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# Occurrence of biofilm forming fungal species and *in vitro* evaluation of anti-biofilm activity of disinfectants used in drinking water

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## Abstract

Fungal contamination in drinking water has garnered considerable attention over the past few decades, especially considering the detrimental consequences of pathogenic fungal species on both human and animal health. The formation of biofilms by certain species is a considerable factor contributing to the emergence of severe fungal infections. This research was designed to isolate and identify fungi, particularly those capable of forming biofilms from 150 samples of drinking water sourced from various locations. The isolated fungal species were tested for them *in vitro* biofilm formation using a microtitration plate method and the crystal violet assay was applied to quantify the established biofilms. The effectiveness of three disinfectants, namely ozone, chlorine, and hydrogen peroxide, in preventing the formation of biofilms by the most isolated fungal species was monitored. The findings indicated that *Aspergillus* species were the most prevalent in drinking water, comprising 63.33% (95/150) of the total number of fungal species identified. *Aspergillus fumigatus* and *Aspergillus flavus* were identified as the primary contributors to biofilm formation in drinking water distribution systems with prevalence rates of 41.00 and 34.00%, respectively, among all *Aspergillus* species. The outcomes of the *in vitro* studies demonstrated that the ozone disinfectant exhibited promising results in inhibiting fungal biofilms compared to chlorine and hydrogen peroxide. In conclusion, these findings provided valuable insights for water distribution authorities to develop effective regimens for controlling biofilm-forming fungal species using suitable antifungal biofilm disinfectants.



## Introduction

Drinking water is an essential requirement for both humans and animals and it must be of the highest quality and be safe for consumption free from any harmful substances and pathogens.[1,2] Various kinds of microorganisms infiltrate the drinking water distribution system (DWDS) and thrive in the favorable environment leading to numerous issues including bad water quality, water pipe blockage and public health concerns[3,4] Despite various elements, microorganisms like bacteria and fungi are a significant contributor to the poor quality of drinking water.[5,6] The quality of tap water is also significantly compromised when microbes access the DWDS.[7] Fungi including *Phialophora spp., Penicillium spp.,* and *Acremonium spp*. are typically regarded as the primary reason for the unpleasant taste and odor of drinking water. These genera are known to produce various chemicals such as 2,4,6-trichloroanisole which imparts an earthy smell to the water.[8] Additionally, the formation of biofilm in DWDS adversely affects the organoleptic properties of drinkable water such as color, taste, and smell.[9-11] Interestingly, filamentous fungi including *Aspergillus* possess the ability to form biofilms. Their mechanism of biofilm formation is similar to that in bacteria and yeast.[12,13] Biofilm formation presents various challenges including clogging and corrosion in water distribution systems or pipelines and causes several infections in humans and animals.[13] *Aspergillus flavus* and *A. fumigatus* are two major species that cause life-threatening infections in humans and animals.[14,15] Aspergillosis is considered to be one of the major disease conditions in immuno-compromised persons.[16,17]

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A diverse range of disinfectants are commonly used to control the fungal biofilm formation in DWDS.[18,19] Chlorine is considered an important biocide that is effective in combating fungal and bacterial biofilm growth due to its minimal detrimental impact on the environment.[20] Many other disinfectants are also used in controlling biofilms such as ozone, monochloramine and chlorine dioxide.[21] They are considered effective against a wide range of bacteria and fungi and inter-kingdom biofilms. They are extensively applied in cooling water towers, cosmetics and textiles industries.[18,22] The current study aimed to isolate and identify the biofilm-forming fungal species from the drinking water sources. The *in vitro* biofilm formation potential of various fungal species were assessed and the effectiveness of anti-biofilm activity of selected disinfectants in drinking water was also evaluated.

