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Research Article

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Fungi as a purifying species in an Integrated Fixed Film Activated Sludge (IFAS) Bioreactor as a Biological Nitrogen Reduction System

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ABSTRACT

Background: Fungi are free-living heterotrophic microorganisms, which absorb nutrients for growth and reproduction. The integrated fixed film activated sludge (IFAS) system is a beneficial technology for developing conventional activated sludge plants, which consisted of a wide range of microorganisms. Methods: Twelve wastewater samples were collected from four IFAS sites during 3 days. The samples were prepared for count and identification. The genera of fungi were identified by macroscopic and microscopic features. The physical-chemical analyses were done according to the standard method protocols. To prevent and reduce the growth of bacteria, 0.05 g/l of chloramphenicol was added to the synthetic wastewater. Results: The highest count of fungi with a mean concentration was found in the Aeration tank (149 CFU/ml), whereas the lowest count of fungi was found in the Effluent tank (3.3 CFU/ml). The highest diversity of genera (17) was isolated in the Aeration tank, while the lowest diversity of genera (3) was isolated from the Effluent tank. The results of the physical-chemical tests showed that the average removals of COD, TN, and TP in the IFAS fungal system were 91.76%, 91.43%, and 80.23%, respectively. Conclusions: This study on fungi and their ability in reducing nutrients called attention to the role of the IFAS system and its potential for developing a new biological nitrogen removal technology based on fungal treatment.

Key words: *IFAS, Fungi, Wastewater treatment, Nitrogen Reduction.*

INTRODUCTION

Nitrogen is a primary concern and one of the most important elements in modern wastewater treatment. It causes the growth of harmful microorganisms and aquatic plants in rivers and water reservoirs, which is an important problem with respect to public and environmental health in the world [1]. In order to protect the quality of surface waters, limiting the discharge of this nutrient has been recognized as an effective method. On the other hand, new environmental regulations with regard to discharging the effluents of wastewater treatment plants into receiving water bodies have become very strict [2]. Therefore, developing conventional wastewater treatment plants with better technologies is necessary to improve the system performance including nitrogen removal [2]. The IFAS technology with an attached biofilm that grows on the fixed media increases the Solid Retention Time (SRT) without overloading solids to the Clarifier tank or expanding the Aeration tank. Thus, it seems to be an economical choice for biological nitrogen removal and upgrading the treatment capacity of Conventional Activated Sludge (CAS) Plants [3-5]. The biological treatment of nitrogen in wastewater involves the removal of this element with

microbial communities such as fungi and bacteria [1]. Fungi penetrate into wastewater treatment plants through sedimentation from atmospheric air, polluted waters, and diseased animals and humans [6, 7]. Previous studies have shown that fungi are heterotrophic organisms and play an important role in wastewater treatment due to their capability in degrading various organic compounds. They also act as bioindicators in microbiological analysis and as water purity indicators [8-10]. These microorganisms facilitate biochemical reactions and transformations that take place in the system as part of the treatment process [11]. Recently, attention is being paid more to the role of fungi than bacteria in biological nitrogen removal. In addition, due to their higher resistance to a variety of inhibitory chemicals, fungi have a promising potential for nitrogen removal [2] especially in wastewater treatment plants where the IFAS technology will be used. In this research, we focus on the performance of the IFAS bioreactor in biological nitrogen removal and the identification of the filamentous fungi and yeasts, which grow in different stages of the IFAS system and participate in the reduction of nutrients from the synthetic wastewater. Moreover, chloramphenicol (a broad-spectrum antibiotic) was added to the synthetic wastewater to prevent bacterial growth.

MATERIALS AND METHODS

Laboratory-Scale IFAS

This research was carried out in a laboratory-scale IFAS system (Figure 1). One liter of activated sludge containing fungi from the Return Activated Sludge (RAS) line of a wastewater treatment plant was injected into the IFAS bioreactor under non-aseptic conditions. The inoculated fungi in the bioreactor were fed with synthetic wastewater. In order to inhibit bacterial growth, 0.05 g/l of chloramphenicol was added to the synthetic wastewater (Table 1). The sampling sites in the Integrated Fixed Film Activated Sludge (IFAS) system were the Anoxic tank, Aeration tank, Clarifier tank, and Effluent tank (Figure 1).

