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The Study of Filamentous Fungi in Potable Water and Its Biofilm Formation in Water Pipeline System

S. Asha and G. Sangeetha Vani†
Department of Microbiology, Ethiraj College for Women, Chennai-600008, Tamil Nadu, India
†Corresponding author: G. Sangeetha Vani; sangeethavani_g@ethirajcollege.edu.in

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ABSTRACT

Water is essential for life and it is an inorganic constituent of living matter. Water pipeline systems are sighted as problematic in aquatic habitats in which multiple pathogens are occupied including fungi. They have rigid cell walls containing glucans and chitin. The bodies of fungi comprise filaments called hyphae. These hyphae are split into a mat of interwoven single cells made of mycelium. Fungi can pollute the drinking water system and are responsible for biofilm formation. Biofilms are complex polymers containing many times their dry weight in water. Moisture is essential for biofilm formation. The occurrence of biofilms affects the quality of drinking water. Hence, the present study is aimed at recovering the fungi from drinking water samples and their biofilm formation in the water pipeline system. Drinking water samples such as mineral water, tap water, and RO-purified water are collected from different places. Fungi such as *Aspergillus*, *Penicillium* and *Mucor* were recovered from these samples and most species belong to *Aspergillus* and *Penicillium.* Further, the biofilm formation of fungi from cast iron in the pipeline system was detected using fluorescence microscopy and fluorescent *in situ* hybridization analysis.

INTRODUCTION

Fungi are eukaryotic and they can occur as unicellular yeast or multicellular filamentous fungi or molds (Yamaguchi et al. 2007). Fungi are widely distributed in nature and it is also present in the soil, organic material, etc. A broad range of filamentous fungi has been discovered or isolated from drinking water. Among the isolated filamentous fungi, possibly contagious, harmful, and allergenic species have also been found (Hageskal et al. 2009). The presence of fungi in drinking water can cause various fungal infections in immunocompromised patients. Mostly, *Aspergillus* and *Penicillium* species are found in water and (Grabinska-Loniewska et al. 2007) causes allergy, ear infections, lung and kidney failure, respiratory problems, and increased levels of invasive infections. Fungi invade into drinking water pipeline system through various routes which include, water treatment, insufficient stored water facilities, cracks in pipelines, main breaks, and installation (Sonigo et al. 2011, Afonso et al. 2019). Filamentous fungi have the potential to grow on surfaces, thus leading to the formation of biofilms. Biofilm is a thin layer of microorganisms adhering to the surface of the structure (Simoes & Simoes 2013). Biofilms form slimy extracellular polymeric substances (EPS), which include polysaccharides, proteins, lipids, carbohydrates, and DNA (Flemming et al. 2002). It is a collection of organic and inorganic, living and dead material present on a surface. It can alter the surrounding environment such as microbial community, pH, and aerobic and anaerobic conditions. These are established on moist surfaces such as water pipelines, food processing equipment, industrial piping, medical devices, and so on (Huq et al. 2008).

There is no conventional method for analyzing fungi in drinking water. As a consequence, diverse isolation procedures are handled. Membrane filtration techniques, spread plate techniques, and direct microscopic observations are relevant methods. High nutritional media such as Sabouraud Dextrose Agar (SDA), Dichloran Glycerol Medium Base (DG18), Corn Meal Agar (CMA), Potato Dextrose Agar (PDA), Czapek Dox Agar (CZ), Malt Extract Agar (MEA), Dichloran Rose Bengal Chloramphenicol Agar (DRBC) are widely used (Afonso et al. 2021). The morphology and etiology of filamentous fungal biofilms are distinguished by *in situ* microscopic examinations (Douterelo et al. 2016) and by using sequencing techniques, such as flow cytometry, pyrosequencing, etc. These methods are outlined to address knowledge gaps concerned to the formation of biofilms in the drinking water system. This study is one such attempt to confirm the existence of fungi in drinking water and to emphasize the importance of fungal biofilms in affecting the quality of water.

Table 1: Filamentous fungi in drinking water samples.

