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Molecular Identification of Fungal Strains Using 16s rRNA Sequencing and A Comparative Assessment of their Efficiency on Reduction of Biological Oxygen Demand in Textile Industry Effluent

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Abstract: *Textile industry effluents contain considerable volumes of dyes, chemical oxygen demand (COD), biological oxygen demand (BOD), total suspended solids (TSS), organic and inorganic chemicals, all of which, if not managed appropriately, can cause threats to the environment.. Many treatment technologies are already in use, but due to their drawbacks standard biological treatment methods are acceptable. The objective of the present study was to isolate and conduct molecular identification of fungal strains and assess the reduction in biological oxygen demand (BOD). Using the pour plate technique, two microbial strains were extracted from textile industry contaminated soil and textile industry effluent. Based on their molecular analyses, the isolates were identified as Aspergillus flavus and Aspergillus aculeatus, and they were deposited at the National Center for Biotechnology Information (NCBI) under accession numbers (MZ544387) and (MZ569631), respectively. They were tested to see if they could reduce high amounts of biological oxygen demand (BOD) from textile industry wastewater. According to the findings, Aspergillus flavus and Aspergillus aculeatus have a good ability to reduce BOD levels from textile industry effluents, with percentages ranging from 82.64% to 95.10% and 84.93% to 96.77%, respectively. The isolated fungi have been shown to be promising candidates and can used in reduction of BOD concentration in textile industry effluent.*

Keywords: Textile industry effluent, fungi, *Aspergillus flavus*, *Aspergillus aculeatus*, BOD, NCBI, sequencing

Introduction

Industrial wastewater discharge is one of the most significant sources of pollution in the environment, particularly in developing countries. When industrial effluents contaminate our environment on a regular basis, they constitute a major threat to human existence. **(Ademakinwa & Agboola, 2015).** Some of the major sectors that create and release brightly coloured wastewater include paper and pulp mills, molasses-based alcohol distilleries, tanneries, dye-making facilities, and textiles **(Santhosh *et al.*, 2020)**. Each of these industrial effluents create some specific problems besides producing aesthetically unacceptable intense coloring of soil and water bodies. These obstruct light from reaching the lowest depths of the aquatic system, causing photosynthesis to stop and anaerobic conditions to develop, resulting in the death of aquatic life and foul-smelling poisoned waters. **(Plácido *et al.*, 2016 and Santhosh *et al.*, 2020).**

Textile industry is amongst the major contributors to the environmental pollution but it is very important for nation's economy because clothes are the basic needs of human beings. The textile industry is considered one of the major worldwide industries which produce vast amount of effluent **(Mondal *et al.*, 2017).** In almost every developing nation, textile manufacturing is among the first industries to be established **(Henagamage, 2019).** Within the industry, the majority of energy, water, and chemicals consumed are for wet processing. Most wet processing involves treatment with chemical baths, which often require washing, rinsing, and drying steps between key treatment steps. Consequently, wastewater is generated, having a very diverse range of contaminants that must be treated prior to disposal **(Kousar et *al.*, 2021).** The textile manufacturing has been recognized as one of the world's largest polluters since it needs a substantial amount of two components:

1. Chemicals: the textile industry utilizes up to 2,000 different chemicals, and
2. Water: it is used at each step of the process. The water becomes contaminated with chemical additives and is subsequently discharged as wastewater, which pollutes the environment due to the effluent's heat, increased pH, and saturation with dyes, de-foamers, bleaches, detergents, optical brighteners, equalizers, and other chemicals used during the process.