Isolation and Identification of Fungi

During the experiment, four fungal samples were gathered daily from the IFAS sampling sites. To isolate and count the number of fungal colonies (CFU/ml), one ml of mixed wastewater samples of each site was aseptically pipetted into 4 Sabouraud Dextrose Agar (SDA) media (Merck, Germany) immediately after collection. The composition of the media was dextrose (40 g/L), casein peptone (10 g/L), agar (15 g/L), chloramphenicol (0.05 mg/L), and distilled water (1000 mL). Then all the Petri dishes were incubated at room temperature for 48-72 h and subjected to identification processes. During the research days, the average number of colonies was calculated. Fungal isolates were identified at the genus and/or species levels. The macroscopic features of the fungi were diagnosed based on the culture (diameter, color, aerial hyphae, and colony texture), while their microscopic specifications were stained with lactophenol aniline blue stain and identified by conidiophores characteristics, and the shape, size, and color of the conidia. [12, 13]. The differentiating media CHROMagar Candida (Paris, France) and Urease (Merck, Germany) were used for identification. Slide cultures were prepared for filamentous fungi and yeasts. Finally, the fungi were diagnosed according to mycological atlases [13-19].

Physical-Chemical Analyses

To perform physical-chemical tests, the Chemical Oxygen Demand (COD) was measured according to method 5220B in Standard Methods for Water and Wastewater Examination (APHA, 2005). The temperature was measured and also a pH meter (inoLab – Series WTW pH 720, Made in Germany) was used to measure pH. Dissolved Oxygen (DO) was measured by a DO meter (Oxi 3210 SET 1, WTW, Made in Germany). Total Nitrogen (TN) and Total Phosphorous (TP) were measured by a HACH DR5000 spectrophotometer (Merck, Germany), using reagent powder pillows.

RESULTS AND DISCUSSION

The experimental results of nitrogen reduction by fungi in the IFAS bioreactor, as well as the identified filamentous fungi and yeasts considering 0.05 g/l chloramphenicol applied to the synthetic wastewater, are presented in Tables 2 and 3 and Figure 2.

The pH and dissolved oxygen concentrations in Table 2 demonstrate that fungi can tolerate different conditions with different pH values. They have the ability to use all forms of oxygen supply available adapting themselves to new environments. Therefore, fungi can grow fast and utilize the essential nutrients when chloramphenicol (as a broad-spectrum antibiotic) is used to prevent the growth of bacteria [10, 20]. Thus, it could be concluded that

under these conditions the performance of the IFAS bioreactor in the removal of TN, TP, and COD is the result of fungal activity. In 2010, Hai and Yamamoto reported a membrane bioreactor with a mixed microbial community that was dominated by fungi [21]. These organisms, which participate in the degradation of organic compounds in wastewaters use nitrogen and phosphorous sources to produce protein, cell walls, and nucleic acids [2, 10, 11, 20].

Table 2 shows seventeen genera of yeasts and filamentous fungi, which were recovered from 12 wastewater samples collected daily from 4 sites of the (IFAS) bioreactor. As can be observed in Table 2, the filamentous fungi, which were identified in the IFAS system in this study were mostly of the genus *Aspergillus sp.*, *Penicillium sp.*, *Fusarium sp*., *Cladosporium sp*., *Rhizopus sp.*, *Mucor sp.*, *Alternaria sp.*, *Paecilomyces sp., Stachybotrys sp., Scopulariopsis sp*., *Aurobasidium sp*., and *chaetomium sp.*; and the yeasts included *Geotrichum sp.*, *Candida albicans*., *Rhodotorula sp.*, and *Trichosporon sp*. Thus, our findings are comparable to those reported by previous studies [22-29]. Figure 2 presents the microscopic pictures of some of the most frequent and important fungi identified in the IFAS bioreactor.