Sample location	Water source	Total water samples	Isolation method	Frequent fungal species
Chennai	Tap water	15	Direct plating	*Aspergillus* sp., *Penicillium* sp.
Chennai	Mineral water	15	Direct plating	*Aspergillus* sp., *Penicillium* sp.
Chennai	RO purified water	15	Direct plating	*Aspergillus* sp., *Penicillium* sp.

MATERIALS AND METHODS

Water samples (45) were collected aseptically from various places in Chennai (Table 1). All the samples were stored in sterile bottles and processed on the day of the collection. The media used for the isolation of fungi are SDA (Sabouraud Dextrose Agar) and DG18 (Dichloran Glycerol Medium Base). The medium was prepared by standard procedure and autoclaved at 121°C for 15 min. The sterilized medium was flooded onto the sterile plate and allowed to solidify. 0.1 mL of the water samples was added directly and plated on the prepared medium plates. A spread plate technique was performed and plates were incubated at room temperature for one week. Fungal colonies were observed after 7 days. Each unique colony was sub-cultured on SDA and DG18 medium to obtain unique individual isolates. The preliminary identification of the fungal isolates was observed using lactophenol cotton blue (LPCB) mount staining. Macroscopic features such as size, shape, color, and appearance were studied from the fungal isolates.

Biofilm sampling has been performed using cast iron from water pipeline distribution systems. Cast iron was plated on DG18 medium and it was suspended in a sterile saline solution before being plated. The prepared DG18 medium plates were incubated at room temperature for the fungal isolates to grow. Fungal colonies that emerged after incubation were detected using fluorescence microscopy and fluorescent *in situ* hybridization (FISH) probe analysis.

RESULTS

A total of 45 water samples were collected. Out of 45 water samples, fungi were retrieved from 20 samples (Fig. 1 to Fig. 5). The most abundant genera were identified as *Aspergillus* and *Penicillium*. The macroscopic examination was studied based on morphological characteristics and color of mycelium and the microscopic analysis was performed using lactophenol cotton blue staining (Fig. 6).

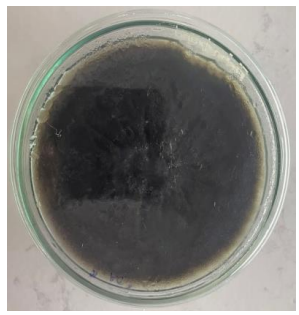


Fig. 1: *Aspergillus niger* on SDA.

Fig. 2: *Penicillium verrucosum* on DG18. medium.

Fig. 3: *Colletotrichum gloeosporioides* was grown on DG18 medium.

Fig. 4: *Penicillium notatum* on SDA medium.

Fig. 5: *Mucor mucedo* on DG18 medium.

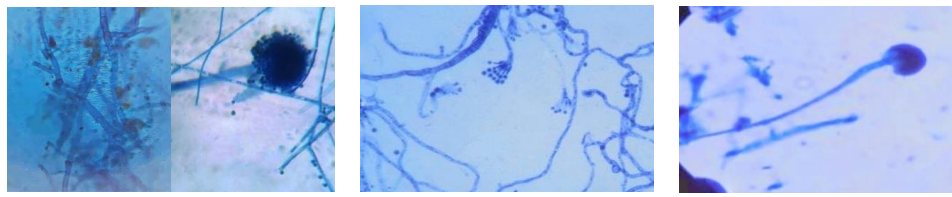
Sampling Biofilms

After incubation, fungal isolates were reclaimed from the cast iron and they were identified as *Aspergillus niger, Penicillium verrucosum,* and *Mucor mucedo* (Fig. 7).

