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QUALITATIVE AND QUANTITATIVE ANALYSES OF FILAMENTOUS FUNGI AND YEASTS AS POTENTIALLY PATHOGENIC SPECIES IN AN INTEGRATED FIXED FILM ACTIVATED SLUDGE (IFAS) SYSTEM

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Abstract

As potentially pathogenic microorganisms in wastewaters, fungi pose an important threat to human health. In the recent decade, despite the hazards which these microorganisms have caused for the human population, relatively few studies have been conducted on fungi as contaminants in wastewaters. This study which was conducted on the diversity and occurrence of filamentous fungi and yeasts highlighted the role of the Integrated Fixed Film Activated Sludge (IFAS) system in wastewater purification from potentially pathogenic fungi. Eighty-one samples were taken from nine IFAS sites. The samples were prepared for count and identification. The genera of fungi were identified by macroscopic and microscopic features. The results showed that the highest frequency of filamentous fungi was related to the genus of *Aspergillus sp*. (45.53%) while the lowest was related to *Aureobasidium sp.* (0.39%). The greatest frequency of yeasts belonged to *Geotrichum sp*. (18.43%) while the lowest frequency was recorded for *Trichosporon sp*. (6.75%). The highest count of fungi with a mean concentration was observed in the RAS tank (63.6 CFU/ml), whereas the lowest count of fungi was found in the Effluent tank (7.8 CFU/ml). The highest diversity of genera (17) was isolated from the Aeration tank while the lowest diversity of genera (5) was isolated from the Effluent tank. This study has provided a useful picture of the IFAS process by significantly reducing the number and genera of potentially pathogenic fungi which is beneficial to public and environmental health.

***Keywords*:** IFAS, filamentous fungi, Yeasts, pathogenic, wastewater.

Introduction

Wastewater treatment involves the removal of organic pollutants and pathogenic microorganisms such as fungi that are hazardous to people and animals (Magwaza *et al.*, 2017; Abbaszadeh et al., 2019; AS *et al*., 2019).). These pathogenic fungi penetrate into wastewater treatment plants through different ways including sedimentation from atmospheric air (fungal bio-aerosols) or through polluted waters (Dynowska, 1997; Biedunkiewicz and Ozimek, 2009). Previous studies have shown that fungi are heterotrophic organisms. (Al-Rejaboo & Jalaluldeen, 2019). Due to their capability in degrading various organic compounds by secreting different enzymes and toxins besides producing antibiotics, fungi play an important role in wastewater treatment. They are also used as bioindicators in routine microbiological analysis and as water purity indicators (Kwasniewska 1988; More *et al.*, 2010; Biedunkiewicz and Baranowska, 2011). On the other hand, because many of these fungi are pathogenic to humans and since they exist in wastewaters, recognizing and purifying them from wastewaters are important in terms of public and environmental health (Navi *et al.*, 1999; Zafar *et al.*, 2017). In 2003 and 2005, Kacprzak and fellow workers showed that some fungi (moulds and yeasts) were very common in the wastewater samples collected from different wastewater treatment plants (Kacprzak *et al.*, 2003; Kacprzak *et al.*, 2005). Therefore, despite the importance of fungi with regard to public health, few studies have been done on them, especially in wastewater treatment plants with the IFAS process.

The aim of this study was to show the role of the Integrated Fixed Film Activated Sludge (IFAS) process in the purification of polluted waters (at the level of municipal wastewaters) from potentially pathogenic fungi. Thus, our study has been done using qualitative and quantitative analyses of yeasts and filamentous fungi at each stage of the IFAS system with a special focus on fungi which are potentially pathogenic for people.