From a total of 1045 fungal colonies recovered from different stages of the IFAS system (Table 2), 716 isolates were filamentous fungi (68.52%) of which 69 isolations belonged to the dematiaceous group and 647 isolations belonged to the hyaline hyphomycetes group. Moreover, from a total of 1045 fungal colonies, 329 isolates belonged to yeasts (31.48%).

About 439 isolates belonging to fourteen genera were detected in the Anoxic tank under anoxic conditions, and 447 isolates belonging to seventeen genera were identified from the Aeration tank under aerobic conditions. The total count of fungi in various sites of the IFAS system showed that the Aeration tank with the mean value of 149 (CFU /ml) and the effluent tank with the mean value of 3.3 (CFU /ml) had the highest and lowest numbers, respectively. The Aeration tank also had the highest diversity of fungal type (Table 2). These results indicate that different aerobic to anoxic conditions are experienced with the existence of biofilm on the media in the Aerobic tank of the IFAS bioreactor. This provides a suitable environment for the growth and reproduction of a variety of fungi that can adapt to these conditions. *Penicillium sp.* and *Fusarium sp.* are some examples of facultative fungi that can grow under these conditions [30].

The results also showed that from the total percentage of filamentous fungi (68.52%), the highest frequency belonged to the genus *Aspergillus* (42.17%) followed by *Penicillium sp.* (31.14%), *Fusarium sp.* (7.26%), *Cladosporium sp.* (5.58%), *Rhizopus sp.* (3.35%), *Mucor sp.* (2.8%), *Alternaria sp.* (2.51%), *Paecilomyces sp.* (2.09%), *Stachybotrys sp.* and *Scopulariopsis sp.* (1.25%), and *Chaetomium sp*. and *Aureobasidium sp.* (0.28%). As was noted above, *Stachybotrys sp.*, *Scopulariopsis sp.*, *Aureobasidium sp.,* and *Chaetomium sp.* were isolated in a rare frequency from less than one percent of all the samples. *Penicillium sp.*, *Aspergillus sp*., and *Cladosporium sp.* were present in all the sampling sites. However, *Aurobasidium sp*., *Chaetomium sp*., and *Trichosporon sp.* were only recovered from the Aeration tank of the IFAS system (Table 2). In addition, about 65.89% of the genus *Aspergillus* was identified at the species level of which the most frequent were *A. flavus* (23.84%), *A. Terreus* (14.9%), *A. fumigatus* (12.91%), *A. niger* (11.92%), and *A. ochraceus* (5.29%), respectively. According to Table 2, from the seventeen genera detected, *Aspergillus sp.*, *Penicillium sp.*, and *Fusarium sp.* had the highest frequency and could be the most effective genera in the biological nitrogen removal from the synthetic wastewater in the IFAS system. This agrees with the findings of Greben *et al*. (2007) who also reported that two of the six hyphomycetes isolates used for biological nitrate removal from the synthetic wastewater were the genera *Penicillium* and *Fusarium*. These fungi were able to remove a significant portion of nitrate from the wastewater [31, 32]. In another research, Akhtar and Ghaffar (1986) showed that from the nine species of fungi, *Aspergillus flavus* was the most effective species in the removal of ammonia nitrogen (with 92% reduction at the pH of 8.0 and the temperature of 20 ºC) [33]. Different studies have also shown that various fungi have the ability to oxidize the reduced forms of nitrogen [34, 35].

From the total percentage of yeasts (31.48%) about 59.59% were not identified and from the 40.41% of the identified isolates, *Geotrichum sp*. (15.5%) was the most frequent genus, followed by *Candida albicans* (12.15%), *Rhodotorula sp.* (9.42%), and *Trichosporon sp*. (3.34%). In this case, the results in Table 2 show that from the total number of 439 fungi isolates in the Anoxic tank, 197 yeast isolates, and from the total number of 447 fungi isolates in the Aerobic tank, 138 yeast isolates, have contributed to the removal of nitrogen and COD. To confirm this finding, we can refer to Thanh and Simard's studies (1971, 1973) in which all the tests were carried out in a shake flask at 26-28 ºC for 3 days. Their results showed that the yeasts of the genus *Rhodotorula sp.* (with 85% and 67% removal of NH3-N and COD, respectively), and *Candida sp.* (with 91% and 72% removal of NH3-N and COD, respectively) had high efficiency in the removal of nitrogen and COD [36].