## Materials and Methods

**Collection of samples.** A total number of 150 water samples were collected from different locations in Faisalabad city, Pakistan. Fifty samples were taken from random places such as canteens, chillers, laboratories, household taps and hostels of Agriculture University, Faisalabad, Pakistan. In addition, ten samples were collected from water treatment plants. Fifty samples were taken from public water tanks and hospitals while forty samples were collected from animal and poultry sheds around Faisalabad city.[20]

**Sampling procedure.** Samples were collected under sterile conditions and their sterility check was done for aerobic bacterial contamination. 150 mL of water sample was collected in a clean sterile glass bottle with screw cap. The taps were allowed to run for at least 1 min to discard the first few streaks of water. While collecting samples from water treatment plants, glass bottles containing sodium thiosulfate (Thermo Fisher Scientific Inc., Waltham, USA) were used to neutralize residual chlorine present in the water.[7] Proper care was taken to open and fill the glass bottles to check for any kind of unnecessary contamination. Samples were preserved at 4.00 ˚C for further processing.[12]

**Selection of culture media.** Two different culture media *i.e.,* sabouraud dextrose agar (SDA; Oxoid, Basingstoke, UK) and potato dextrose agar (PDA; Oxoid) were used to isolate the fungal species from the water samples collected from various sources. A 0.50 mL of each water sample was inoculated on the surface of SDA and PDA using a spread plate method.[12] Overall, 300 petri plates were inoculated with random water samples. Out of 300 petri plates, 150 were incubated at 25.00 ˚C while the other 150 were incubated at 37.00 ˚C for 5 - 7 days in two incubators to check the comparative growth of fungi at different incubation temperatures. The presence of fungal colonies was observed every 24 hr on a regular basis.[23,24]

**Preliminary macroscopic and microscopic examination of fungal species isolated from water samples.** Isolated fungal species were primarily identified based on macroscopic and microscopic characteristics.[23] Macroscopic characteristics *i.e.,* surface elevation, borders, color, size of the colonies, texture of the colonies, pigmentation and opacity of colonies were observed using the naked eye for the identification of fungal isolates.[15]

**Slide culture technique.** Microscopic characteristics of fungi were also observed by using the slide culture technique. Under the laminar air flow (sterile conditions) an intact, grease-free, neat and clean slide was taken and a block of molten nutrient agar of desirable size was placed on it. After that, a suspected fungal colony was picked carefully by a sterile disposable inoculating loop. After sterile inoculation, the slides were incubated at 25.00 ˚C for 3 - 5 days. The visible fungal colonies on agar slides were observed after 3 - 5 days.[25]

**Mycological staining.** Subsequently, a mycological staining procedure (Lactophenol Cotton Blue staining; Thermo Fisher Scientific Inc.) was performed to stain the fungi to examine the further details under the microscope (Thermo Fisher Scientific Inc.) at 4 and 40 ×. Briefly, a drop of Lactophenol Cotton Blue was put on the slide after the coverslip was placed over it. Several microscopic features including an arrangement of spores, hyphae and other characteristics were observed under a light microscope. Slides were examined at low magnification to higher magnification.[26]

**Preparation of fungal spores.** Spore suspension of isolated fungal species *(A. flavus,* and *A. fumigates*) was made by harvesting each fungus from a 7-day-old pure culture in PDA by putting 2.00 mL of physiological saline solution at a concentration of 0.85% into the petri plates. The spore suspension was subjected to re-suspension and vortex mixing before the quantification. The final concentration of spores (after standardization) was kept at $1.00 \times 10^5$ spores *per* mL by diluting suspensions with the saline solution (0.85%). The spores were quantified using Neubauer counting chamber.[17]

**Determination of biofilm formation.** For the biofilm determination, 100 µL previously prepared spore suspension, 100 µL of culture broth media (potato dextrose; Thermo Fisher Scientific Inc.) and 100 µL of distilled tap water were dispensed *per* well into a 96-well polystyrene microtiter plate (Kartell, New York, USA) with a flat bottom. Negative blank well with only media was also included. Plates were incubated at 37.00 ˚C for 24 hr to give the optimal conditions for biofilm formation. The Plates were monitored after every 4 hr as 4, 8, 12, 16, 20, and 24 hr). Biofilm, extracellular polymeric substances and other possible structural parameters were examined under a stereomicroscope (DFC450; Leica, Wetzlar, Germany).