Materials and Methods

Laboratory-Scale IFAS

The surveys were carried out in a bench scale of the IFAS system (Fig. 1). The system was cultured with activated sludge microorganisms from Ahvaz wastewater treatment plant of Choneiybeh and fed with synthetic wastewater. Then the fungal samples were gathered monthly from 9 sites with the IFAS system. Two samples in the Anoxic tank, two samples in the Aeration tank, two samples in the Clarifier, one sample in the Effluent tank, and two samples in the Return Activated Sludge Tank were collected. The yeasts and filamentous fungi were isolated and identified in the collected samples in the mycology laboratory.

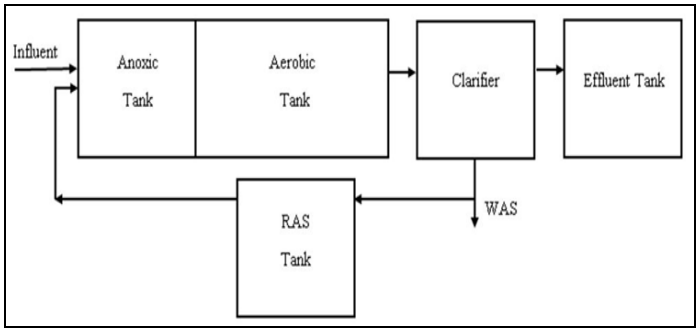

Fig. 1: Schematic diagram of the Integrated Fixed Film Activated Sludge (IFAS) System used in this study.

Isolation and Identification of Fungi

To isolate and count the number of the fungal colonies (CFU/ml), one ml of the mixed wastewater samples of each site (immediately after collection) was aseptically pipette into 9 Sabouraud Dextrose Agar (SDA) medium (Merck, Germany). All petri dishes were incubated for 48-72 h at room temperature and subjected to identification processes. During the research seasons, the average number of colonies was calculated. Fungal isolates were identified at the genus and/or species levels. The macroscopic features of fungi were diagnosed based on culture (diameter, color, aerial hyphae, and colony texture), while their microscopic specifications were stained with lactophenol aniline blue stain and were identified by conidiophores characteristics, and the shape, size, and color of conidia (Yang and Heinsohn, 2007; Watanabe, 2010). The differentiating media of CHROMagar Candida (Paris, France) and Urease (Merck, Germany) were used for identification. Slide cultures were prepared for filamentous fungi and yeasts which were finally diagnosed according to mycological atlases including "introduction to food and airborne fungi" compiled by Samson *et al.* (2004).

Results and Discussion

According to Table 1, seventeen genera of yeasts and filamentous fungi were recovered from 81 wastewater samples collected monthly from 9 sites of Integrated Fixed Film Activated Sludge (IFAS) System for nine months (9 samples/month) from March to December 2017 (Fig. 1). A total of 3785 fungal colonies including 2304 filamentous fungi (60.87%) and 1481 yeasts, and yeast-like fungi (39.13%) were isolated from different stages of the IFAS system during this investigation. From the 2304 isolations of filamentous fungi, 223 isolations were dematiaceous fungi and 2081 isolations were hyaline hyphomycetes. The results showed that from the total percentage of filamentous fungi (60.87%), the highest frequency was related to the genus *Aspergillus* (45.53%) followed by *Penicillium sp.* (32.3%), *Cladosporium sp.* (7.33%), *Fusarium sp.* (5.86%), *Rhizopus sp. (2.13%), Mucor sp.* (1.69%), *Paecilomyces* sp. (1.65%), *Alternaria sp.* (1.21%), *Stachybotrys sp.* (0.74), *Scopulariopsis sp.* (0.69%), *Chaetomium sp*. (0.47%), and *Aureobasidium sp.* (0.39%). Therefore, *Stachybotrys sp.*, *Scopulariopsis sp.*, *Aureobasidium sp.*, and *Chaetomium sp.* were isolated in a rare frequency in less than one percent of all the samples. *Penicillium sp.*, *Aspergillus sp*., *Fusarium sp., Cladosporium sp.*, and yeasts were present at all the sampling sites. However, *Aurobasidium sp*. was only recovered from the Aeration tank stage of Integrated Fixed Film Activated Sludge (IFAS) system (Table 1).