As can be calculated from Table 3, the average percentages of TN, TP, and COD removal reached 91.43%, 80.23%, and 91.76%, respectively. These results are probably due to the powerful extracellular enzyme system of the fungi identified in the IFAS bioreactor. These fungi can enhance the organic matter degradation and utilize the nitrogen and phosphorus compounds for growth and reproduction [10], resulting in the high biological treatment efficiency of the IFAS system under fungal treatment conditions.

As is clearly illustrated in Table 2, the mean fungal counts in the wastewater of different sites of the IFAS bioreactor were significantly decreased from 144.3 to 3.3 (CFU/ml) from the first to the final steps. In addition, the number and genera of fungi decreased, which indicates the reduction of organic compounds and nutrients in the final steps of the treatment. This again demonstrates the high efficiency of the IFAS system. Table 4 shows the examples of the fungi used to treat different wastewaters, the optimal culture conditions, the effects of fungal treatments reported in previous studies, and the results of the current study.

Considering the existence of the attached biofilms in the IFAS fungal bioreactor, the possibility to obtain a biochemical pathway to accomplish both nitrification and denitrification processes simultaneously was observed. Hence, the IFAS fungal bioreactor may have significant advantages over other bacteria denitrifying systems including higher rates of nitrogen removal, higher resistance to toxics, and lower oxygen and lower carbon source requirements.

CONCLUSION

To sum up this study, the experimental results showed the positive role of the IFAS system in nitrogen removal efficiency. Using the fungal IFAS bioreactor, which to the best of the authors' knowledge has not been used before in our country, could be regarded as an important development in the biological treatment of wastewaters. This research also provided the foundation for further studies on the specific species of fungi and their effects on biological nitrogen removal in the treatment of wastewaters.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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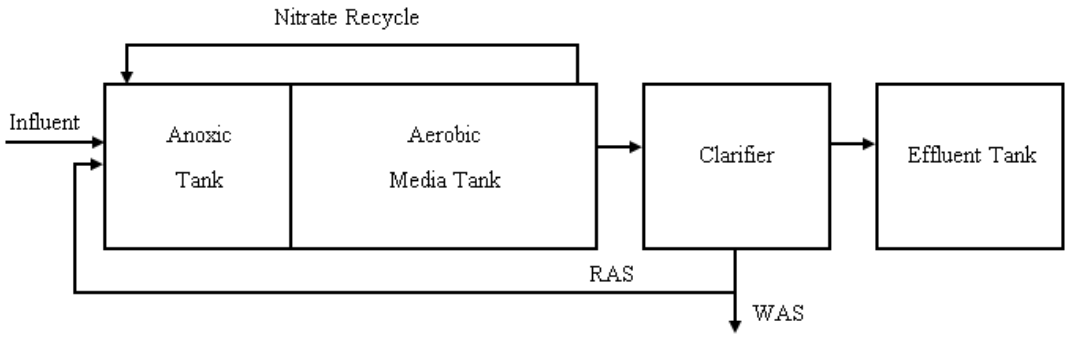

Figure 1. Schematic diagram of the Integrated Fixed Film Activated Sludge (IFAS) System used in this study.