While conventional chemical and physical degradation techniques were used effectively for a long time, alternatively, traditional biological approaches (Bioremediation) are increasingly being used to remediate effluents and wastewater containing colours and harmful compounds **(Rani *et al.*, 2014).** Bioremediation is getting prominence because to its low cost, effectiveness, and environmental stewardship, and the metabolites formed during biodegradation are typically nontoxic or comparatively less hazardous in nature. **(Dewi *et al.*, 2018 and Alaguprathana & Poonkothai, 2017).** In the polluted water system, suitable microbes undergo numerous physical and chemical processes, and the pollutants are reduced and eliminated during the microbial metabolism. Microbial bioremediation has recently emerged as a promising alternative to existing chemical treatments. Bacteria, algae, and fungi are among the microorganisms used in biological degradation approaches. Microbial treatment systems have advantage of being simple in design and low in cost **(Kousar *et al.*, 2020 and Sghaier *et al.*, 2019).**

Mycoremediation is a kind of bioremediation that involves the use of fungi to decontaminate a site. The primary role of the ecosystem is decomposition, which is performed by the mycelium of fungi. Fungi are distinctive among microorganisms in their ability to create a wide range of extracellular proteins, organic acids, and other metabolites, as well as their ability to adapt to extreme environmental conditions (**Jwieli *et al.*, 2021 and El Enshasy *et al.*, 2017).** The extracellular nature of fungal enzymes is also beneficial in tolerating high toxicant concentrations. The importance of fungi in the environment is to decompose and transform both organic and inorganic substrates **(Medfu Tarekegn *et al.*, 2020)**. Fungi are involved in the biodegradation of toxic substances or compounds into harmless, tolerable, or beneficial products. Fungal cell walls and their components play an important role in biosorption of hazardous chemicals during wastewater treatment, in addition to extracellular enzyme synthesis. Fungi have been found to be effective organisms for treating textile effluents. *Aspergillus species* are a ubiquitous group of filamentous fungi **(Selvarajan *et al.*, 2019 and Barrech *et al.*, 2018).**

In the present study, an attempt has been made to bring out the effectiveness of fungi for reducing Biological Oxygen Demand in textile industry effluent and the efficiency of bioremediation was validated with the two fungal isolates.

Materials and Methods

Effluent collection

Effluent for the present study was collected from a Textile Mill located in Banglore, Karnataka, India. A sterile container was used to take a sample from the effluent treatment plant's inlet. Samples were collected in dry cans that were properly labeled. The wastewater was then taken to a laboratory and preserved at 4^0 Celsius for further research.

Isolation of fungi from effluent sample and soil sample

Fungi from effluent was determined by serial dilution of 10^{-1} to 10^{-10} and plating in Potato Dextrose Agar (PDA) media. Further dilutions were done after the soil sample (1g) was suspended in 10ml of sterile distilled water. To isolate fungal strains, 10^{-1} to 10^{-10} soil dilutions were prepared. Subsequently, for isolation, 10^{-8} and 10^{-9} were used to avoid over crowding of fungal colonies in soil as well as for effluent. In petriplates containing 30ppm streptomycin and 20ml of sterile potato dextrose agar (PDA) medium, 100ul of soil suspension of each

concentration was introduced. The culture plates were kept in the laboratory up to 7 days. To obtain pure cultures, each developing colony was sub-cultured.

Preparation of inoculum

All fungal strains were initially grown at 28- 30^0C on potato dextrose agar (PDA) slants. After sporulation, 10 mL of sterile distilled water was added to each strain's culture slant and vortexed. Using sterile distilled water, each spore suspension was adjusted to a final concentration of 10^8spores/ml. Homogenized spore suspension of each fungal strain (1ml) was directly used for inoculation. i.e. for preparation of mycelia biomass; 1ml spore suspension (10^8 spores/ml) injected in 100ml potato dextrose broth (PDB) and incubated for 4 days on a rotary shaker (150 rpm/min) at 28-30^0C **(Mazaheri Tehrani *et al.*, 2014).**

Identification of the fungal isolate

The methodology of **Aneja, 2018** was used to identify isolated fungal colonies based on colony features on PDA (colony morphology, colour, appearance) and microscopic characteristics (septation of mycelium, shape and texture of reproductive structure i.e. conidia). The Basic Local Alignment Search Tool (BLAST) in GenBank was used to verify ITS sequence fragment identity against the National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/) database and MEGA version 5. The nucleotide collection is commonly used in BLAST searches (BLASTn). Phylogenetic trees were generated.