Table 1: Distribution of number (CFU/ml) & percentage frequency (%F) of various fungal genera and species recovered from different sites (I to IX) of IFAS system during 9 months from March to December 2017.

No.	Genera and or species	Number and percentage of Fungi	Total, N. (%F)	Remarks*							
		I	II	III	IV	V	VI	VII	VIII	IX	
1	*Aspergillus sp.*	17(4.23)	36(11.28)	22((3.94)	38(6.87)	23(6.00)	44(11.79)	17(23.94)	24(4.18)	36(6.50)	257(6.78)	Path, sap
2	*A. flavus*	29(7.21)	22(6.89)	34(6.09)	33(5.96)	31(8.09)	21(5.63)	13(18.30)	42(7.33)	32(5.78)	257 (6.78)	Path, sap
3	*A. fumigatus*	22(5.47)	13(4.07)	35(6.27)	24(4.34)	12(3.13)	4(1.07)	9(12.67)	33(5.76)	16(2.89)	168(4.43)	Path, sap
4	*A. niger*	27(6.72)	5(1.56)	28(5.02)	23(4.15)	9(2.35)	5(1.34)	1(1.40)	14(2.44)	24(4.33)	136(3.59)	Path, sap
5	*A. ochraceus*	3(0.75)	6(1.88)	10(1.79)	11(1.98)	8(2.08)	2(0.53)	-	4(0.69)	-	44(1.16)	Path, sap
6	*A. Terreus*	20(4.97)	21(6.58)	27(4.83)	15(2.71)	23(6.00)	26(6.79)	2(2.81)	29(5.06)	24(4.33)	187(4.94)	Path, sap
7	*Penicillium sp.*	84(20.89)	78(24.45)	91(16.30)	103(18.62)	82(21.40)	104(27.88)	20(28.16)	79(13.78)	103(18.62)	744(19.65)	Path, sap
8	*Fusarium sp.*	10(2.48)	12(3.76)	20(3.58)	20(3.62)	21(5.48)	24(6.43)	1(1.40)	12(2.09)	15(2.71)	135(3.56)	Path, sap
9	*Cladosporium sp.*	20(4.97)	5(1.56)	23(4.12)	21(3.79)	21(5.48)	10(2.68)	3(4.22)	33(5.76)	33(5.96)	169(4.46)	Path, sap
10	*Alternaria sp.*	7(1.74)	8(2.50)	5(0.89)	6(1.08)	-	-	-	-	2(0.36)	28(0.73)	Path, sap
11	*Mucor sp.*	12(2.98)	4(1.25)	4(0.72)	7(1.26)	6(1.56)	6(1.60)	-	-	-	39(1.03)	Path, sap
12	*Rhizopus sp.*	12(2.98)	9(2.82)	6(1.07)	6(1.08)	5(1.30)	3(0.80)	-	4(0.69)	4(0.72)	49(1.29)	Path, sap
13	*Stachybotrys sp.*	3(0.75)	3(0.94)	3(0.54)	4(0.72)	2(0.52)	2(0.53)	-	-	-	17(0.44)	Path, sap
14	*Scopulariopsis sp.*	3(0.75)	4(1.25)	3(0.54)	2(0.36)	2(0.52)	2(0.53)	-	-	-	16(0.42)	Path, sap
15	*Paecilomyces sp.*	8(1.99)	6(1.88)	8(1.43)	7(1.26)	3(0.78)	2(0.53)	-	4(0.69)	-	38(1.00)	Path, sap
16	*Aureobasidium sp.*	-	-	3(0.54)	6(1.08)	-	-	-	-	-	9(0.23)	Path, sap
17	*chaetomium sp.*	-	-	2(0.36)	5(0.90)	-	-	-	4(0.69)	-	11(0.29)	Sap
18	*Yeasts*	82(20.39)	52(16.30)	147(26.34)	142(25.67)	66(17.23)	57(15.28)	5(7.04)	139(24.25)	131(23.68)	821(21.69)	Path, sap
19	*Trichosporon sp.*	-	-	13(2.33)	14(2.53)	16(4.17)	10(2.68)	-	29(5.06)	18(3.25)	100(2.64)	Path, sap
20	*Geotrichum. Sp.*	26(6.46)	10(3.13)	48(8.60)	35(6.33)	24(6.26)	22(5.89)	-	61(10.64)	47(8.49)	273(7.21)	Path, sap
21	*Candida albicans*	5(1.24)	16(5.01)	16(2.86)	21(3.79)	20(5.22)	18(4.82)	-	37(6.45)	50(9.04)	183(4.83)	Path
22	*Rhodotorula sp.*	12(2.98)	9(2.82)	10(1.79)	10(1.80)	9(2.35)	11(2.95)	-	25(4.36)	18(3.25)	104(2.74)	Path
23	Total N of G&S	402(10.62)	319(8.42)	558(14.74)	553(14.6)	383(10.11)	373(9.85)	71(1.87)	573(15.1)	553(14.6)	3785(100)	-
24	Mean (CFU/ml) (Min-Max)**	44.6 (34-65)	35.4 (24-45)	62 (55-76)	61.4 (54-75)	42.5 (34-53)	41.4 (33-56)	7.8 (5-11)	63.6 (50-70)	61.4 (57-72)	64.6 (35-58)	-