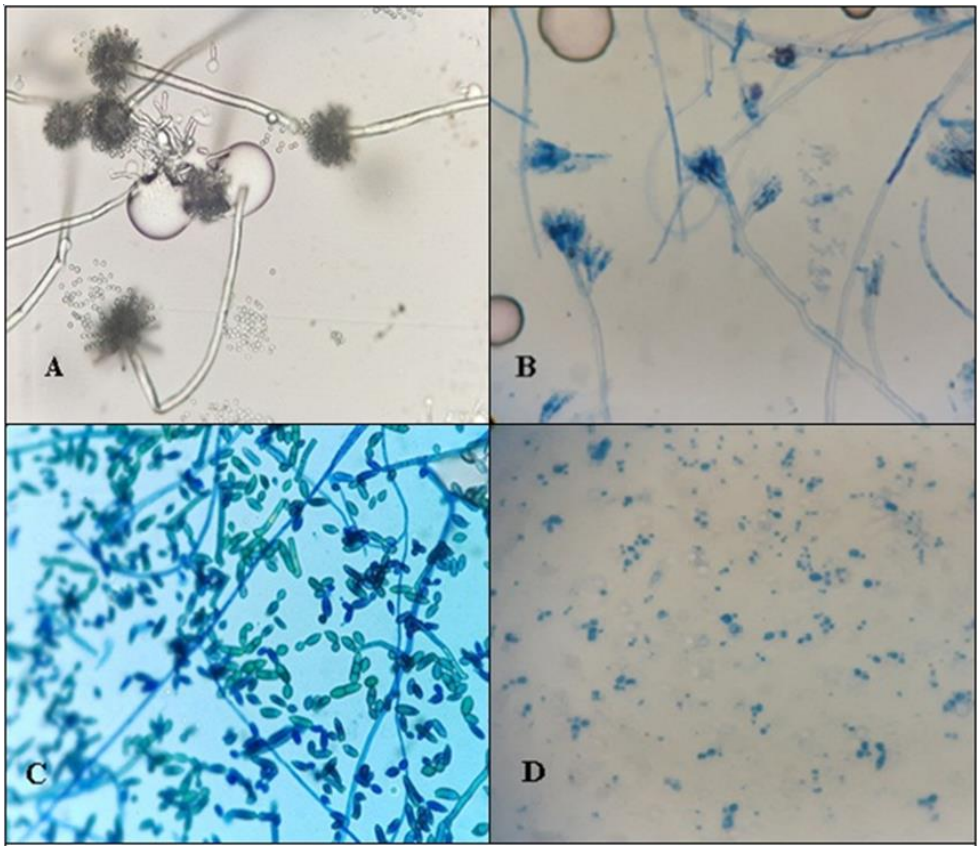

Figure 2. A: *Aspergillus sp*., B: *Penicillium sp*., C: *Cladosporium sp*., D: Yeast.

Table 1. Composition of synthetic wastewater used in this study.

Chemical Constituent	Concentration
Dry milk (g/l)	1.2
Chloramphenicol (g/l)	0.05
$MgSO_4.7H_2O$ (mg/l)	50 mg/l
$MnSO_4.7H_2O$ (mg/l)	5
$FeSO_4.7H_2O$ (mg/l)	2.2
KH_2PO_4 (mg/l)	68
K_2HPO_4 (mg/l)	185
Na_2HPO_4 (mg/l)	169.6
NH_4CL (mg/l)	138.3
$CaCL_2$ (mg/l)	3.8
Resultant COD (mg/l)	330±20

Table 2. Distribution of the number (CFU/ml) and percentage frequency (%F) of various fungal genera and species recovered from different sites (I to IV) of the IFAS system.

No.	Genera of fungi	Number of Fungi				Total, N. (% F)	Remarks*
		I	II	III	IV		
1	*Aspergillus sp.*	110	128	61	3	302 (28.9)	Pur, sap
2	*Penicillium sp.*	73	99	47	4	223 (21.34)	Pur, sap
3	*Fusarium sp.*	16	24	12	-	52 (4.97)	Pur, sap
4	*Cladosporium sp.*	11	15	11	3	40 (3.83)	Pur, sap
5	*Alternaria sp.*	11	6	1	-	18 (1.72)	Pur, sap
6	*Mucor sp.*	8	9	3	-	20 (1.91)	Pur, sap
7	*Rhizopus sp.*	11	11	2	-	24 (2.29)	Pur, sap
8	*Stachybotrys sp.*	5	3	1	-	9 (0.86)	Pur, sap
9	*Scopulariopsis sp.*	5	4	-	-	9 (0.86)	Pur, sap
10	*Paecilomyces sp.*	6	6	3	-	15(1.43)	Pur, sap
11	*Aureobasidium sp.*	-	2	-	-	2(0.19)	Pur, sap
12	*chaetomium sp.*	-	2	-	-	2(0.19)	Pur, sap
13	*Yeasts*	124	72	-	-	196(18.76)	Pur, sap
14	*Trichosporon sp.*	-	11	-	-	11(1.05)	Pur, sap
15	*Geotrichum. sp.*	17	28	6	-	51(4.88)	Pur, sap
16	*Candida sp.*	30	9	1	-	40(3.83)	Ind, pur
17	*Rhodotorula sp.*	12	18	1	-	31(2.96)	Ind, pur
18	Total N of G	439	447	149	10	1045(100)	-
19	Mean (CFU/ml) (Min-Max)**	146.3 (5-124)	149 (2-128)	49.7(1-61)	3.3(3-4)	-	-

