
14. Water bath maintained at 50 °C for tempering agar medium
15. Incubator maintained at 15, 20, or 25 °C, 90% relative humidity
16. Vortex
17. Heating stirrer
18. Colony counter with magnifying glass

Techniques

Fungi tend to spread unevenly in water bodies. It is therefore important to mix the water in the sample bottle vigorously prior to examination. When using a low volume of sample, it is recommended that several repeats (optimally five) be examined [34].

Spread Plate Technique

Streak 0.1–0.05 mL water sample onto the center of pre-solidified agar medium (10–20 mL medium) in a 90-mm Petri dish using a sterile pipette. Spread the sample with a spreading rod (available as disposable plastic rods, but glass rods, which can be ethanol-sterilized, are preferred). Streak back and forth across the plate, working up and down several times to distribute the fungal units as evenly as possible. Use a single

rod per sample. Cover the plate and wait several minutes before inverting it and incubating.

Pour Plate Technique

Water sample (usually 1 mL, but as little as 0.1 mL can be used) is added to an empty 90-mm Petri dish. Then, 10–15 mL of molten propionate medium is poured at 45 °C. The medium should not be poured directly on the water sample. Mix water sample and medium and let the mixture solidify. In some cases, antibiotics may be added to the Petri dish as well before pouring the molten medium. Incubate plates noninverted.

MF Technique

Shake the sample bottle to distribute the fungal units uniformly. Filter the sample (10–1,000 mL, ideally 100 mL) through the MF. Rinse the sides of the funnel with 20–30 mL sterile dilution water. Turn off the filtration system vacuum and aseptically remove the MF from the filter base using sterile forceps. Overlay the MF on the agar medium surface in a 50-mm Petri dish. Close the dish and incubate, either inverted or noninverted. If the fungal counts exceed 80CFU/filtered volume, lower volumes of water should be used, or the water source can be diluted 1:10 with dilution water and filtered.

Incubation Conditions

Petri dishes are either incubated inverted or noninverted. Incubating the plate in the inverted position prevents dripping of the condensed water onto the agar medium surface. Incubation is performed in the dark to avoid overproduction of conidia that might spread and recontaminate the agar medium surface. In some cases, however, incubation in the light, but not direct sunlight, is preferred to increase (colored) conidiation. The incubation temperature is usually relatively low (15–20 °C) to avoid overgrowth by fast-growing fungi, allowing the slower fungi to germinate and grow as well. In this case, a longer incubation may be needed to recover higher counts. It is recommended that the plate be observed as soon as possible. However, for the purpose of standardization, it is recommended that the results be read after defined incubation periods. The SMEWW [34] recommends incubation of spread plates at 15 °C for 7 days, or 20 °C for 5–7 days. Slow-growing fungi may not produce noticeable colonies until 6 or 7 days of incubation. For the pour plate method, it is recommended that plates be incubated at 20–24 °C for 3–7 days. For the MF technique, the recommendation is 15 °C for 5 days or 20 °C for 3–7 days. In all cases, plates should be incubated in a humid (90–95% RH) atmosphere.

Counting

The count can provide an estimate of cultivable fungi extracted from the water sample. All filamentous fungi and yeast colonies may be counted together or separately. The number of fungi in the water sample is calculated as CFU/mL of water. The calculation should take into account the dilution factor. If counting cannot be performed immediately, the culture plate can be kept at 4 °C for 24 h. It is advisable to count colonies in plates that have the optimal number of 20–150 colonies per 90-mm plate for the spread plate technique, and up to 300 colonies for the pour plate technique. For the MF technique, it is suggested that a magnifying binocular microscope be used to count all of the colonies, which may be hard to see on the white filter background. Ideal plates for counting should have 20–80 colonies per filter.

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