Site of Sampling: I-Anoxic tank point1, II-Anoxic tank point2, III-Aeration tank point1, IV-Aeration tank point2, V- Clarifier point1, VI- Clarifier point2, VII- Effluent tank, VIII-Return Activated Sludge tank point1, IX- Return Activated Sludge tank point2.
*Path: pathogenic- sap: saprophytic
**Mean, Min and Max: The average, minimum and maximum CFU/ml of fungi from 9 sites of IFAS.
N=Number, G=Genera, S= Species

Seventy-six percent of the *Aspergillus* genus was identified at the species level. However, about 24% of this genus was not identified at the species level. The most frequent *Aspergillus* species were *A. flavus* (24.49%), *A. Terreus* (17.82%), *A. fumigatus* (16.01%), *A. niger* (12.96%), and *A. ochraceus* (4.19%), respectively. Four yeasts (*Geotrichum. Sp.,Candida albicans*, *Rhodotorula sp.* and *Trichosporon sp.*) were isolated and identified from the 81 samples. From the total percentage of yeasts (39.1%) identified, *Geotrichum sp*. (18.43%) was the most common genus and was recovered in high frequency of occurrence, followed by *Candida albicans* (12.35%), *Rhodotorula sp.*

(7.02%) and *Trichosporon sp*. (6.75%). About 55.43% of the yeasts were not identified (Table 1).

The total count of fungi in the various sites of the IFAS system showed that the Return Activated Sludge Tank with the mean value of 63.6 (CFU/ml) and the Effluent tank with the mean value of 7.8 (CFU/ml) had the highest and lowest numbers, respectively (Table 1). Even though the Return Activated Sludge tank contained the greatest amount of fungi, the Aeration tank had the highest diversity of fungal type. Fig. 2 shows the microscopic pictures of some of the most important and most potentially pathogenic and allergenic fungi.

