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Determination of Filamentous Fungi Associated with Biofilm Production in Stored Drinking Water

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ABSTRACT

Microbial biofilms are dynamic community of microbes strongly attached to biologic and non-biologic substrates enclosed in self-produced protective exo-polymeric matrix or primary polysaccharide material. Bacteria are the probable most frequently studied microbe in biofilms. This study determined the filamentous fungi associated with biofilms in stored drinking water. Filamentous fungi involvement in biofilm production was determined using Congo Red Agar (CRA) method, complemented with wet mount method for examination of fungal isolates of the culture. Results obtained revealed that five filamentous fungi species were associated with biofilm production in the stored drinking water samples. The fungi included: *Fusarium oxysporium, Rhizopus stolonifer, Alternaria alternata, Aspergillus niger* and *Penicillium* species. Results also revealed that *A. alternata* had the highest frequency of occurrence (40%) than other fungal species. The mycelia of *F. oxysporium* were in pellets, a possible induction of sporulation. This pelletization is attributed to the medium of isolation which contained polysaccharide starch and inulin. In all, this study has shown that filamentous fungi are associated with biofilm formation in stored drinking water. Drinking water should therefore be handled with utmost care to exclude human fungal infections.

Keywords: Stored Drinking Water, Biofilm, Filamentous Fungi, Fungal Infections, Exo-polymeric Matrix.

Introduction

Microbial biofilms are dynamic communities of micro-organisms strongly attached to biologic and non-biologic substrates that are enclosed in a self-produced protective exopolymeric matrix (EPM), (Costerton *et al*., 1995). It can also be perceived as an assemblage of microbial cells associated with a surface and enclosed in a matrix of primary Polysccharide material (Herrling and Guthausen, 2019).

Development of biofilms occurring in inner surface of storage vessels offers a suitable medium for the growth of microorganisms and as such contributes to the deterioration of treated drinking water quality in homes. According to Herrling and Guthausen (2019), biofilms can alter dissolved oxygen level, taste and production of odour and colour change, ranging from red to black, due to increased bacteria level in the stored water.

It is estimated that approximately 80% of all bacteria in the environment exist in biofilm communities and more than 65% of human microbial infections are involved in biofilms (Dolan, 2008).

Bacteria are probably the most frequently studied group of micro-organisms with respect to the quality of drinking water; possibly because many pathogenic bacteria and other parasites and their occurrence in drinking water often lead to relatively acute symptoms and disease in humans (Mara and Horan, 2006). Fungi occurrence in water has often been over looked, but may be regarded as a chronic problem in drinking water distribution systems and possibly an undermined problem.

A broad range of filamentous fungi have been reported to be associated with drinking water, amongst which could possibly be contagious, harmful and allergic (Yamaguchi *et al.*, 2007; Hageskal *et al.*, 2009).

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The presence of fungi in drinking water can cause various fungal infections in immuno-compromised individuals, and such fungi are reported to include *Aspergillus* and *Penicillium* species which could cause allergy, ear infections, lung and kidney failure, respiratory problems, and increased levels of invasive infections (Grabinska-Loniewska *et al.*, 2007). These fungi can invade drinking water pipeline systems through various routes such as water treatment part, insufficient storage water facilities, cracks in the pipelines, main breaks and installation; hence filamentous fungi have the potential to grow on surface that may lead to formation of biofilms (Sonigo *et al.*, 2011; Afonso *et al.*, 2019).

Fungal biofilms are communities of adherent cells surrounded by an extracellular matrix, which are commonly found during infections caused by a variety of fungal pathogens. Clinically, biofilm infections can be extremely difficult to eradicate due to their resistance to antifungal agents and host defenses. The growth of biofilm communities allows microbial cells to survive in hostile environments which enhance their resistance to physical and chemical pressures and promote metabolic co-operation (Jabra-Rizk *et al.*, 2004). Since in the 1970s, there have been several researches, reporting on a wide diversity of filamentous fungi, being detected from drinking water and the most frequently isolated belong to the fungal genera, *Aspergillus, Cladosporium* and *Penicillium*. This might be related to their ability to secrete a pigment known as melanin which confers protection to spores against a variety of stresses; providing the organisms with a competitive advantage and greater resistance to water treatments (Sonigo *et al.*, 2011). Additionally, the hydrophobicity property of their spores further protects them against water disinfection as their spores tend to aggregate between each other with other particles.

Filamentous fungi, as mentioned above can also enter drinking water from various locations though, this is considered as unnatural habitat for them. According to Wang *et al*. (2024), filamentous fungi can form mycelia pellets during biofilm study due to the medium of isolation.

The presence of biofilm in water distribution system can pose health threat since they permit a high concentration of organisms to occur and play a role in the accumulation, protection and dissemination of pathogens (Hug et al., 2008, Hageskal *et al*., 2009). Previously, there was dearth of satisfactory demonstration of involvement of filamentous fungi in biofilms, but knowledge has increased on the occurrence of fungi in drinking water due to increased studies. Fungal biofilm production occurs by interfacial adhesion, growth, maturation and diffusion (Wang *et al*., 2024).