Experimental setup

Aspergillus flavus and *Aspergillus aculeatus* were tested for their biodegradation ability under laboratory condition. The effluent sample was diluted to various dilutions for the treatment: 25%, 50%, and 75%. Dilution was used to examine the organism's degrading capability at various effluent dilutions. The organism was inoculated into each effluent dilution and treatment was conducted in laboratory for 5 days with 150rpm/ minute in rotary shaker at 30^0 C.

Analysis of BOD

The standard technique of **APHA, 2017** was used to determine the BOD of wastewater. The method consists of filling an air tight bottle with sample and incubating at 20^0C for 5 days. Before and after the incubation, the dissolved oxygen (DO) was measured. The difference in DO was calculated, and the BOD was estimated as well.

Result and Discussion

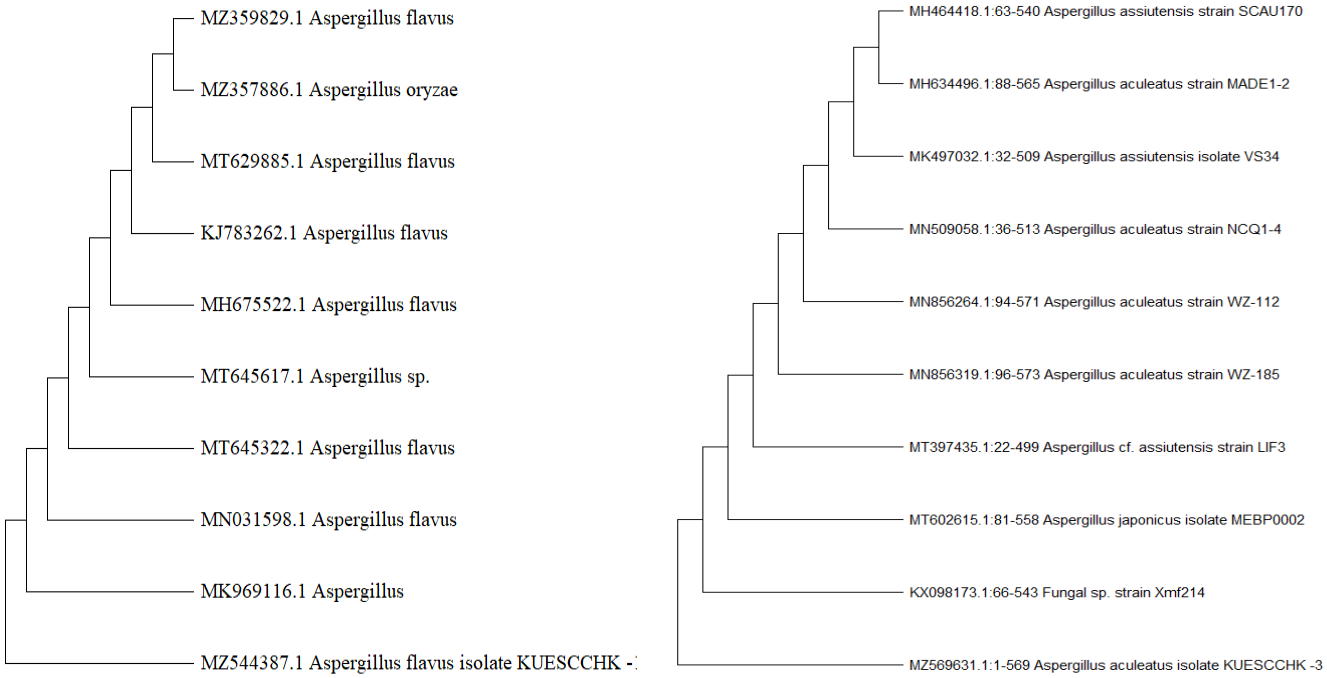
Two fungal strains were identified from textile industry wastewater polluted soil and effluent for the present study. The internal transcribed spacer (ITS) regions are well maintained in most species, with intraspecific resemblances greater than 100%, yet there is diversity between species, making it appropriate for classification. The initial PCR primer sets used to duplicate the fungal ITS sections are ITS1 (fungal specific primer) and ITS4 (fungal general primer). The ITS rRNA analysis using ITS1 and ITS4 primers confirmed the organisms to be 100% identical. When the 18S partial gene, ITS1 region, ribosomal 5.8S gene, ITS4 region, and ribosomal 28S partial gene sequences were examined, percent identities of 98-100 percent were obtained. Figure 1 shows the phylogenic tree of two isolated fungus strains. Based on the genetic and phylogenetic analysis, the new strains were identified as *Aspergillus flavus* and *Aspergillus aculeatus*. Two of these fungal isolates had 100% similarity in their 16s rRNA sequences, which were deposited in GenBank (Table 1).