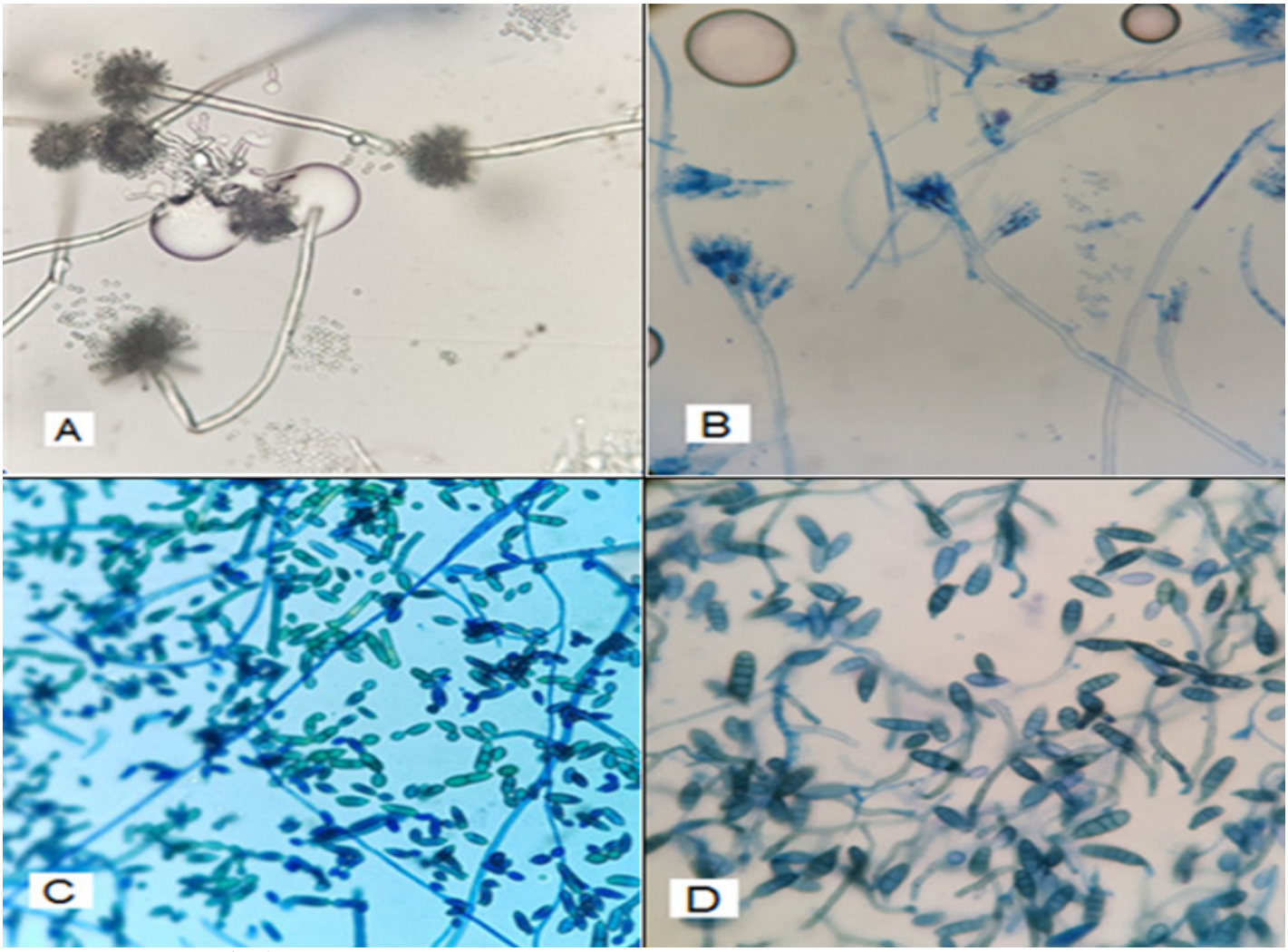

Fig. 2: A: *Aspergillus sp*., B: *Penicillium sp*., C: *Cladosporium sp*., D: *Alternaria sp*.