Biofilm Detection

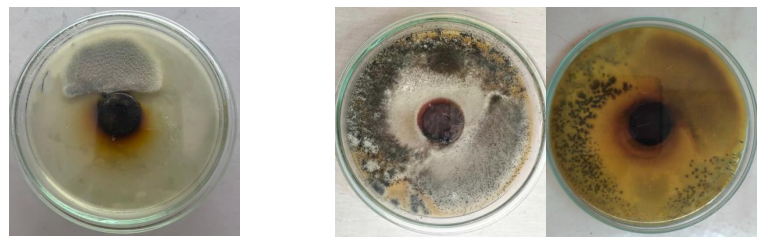
For monitoring microbial biofilm formation, fluorescence microscopy was enabled. Fluorescence microscopy utilizes an elevated level of light intensity to brighten the sample (Simoes et al. 2015). Radiant objects in opposition to black backgrounds are seen better. Hence the habitual of fluorescence microscopy is vastly specific and sensitive. Morphological characters were visualized using Calcofluor White MR2 (CW) staining (Fig. 8). CW stain is an indefinite fluorochrome that is attached with chitin and cellulose accommodated in the fungal cell walls (Goncalves et al. 2006).

To confirm the filamentous structure, samples were submitted for fluorescent *in situ* hybridization (FISH) analysis. FISH is an important machinery to examine eukaryotic chromosomes and genomes in molecular biology, cytogenetics, etc. For the identification from fungal culture, the cells are fixed in the first stage. After fixation, the sample was denatured for the hybridization process. After hybridizing, the washing and mounting step proceeded. Hybrids formed between the probes and the targets can be detected using a fluorescent microscope. (Fig. 9). Filamentous fungi with biofilm formation were identified between 24 h and 48 h of FISH probe analysis. Overall, a comprehensive association between the Calcofluor White (CW) filamentous fungi in biofilm is effective for fluorescent *in situ* hybridization analysis.



(a) (b) (c)

Fig. 6: a) *Aspergillus niger,* b) *Penicillium verrucosum,* and c) *Mucor mucedo* was microscopically observed using lactophenol cotton blue stain.



7 days of incubation. 30 days of incubation.

Fig. 7: Fungal growth on DG18 medium using cast iron.

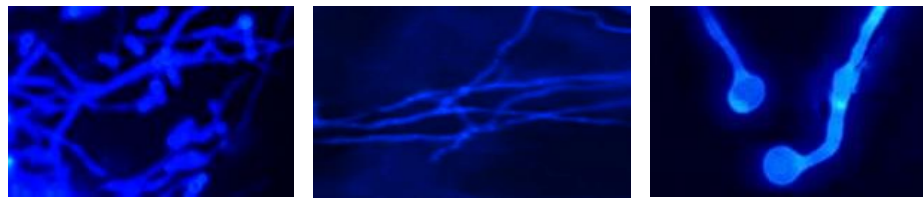


Fig. 8: Filamentous fungi structure visualized using CW staining.

DISCUSSION

Examination of fungi in drinking water was accomplished in the least number (Nagy & Olson 1982). Most authors agreed fungi generally exist, but the importance of cleanliness and health risks are noticed to be in less number. The pervasiveness of fungi was scrutinized in 45 drinking water samples from mineral water, tap water, and RO-purified water in workplaces and residences. Mineral water was more polluted than tap water. The prevailing genera were mostly *Aspergillus* and *Penicillium spp*., and it can be considered a feasible transmission path for filamentous fungi. Fungi are eukaryotic creature and they possess unusual features which includes nourishment by heterotrophic absorption, growth of vegetative and reproductive formation (i.e., spores and hyphae), and reproduction by both sexual and asexual (Siqueira & Lima 2013). Drinking water biofilms are devised as complex infectious groups of several microbes (bacteria, protozoa, fungi, algae, and viruses), however, all are modified to develop under oligotrophic circumstances (Gonclaves et al. 2006). The laboratory investigations have also displayed that fungal hypha could facilitate bacterial assembly in the surroundings and thus establishing new bacteria. But this theory and the environmental interconnection between them will require further research. Secondary metabolites produced by fungi can provide microbial attribution in water pipes (Arvanitidou et al. 1999, Giuseppina et al. 2020). As a consequence, alterations in proper sanitization can take place and the remaining chlorine in the treated water pipeline system can be modified. The elevated popularity of *Aspergillus spp., Penicillium spp*. and *Cladosporium spp*. are considered as dark colored fungi and it gives rise to mycoses and many other causes, such as skin diseases, allergies, and transmissible infections. Moreover, the genera are also linked with the manufacture of mycotoxins. Fluorescence microscopy is commonly used to acquire information on the cell morphology of fungi. Siqueira et al., 2011, stated that Calcofluor White MR2 (CW) staining enables the vision of cell walls in fungi due to its bond between the carboxylated polysaccharide and beta (1-3), (1-4) polysaccharides in cellulose. The main heterogeneity of fungi established in this work recommends that further deliberation must be given to certain microorganisms in water distribution system analysis. The predominance of fungi in the drinking water distribution system might be difficult as they can produce spores to accumulate with one another along with distinct particles expanding their resistance to purification and they can be more resistant than bacteria.