**Biofilm quantification assay.** Biofilm quantification was done with the help of crystal violet assay.[19,27] All staining steps were performed at 20.00 ˚C in the Mycology laboratory (BSL-III), Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan. The biofilms were fixed with 100 µL methanol (Honeywell - Burdick & Jackson, Muskegon, USA) at 99.00% concentration. For the negative control wells were prepared with the same medium without inoculation of microorganisms. Subsequently, the supernatant was discarded and plates were allowed to be air-dried for 10 min. After that 100 µL solution of crystal violet dye was prepared. Soon after that, the crystal violet dye was dispensed in all wells of the microtiter plates. After 20 min of incubation at 20.00 ˚C, an excessive amount of crystal violet dye was removed or washed by subjecting the plates to running tap water. Then, 150 µL of acetic acid with 33.00% concentration was used to free the bound crystal violet.[28-30] The experiment was replicated four times.

**Determination of optical density (OD) value.** The OD570 value was measured by observing the absorbance at 590 nm with the help of the microtiter plate reader (Synergy HT; BIO-TEK, Winooski, USA).[14] In evaluating biofilm formation, absorbance data were obtained and depicted as the mean ± standard deviation (SD). To determine the strength of biofilm formation, a cutoff OD590 value was established, which was set at three times the SD above the mean absorbance of the negative control. Samples with an OD595 value surpassing this cutoff were categorized as strong biofilm formers. Those with values above the mean but below the cutoff were considered medium biofilm formers and samples with values slightly above the mean of the negative control but below the cutoff were classified as weak biofilm formers.

**Antibiofilm activity of commercially available disinfectants.** The microtiter plate method was used to assess the anti-biofilm effect of the selected disinfectants like chlorine, ozone, and hydrogen peroxide.[21] The biofilms of isolated fungal strains were developed on commercially available pre-sterilized, polystyrene, flat-bottomed, 96-well microtiter plates.[30] Four plates were prepared for each isolate with time protocol of 6, 12, 24, and 48 hr. Ozone, chlorine and hydrogen peroxide (Roam Technology, Genk, Belgium) were the selected dis-infectants that were used in this study to evaluate their *in vitro* fungal antibiofilm activities. The microtiter plates odd-numbered columns (1, 3, 5, 7, 9, and 11) were designated as controls, while the even-numbered columns (2, 4, 6, 8, 10, and 12) were designated for disinfectants. In even-numbered columns, the first three wells were selected as control *i.e.,* without disinfectant and the next three wells were used for a disinfectant in each column. Dilutions of disinfectant were freshly prepared on the same day when antibiofilm testing was done. Before the experiment, isolated fungi were grown on SDA at 35.00 ˚C for 24 hr and saline-washed suspensions of each strain of fungal species were prepared. The turbidity of each suspension was adjusted to the equivalent of $3.00 \times 10^7$ colony forming unit *per* mL with SDB supplemented with glucose concentration (30.00 mg $L^{-1}$). Each well was inoculated with aliquots of 20.00 µL of fungal suspension and 180 µL of SDB with glucose. Each plate was incubated for the above-described time points at 35.00 ˚C. After biofilm formation, the medium in the wells was aspirated and planktonic cells were removed by thoroughly washing three times with sterile phosphate-buffered saline. A volume of 200 µL aliquot of disinfectants was then added except for the control wells. According to the manufacturer's recommendations, the contact times of ozone, hydrogen peroxide and chlorine was designated as 10 min.[28,29] All experiments were performed in four replicates.[13]

## Results

Out of 150 samples, 95 samples were positive for *Aspergillus spp*. Out of them, 39 samples were positive for *A. fumigatus,* 33 samples for *A. flavus* and 23 samples for *A. niger.* Other 55 samples were positive for miscellaneous fungi such as *Penicillium, Trichoderma* and *Cladosporium* etc. The comparative frequency in terms of positive water samples with different *Aspergillus* species including *A. niger, A. fumigatus,* and *A. flavus* from different water sources is given in Table 1. It shows that the most commonly occurring biofilm-forming fungal species are *A. fumigatus, A. flavus,* and *A. niger*. Out of them, *A. fumigatus* has comparatively greater biofilm formation capacity compared to other related species. *Aspergillus species* were 63.00% of total fungal species isolated from drinking water. Out of that, *A. fumigatus* was 41.00%, *A. flavus* 34.00%, and *A. niger* 4.00%, respectively.