Wastewaters contain microorganisms such as fungi that could be harmful to humans and animals especially to the people who work in wastewater treatment plants. Fungi can adapt and grow fast due to their ability to use all forms of available oxygen and also their tolerance to environments with highly oxidizing conditions and different pHs (Coulibaly *et al.*, 2003; More *et al.*, 2010). These microorganisms contribute to biochemical reactions and transformations that take place in the system (Andersson *et al.*, 2005). The fungi recorded from the wastewater of the IFAS system in this study were mostly of the genus *Aspergillus sp.*, *Yeas*ts, *Penicillium sp.*, *Cladosporium sp*., *Mucor sp.*, *Rhizopus sp.*, *Geotrichum sp.*, *Aurobasidium sp*., *Scopulariopsis sp*., *Fusarium sp*., *Chaetomium sp.*, *Alternaria sp*., *Rhodotorula sp.*, *Trichosporon sp*., and *Candida albicans*. These results are similar to those of previous reports (Cooke, 1970; Bux and Kasan, 1994; Fakhru'l-Razi *et al.*, 2002; Jamal *et al.*, 2005). As was noted above, the dominant genus in all the stages of the IFAS system was *Aspergillus*. This genus causes *Aspergillosis* infections (pneumonia) and allergenic reactions by producing secondary metabolites such as volatile organic compounds (VOCs) and aflatoxins, ocratoxins, and ochratoxin (Nurmatov *et al.*, 2015). Several studies have shown that wastewaters are a crucial source for the growth of this genus which spreads its spores to the air. The most important species of yeasts isolated from this system was *Candida albicans* which is a normal flora while it is in the alimentary system of humans and mammalian animals but can cause pathogenic properties in immunocompromised individuals. Hence, the presence of potentially pathogenic fungi of the species *Candida albicans* in wastewaters indicates its direct source from human or animal bodies (De Toni and Reilly, 2011). Moreover, this species is responsible for a wide range of infections from superficial to systemic candidiasis causing a high mortality rate (Wang, 2015). In 2000, Doggett found that *Candida sp.* (yeast) has the capability of biofilm formation in water pipes (Doggett, 2000). For this reason, wastewater treatment plants play an important role in reducing these genera as potentially pathogenic fungi. Our quantitative analysis has also shown that the number of the species *Candida albicans* was significantly reduced in the treated wastewater in the Effluent tank.

Other fungal taxa recovered from this research trigger a range of allergic fungal rhinitis, hypersensitivity pneumonitis, and skin irritation in the general population. Furthermore, some of them have the ability to infect humans especially immunocompromised individuals in different ways such as inhalation of waterborne fungi, contact, drinking, and showering. They lead to severe infections such as meningitides and rhinocerebral mucormycosis with a high risk of death within 48 hours (Kurup et al., 2006; Spellberg *et al.*, 2012; Baumgardner, 2016; Babič et al., 2017). Despite the pathogenic characteristics of fungi discussed in this research (filamentous fungi and yeasts) as heterotrophic organisms, fungi are also capable of organic compound degradation and have purifying properties.

It can be noted that the total number of fungi observed in the Return Activated Sludge tank was higher than those of other sampling points in the IFAS system. This finding is probably due to an anoxic condition which took place in the Return Activated Sludge tank increasing the number of yeasts. This condition also took place in the pre-anoxic tank of the IFAS system in which the yeast count was relatively more than that of other fungi except *Penicillium sp.* which highly adapts itself to such environments. However, the Aeration tank had the highest diversity of fungi compared with the other stages of the IFAS system. The major reason for this diversity is the possibility that most of the fungi are aerobic microorganisms. Moreover, the total fungi count in the Effluent tank has decreased dramatically to 71 (CFU/ml), illustrating the successful performance of the IFAS system.

Conclusion

To the best of the authors' knowledge, this is the first study conducted on the IFAS system and the profiles of pathogenic fungi isolates in Iran. To summarize this study conducted on the IFAS system, it should be concluded that these results are important in understanding the role of the IFAS purification process in decreasing pathogenic fungi and the risks which they pose to human health. Therefore, our findings suggest that upgrading conventional wastewater treatment plants with the IFAS technology can be very beneficial to public and environmental health.

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