There is no conventional method for analyzing fungi in drinking water, hence diverse procedures may be involved; such as membrane filtration, spread plate and direct microscopic observations techniques relevant to the study (Fujita *et al*., 1994; Zhou *et al*., 2000; Saraswathy and Hallberg, 2005; Liu *et al*., 2008; Wang *et al*., 2024).

Haven known that the morphology and etiology of filamentous fungal biofilms can be distinguished by *in Situ* microscopic examinations and use of sequencing techniques such Illumina (Solexa); coupled with the concern of the impact of presence of fungi in biofilms. This study was intended to investigate the presence of filamentous fungi associated with stored drinking water and in subsequent study will devise strategy to reduce or stop the impact of such fungal species in water against human health.

Materials and Methods

Source of Water Sample

The water sample for the study was obtained from a running tap at the screen house of plant science and Biotechnology, Rivers State university , Nkpolu-Oroworukwo which lies within the latitude 4^{o} 48”4.67856 N and longitude 6^{o} 58 33.35032” E; located in the tropical rain forest region of Southern part of Nigeria Popularly known as Niger Delta Region.

Sample Collection

Drinking water sample was filled up to three quarter (¾) level in a clean plastic container and stored for nine weeks in the departmental screen house to allow microbial biofilm formation to take place. Thereafter, swap sample from the sides of the container was taken for microscopy.

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Media Preparation

Sabourand Dextrose Agar (SDA) was used for the study. The medium was prepared according to the manufacturer's prescription and instruction.

Cargo Red Agar (CRA): This medium was also prepared based on the manufacturer's instruction. The CRA medium was prepared with Brain Heart Infusion Agar (TM 361), 52g/L, Sucrose, 50g/L and Congo Red indicator 8g/L. Congo Red stain was prepared as a concentrated aqueous solution and autoclaved at 121^{o}C for 15mins separately from the other medium constituents; then this was added to the autoclaved Brain Heart infusion agar with sucrose at 55^{o}C. CRA plates were inoculated with test organisms from swabs sample, and incubated at room temperature for 48hrs (Freeman *et al*., 1989).

Isolation of Filamentous fungi

Swab sample from water containers were taken and inoculated directly on sterile media plates containing Sabourand Dextrose Agar (SDA) by streaking. This was incubated in inverted position at room temperature for 2-7 days; after which, the plates were observed and the colonies that developed were characterized and sub cultured and the media plates autoclaved and discarded.

Macroscopic and Microscopic examination of fungal isolates

The wet mount method as described by Cheesebrough (2006) was used to examine the fungal isolates of the culture. A small portion of the isolates were picked with a sterile wire loop and placed on clean and grease-free microscope slide; then a drop of lactophenol blue was added and smeared. This was covered with a slip and examined under the microscope (x10) for hyphal features and (x40) objective lens for other characteristic features.

Identification of the isolates was based on their cultural morphology and microscopic features. Morphological studies were carried out on different media plates used for the isolation of organisms; while pure cultures were isolated based on colony size, shape, pigmentation, elevation and texture of the individual organisms after 48hours of growth at 30^{o}C.

Determination of fungal species associated with biofilm

The method of Congo Red Agar (CRA) of Freeman *et al*. (1989) was used. The fungi isolated from the drinking water sample were inoculated on Congo Red Agar (CRA) plates and incubated at room temperature for 48hours. The medium was observed for black colouration; an indication of biofilm production.

Results

The results of the macroscopic and microscopic characterization of fungal isolates harvested from the stored water containers and suspected to be filamentous fungi in the biofilm are presented in Table 1. The filamentous fungi identified to be associated with the stored water containers for biofilms included: *Fusarium oxysporium, Rhizopus stolonifer, Aspergillus niger, Alternaria alternata* and *Penicillium* species.

The results shown on Table 2 revealed the percentage Frequency of occurrence (%) of Confirmed Biofilm – Producing filamentous fungi isolated from water storage containers. It revealed that *Alternaria alternata* recorded the highest occurrence than the other fungal species.

The result of filamentous fungi isolates associated with biofilm production revealed that *Fusarium oxysporium, Rhizopus, stolonifer, Alternaria alternata, Aspergillus niger* and *Penicillium* spp produced biofilm in the study.

The results shown on Plates 1a-e revealed the morphological macroscopy of the filamentous fungal isolates with biofilm production. It also revealed that *Fusarium oxysporium* mycelia were in pellets; a possible sign of sporulation.