**Table 1.** Comparative frequency of occurrence of biofilm-forming fungal species from different water sample sources.

| Sources (n) | Positive *Aspergillus* spp. | Positive *A. niger* | Positive *A. fumigatus* | Positive *A. flavus* |
|---|---|---|---|---|
| **Animal sheds (40)** | 30 | 4 | 15 | 8 |
| **Canteens (20)** | 5 | 2 | 1 | 2 |
| **Hospitals (30)** | 25 | 5 | 10 | 11 |
| **Laboratories (25)** | 20 | 3 | 9 | 8 |
| **House (25)** | 10 | 5 | 2 | 3 |
| **Water plants (10)** | 5 | 2 | 2 | 1 |
| **Total (150)** | 95 | 21 | 39 | 33 |

The OD value range of *A. fumigatus* ranged from 0.05 to 0.45 and *A. flavus* from 0.05 to 0.30. It indicated that *A. fumigatus* had stronger biofilm-forming potential compared to *A. flavus.* The range OD values *of A. flavus* and *A. fumigates* is demonstrated in Figures 1 and 2, respectively. The antibiofilm potential of selected disinfectants *i.e.,* ozone, chlorine and hydrogen peroxide showed that ozone had strong oxidative potential to reduce biofilm formation of *A. fumigatus* and *A. flavus* compared to chlorine and hydrogen peroxide. Moreover, hydrogen peroxide had comparatively more antibiofilm inhibition potential than chlorine. The antibiofilm potential of the disinfectants along with their contact time and efficacies are shown in Table 2.

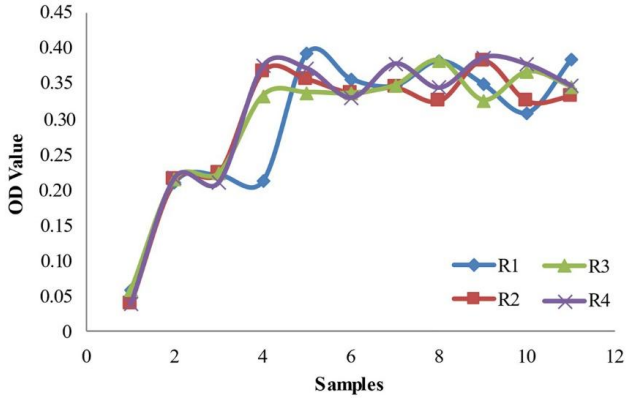


**Fig. 1.** Optical density (OD) value of *Aspergillus flavus.* R1, R2, R3, and R4: replicates 1, 2, 3, and 4, respectively.

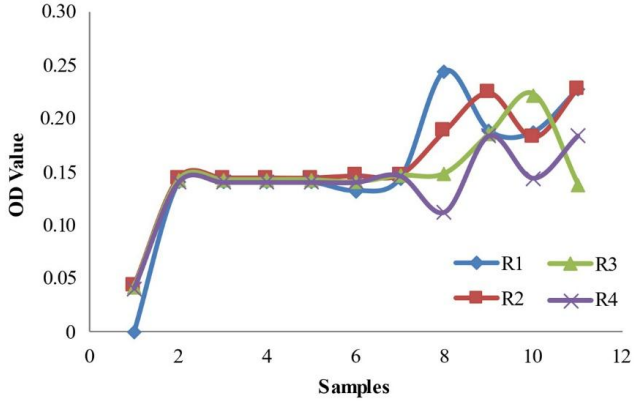


**Fig. 2.** Optical density (OD) value of *Aspergillus fumigatus.* R1, R2, R3, and R4: replicates 1, 2, 3, and 4, respectively.

## Discussion

The presence of fungal biofilms in drinking water poses a high threat to human and animal health. Fungal biofilms also interfere with the drinking water quality (organoleptic properties) and form inter-kingdom biofilm communities along with bacteria. These biofilm communities show resistance to disinfectant treatments and are one of the biggest concerns for drinking water distribution authorities.

In the current study, 150 drinking water samples were collected and processed to monitor the occurrence of the biofilm-forming filamentous fungal species. Isolated species were evaluated based on their microscopic and macroscopic characteristics. Four fungal genera (*Aspergillus, Cladosporium, Trichoderma,* and *Penicillium*) were identified and differentiated based on spore and hyphae formation on the methodology adopted by Abdalla *et al.*[1] Our study indicated that *Aspergillus* species were one of the most frequently found filamentous fungi (63.00%) in drinking water. Out of all *Aspergillus* species, the most important and frequently occurring fungal species were *A. fumigatus* (41.00%) and *A. flavus* (34.00%).