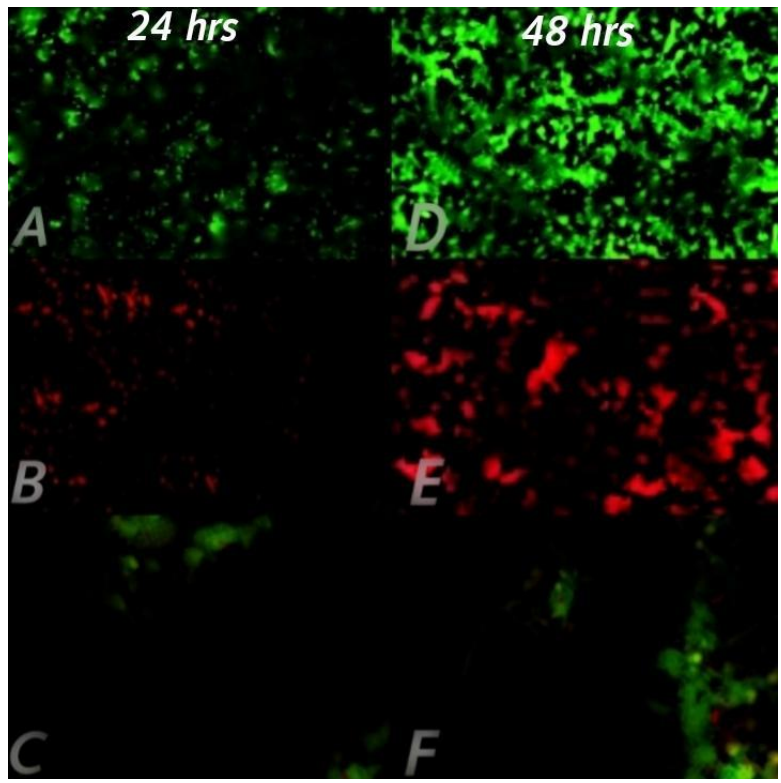


Fig. 9: Biofilm formation of filamentous fungi between 24 and 48 hrs.

CONCLUSION

Fungi are relatively usual in drinking water pipeline systems. This work is an outline concerning the issue correlated with filamentous fungi in drinking water and its relation to the formation of biofilms. Tap water, mineral water, and RO purified water could be a reservoir for fungi, which make public health at threat. For the study of fungi-related biofilms, standard techniques are needed, similar to those that are involved in the contamination of bacteria in water. Fungal identification at the species level is laborious. Therefore, in the future new molecular and epidemiological studies also require to regulate the importance of health. At the same time, we must anxious about fungal presence in drinking water, because increasing levels of fungi can diminish the grade of water and comprise health issues (Boe-Hansen et al. 2003). Fungal species are challenging to analyze, therefore the studies on fungi in drinking water need skill and caution. To overcome the problem, sufficient treatment of water could be a result (Momba et al. 2000), and also the use of sanitizers helps in the control of biofilms. Although, once the biofilms are formed it is not easy to remove because of the reality that the EPS are counteracting sanitizers. It is necessary to identify the threshold levels of fungi from different types of water in various places (hospitals, industries, etc). In conclusion, the results show that filamentous fungi can form biofilms in the water pipeline system.

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