Sites of Sampling: I-Anoxic tank , II- Aeration tank , III- Clarifier, IV- Effluent tank
N: Number, G: Genera, S: Species
* Pur: purifying, Sap: Saprophytic, Ind: Indicatory
** Mean, Min and Max: The average, minimum, and maximum (CFU/ml) of fungi from 4 sites of the IFAS system.

Table 3. Selected Physical and Chemical data recorded at each stage in the IFAS System during 3 days of experiment.

P	DO (mg/l)			pH			COD (mg/l)			TN (mg/l)			TP (mg/l)			Water temperature (ºC)		
	I	II	II	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
1	-	-	-	6.54	6.38	6.67	307	289	275	56.50	58.40	53.24	8.54	8.33	8.08	25.4	25.7	25.5
2	3	2	3	7.98	7.82	7.96	183	192	176	20.35	21.46	20.08	6.54	5.33	6.08	25.8	25.9	25.8
3	0.5	0.3	0.2	7.43	7.49	7.35	28	32	30	6.8	8.2	9.4	2.1	2.8	2.4	25.6	25.5	25.3
4	-	-	-	7.36	7.46	7.52	22	25	24	4.41	5.32	4.56	1.75	1.68	1.25	25.2	25.1	25.2

P: Pattern, 1– Anoxic tank, 2– Aeration tank, 3– Clarifier, 4–Effluent tank, I– 28.05.2018, II– 29.05.2018, III– 30.05.2018.

Table 4. Examples of fungi used to treat different wastewaters, Optimal culture condition and the effect of fungal treatment reported.

Wastewaters	**Fungi**	**Treatment**		**References**
		Reactor and medium handling	Parameters removal	
Domestic sewage	*Penicillium sp., Fusarium sp., Cladosporium sp.,*	Shake-flask	COD (72.3%) Phosphates (97.5%)	Thanh and Simard (1973)

	Scopulariopsis s., Mucor sp., Geotrichum sp., Paecilomyces sp.		N-total (86.8%)	
Domestic sewage	*Aspergillus niger*	Stirred tanks reactor in series	COD (72%) N-total (65.4%)	Coulibaly (2002)
Starch processing effluent	*Aspergillus sp., Rhizopus sp. Trichosporon sp., Geotrichum sp., A. terreus;* Rhizopus sp.	Shake-flask, air lift bioreactor (45l); addition of nutriment (NH4)2SO4; Urea; NH4NO3; NaNO3; K2HPO4; KH2PO4)	TOC (44-88%) SS (95%) COD (97.8%)	Jin et al. (1999abc; 2001)
Activated domestic sludge	*Aspergillus sp.* *Penicillium sp.*	Shake flask (2 days, 33–35ºC, 150 rpm): with wheat flour as a co-substrate.	Turbidity (98.95%) COD (94.4%, 84.4%) Decrease in SRF (93.2%)	Mannan et al. (2005)
Domestic Wastewater	*Mucor hiemalis*	Shake flask: (6 days, 32ºC, and 150 rpm) sludge with and without wheat flour (WF) as co-substrate (2%)	Turbidity (99%) TSS (98%) COD (87%)	Fakhru'l-Razi and Molla(2007)
Synthetic Wastewater	Table 2.	IFAS bioreactor; addition of nutriments: Table 1.	COD (91.76%) TN (91.43%) TP (80.23%)	This study