The biofilm-forming potential of fungal species particularly *Aspergillus* species including *A. fumigatus, A. flavus* was also studied in the research conducted by Góralska *et al.*[10] The microtitration plate method was used to monitor the *in vitro* biofilm-forming potential of *A. flavus* and *A. fumigatus* as described by Siqueira *et al.*[17] The crystal violet method was used to quantify the biofilms formed by the *Aspergillus* species. Subsequently, the OD values were measured to monitor the potential of the two most important biofilm-forming fungal species *i.e., A. flavus* and *A. fumigatus.* A similar method was used by Afonso *et al.*[3] These biofilm fungi are the major source of contamination in drinking water systems and cause severe health-related conditions in immunocompromised patients and animals as reported by Ma *et al.*[16]

The important and unique part of our study was to monitor the antibiofilm activity of three selected commercially available disinfectants including chlorine, hydrogen peroxide and ozone to make the effective regimes to combat these devastated pathogens. In the

**Table 2.** Type of disinfectants and their contact time along fungal species. The contact time was 10 min for all disinfectants.

| Disinfectants | Fungal species | Observation |
|---|---|---|
| **Ozone** | *Aspergillus fumigatus* | A considerable reduction (sensitive) in biofilm formation was observed. |
| | *Aspergillus flavus* | A considerable reduction (sensitive) in the biofilm was observed in many wells. |
| **Chlorine** | *Aspergillus fumigatus* | No inhibition (resistance) in biofilm was observed |
| | *Aspergillus flavus* | A slight inhibition (intermediate) in biofilm was observed |
| **Hydrogen peroxide** | *Aspergillus fumigatus* | No decrease in biofilm was observed. However, in some wells, strong inhibition was seen. |
| | *Aspergillus flavus* | A slight reduction (intermediate) in the biofilm was observed in a few wells |

current study, three disinfectants were used and their effectiveness against fungal biofilms was evaluated with the help of the microtitration plate method. The contact time was kept at 10 min for each disinfectant. Ku *et al*.[18] used the same method to monitor the efficacy of disinfectants against the *Candida* spp. After performing the proper experiment, it was concluded that ozone showed a higher level of effectiveness against the fungal biofilms and inhibits the biofilm formation compared to chlorine and hydrogen peroxide. The type of disinfectants and their contact time along with their potential effect on the fungal biofilms are shown in Table 2. The same effectiveness of the ozone against the fungal spores was reported by Wen *et al*.[7] in a study conducted to monitor the efficiency of ozone against the three most frequently occurring fungal species in drinking water. In their studies, various cellular changes such as plasma membrane damage, changes in reactive oxygen species, leakage of intracellular compounds and esterase activity in the spores were detected after inactivation. It was also stated that the fungicidal performance of ozone was greater than that of chlorine dioxide and chlorine which are commonly used disinfectants in the DWDS. Moreover, the inactivation efficiency of ozone on fungal spores in real water matrices was reduced to a certain limit of the efficiency in phosphate buffer.

In conclusion**,** the safety of drinking water is very important for human beings and animals. There are various diseases which are directly transmitted by the consumption of unsafe water in humans. Microbiological analysis plays an important role in assessing the microbial safety of drinking water. Fungi are indicators of the microbial safety of water. They spoil the quality of drinking water and cause a variety of mild to severe infections in human beings. Additionally, some fungal species have the potential to form biofilms in DWDSs which is the biggest problem for drinking water utilities.

Fungal biofilms become resistant to various disinfection treatments. In the current study, it was concluded that filamentous fungi formed biofilms in the DWDSs. Out of all filamentous fungi, *Aspergillus* species were mainly involved in biofilm formation. All along with that, ozone treatment showed good antibiofilm activity compared to hydrogen peroxide and chlorine. Therefore, it is recommended to use ozone in DWDSs in recommended quantities to check the establishment of biofilms. Further research is needed in this area to monitor the safety concerns of the disinfectants with respect to human and animal health concerns.

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## Conflicts of interest

The authors have no conflicts of interest to disclose.

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