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Table 1: Macroscopic and Microscopic characteristics of fungi isolated from stored water containers

Isolate Code	Macroscopic Feature	Microscopic Feature	Suspected Organism
1	White fluffy growth with yellow on reverse	Canoe-shaped conidia with septate hyphae	*Fusarium oxysporium*
2	Gray with cotton candy like texture from the front, the colony seemed white and later turned gray-yellow	Aseptate hyphae with round conidia heads	*Rhizopus, stolonifer*
3	Gray lawny elevated growth with wrinkled surface and black on reverse.	Ellipsoidal conidia with septate branching hyphae	*Alternaria alternata,*
4	Very light green lawny growth with white wrinkled periphery	Septate branching hyphae with chain like conidia	*Penicillium* specie
5	Initial white growth that changed to black after a few days, showing conidia spores	Filamented hyphae appearing like plant	*Aspergillus niger*

Table 2: Frequency of occurrence (%) of Confirmed Biofilm – Producing filamentous fungi isolated from water storage containers

Isolate Code	Isolated Fungi	Water Storage Container 1	Water Storage Container 2	Frequency of Occurrence (%)	Biofilm Production
1	*Fusarium oxysporium*	0	33.3	20	+
2	*Rhizopus, stolonifer*	50	0	40	+
3	*Alternaria alternata,*	50	33.3	20	+
4	*Penicillium* specie	0	33.3	20	+
5	*Aspergillus niger*	**33.3**	**0**	20	+

Plate 1a: *Rhizopus stolonifer*

Plate 1b: *Alternaria alternata*

Plate 1c: *Aspergillus niger*

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Plate 1d: *Penicillium* species

Plate 1e: Pelletization in *Fusarium oxysporium*

Plate 1: Macroscopic features of biofilm producing filamentous fungi isolated from the drinking water sample

Discussion

The findings of this study revealed that five filamentous fungal species were associated with biofilm production in stored drinking water. It is possible the fungal species attached themselves to the water containers by their self-produced matrix as well as matrix of primary polysaccharide material which would have been present for their attachment to the surface. This finding seems to align with the report of Herrling and Guthausen (2019), who suggested that community of biofilm producing micro-organisms attach to a living surface by self-produced matrix enclosed in a matrix of primary polysaccharide material.

The findings also affirm the report of Yamaguchi *et al*. (2007) and Hageskal *et al*. (2009) who suggested that a broad range of filamentous fungi are associated with drinking water among which are possible contagious, harmful and allergic strains such as *Penicillium* and *Aspergillus*. According to them such filamentous fungi in drinking water could cause various fungal infections in immune compromised individuals, as a result of allergy, ear infections, lung and kidney failure; as well as respiratory problems and increased levels of invasive infections.

These filamentous fungi that formed biofilms in water storage during this study may have gained access through various routes through the air, in the container or present in the water before the water was fetched for the study.

This finding also has a bearing with the submission of Grabinska-Loniewska *et al*. (2007), Sonigo *et al*. (2011) and Afonso *et al*. (2019) who altogether reported that filamentous fungi such as *Aspergillus* and *Penicillium* can invade drinking water pipelines through various routes such as water treatment part, insufficient storage water facilities, cracks in the water pipelines, major breaks and installation; hence filamentous fungi have the potential to grow on surfaces that may lead to formation of biofilms.

And according to the reports of Sonigo *et al*. (2011), it is possible that the hydrophobicity of their spores may have protected them on their surface of attachment by aggregating between each other with other particles.

The findings of this study also revealed that *Fusarium oxysporium* mycelia formed pellets. It is likely because of the medium used for isolation hence there is no conventional method for analyzing fungi from drinking water; which results in the use of diverse procedures. These findings clearly agree and affirm the suggestions of Fugita *et al*. (1994); Zhou *et al*. (2000), Saraswathy and Hallberg (2005), Liu *et al*. (2008), Afonso *et al*. (2019) and Wang *et al*. (2024). All these researchers had reported that since there is no conventional method for analyzing fungi in drinking water, that diverse procedures may be involved. They also reported that the type of medium used in isolation could lead to mycelia pelletization.

Accordingly, the findings of this study agree with their reports which related that *Fusarium* formed pellets due

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to the medium of isolation. In affirmation with their suggestions, it is likely there was polysaccharide starch and inulin in the medium of isolation which may have induced sporulation in *F. oxysporium*.

In conclusion, this study has shown that filamentous fungi being ubiquitous are also present in stored drinking water forming biofilms. These fungi can attach themselves to a surface by self-produced polymeric matrix enclosed in a matrix of primary polysaccharide material. They have the ability to alter dissolved oxygen level in drinking water, change taste, produce unfriendly odour due to increase in microbial activities, fungi inclusive.

This study has also revealed the presence of filamentous fungi that can be contagious, harmful, create ear infections, cause lung and kidney diseases, respiratory infections and increased invasive infections. Therefore, care should be taken to exclude these fungal species from drinking water source.

The study has also shown that detection and isolation of filamentous fungi in stored water is possible and should follow a uniform process in which the methodologies should complement each other as seen from the findings of this study. It has become clearer that indeed filamentous fungi are associated with stored drinking water as such should not be undermined any longer because of the inherent danger in their involvement in drinking water infection; especially on those whose immunities have been compromised who may be in greater health danger.

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