1. The NCBI database BLASTn result

Accession number	Description	Maximum Identity
MZ544387	*Aspergillus flavus*	100%
MZ569631	*Aspergillus aculeatus*	100%

ISSN 2515-8260 Volume 09, Issue 07, 2022

1. Figure : Phylogenic tree of *Aspergillus flavus and Aspergillus aculeatus*



Based on colonial morphology, fungal mycelia appearance and colour on culture plates, and microscopic features viz. mycelia septation, size, shape, diameter, texture and septation of reproductive structure conidia were observed (Table 1 and Figure 1). Microscopic examination was done for the fungal strains at 40X magnification and macroscopic observation was also done (Table 2, Figure 2- I and II).

Table 2. Macroscopic and microscopic characteristics of fungal strains

Isolates	**Macroscopy**	**Microscopy**	
		Nature of hyphae	**Conidia shape**
MZ544387 (*Aspergillus flavus)*	The upper surface of colonies was olive green with white edge, granular surface and white coloration on the reverse side	Non-septate	Rough, irregular
MZ569631 (*Aspergillus aculeatus)*	The colonies were widely spread, black, spongy surface densely packed and brown on reverse side	Non-septate	Ellipsoidal

2. Figure: Macroscopy images of isolated fungal strains. The photos in the figure are the original collection by the author

I. *Aspergillus flavus (A- Surface, B-Reverse & C-Microscopy image)*

A **B** **C**

II. *Aspergillus aculeatus (A-Surface, B-Reverse & C-Microscopy image)*

A **B** **C**

Biological Oxygen Demand

The biological oxygen demand (BOD) is one of the most significant and extensively used parameter for defining organic pollution in water and waste water. It is calculated by estimating how much oxygen aerobic microorganisms require to degrade organic materials in wastewater. One of the most extensively used measures for determining water quality is the Biochemical Oxygen Demand (BOD). It gives data on the readily biodegradable portion of the organic load in water **(Yu *et al.*, 2019 and Prambudy *et al.*, 2019).**

The purpose of the study was to see how efficient specific fungal strains are at reducing concentration of BOD in textile industry effluent. The result revealed that *Aspergillus aculeatus* was more efficient than *Aspergillus flavus* at reduction of BOD in textile industry effluent. After treatment the BOD showed a drastic reduction in concentration. *Aspergillus aculeatus* reduced BOD concentration by 84.93%, 88.36%, 92.99, and 96.77% in 25%, 50%, 75% dilutions and raw effluent respectively. *Aspergillus flavus* reduced BOD concentration by 82.64%, 86.40%, 91.30% and 95.10% in 25%, 50%, 75% dilutions and raw effluent respectively. At a 25% dilution, both fungal strains exhibited their highest reduction rate. These findings are in agreement with Selim *et al.,* (2021), who worked on biological treatment of textile effluent using *Aspergillus flavus*. *Aspergillus flavus* reduced BOD by 55.3% in effluent at rotary shaker (150rpm/minute) for 5 days (30^0C).

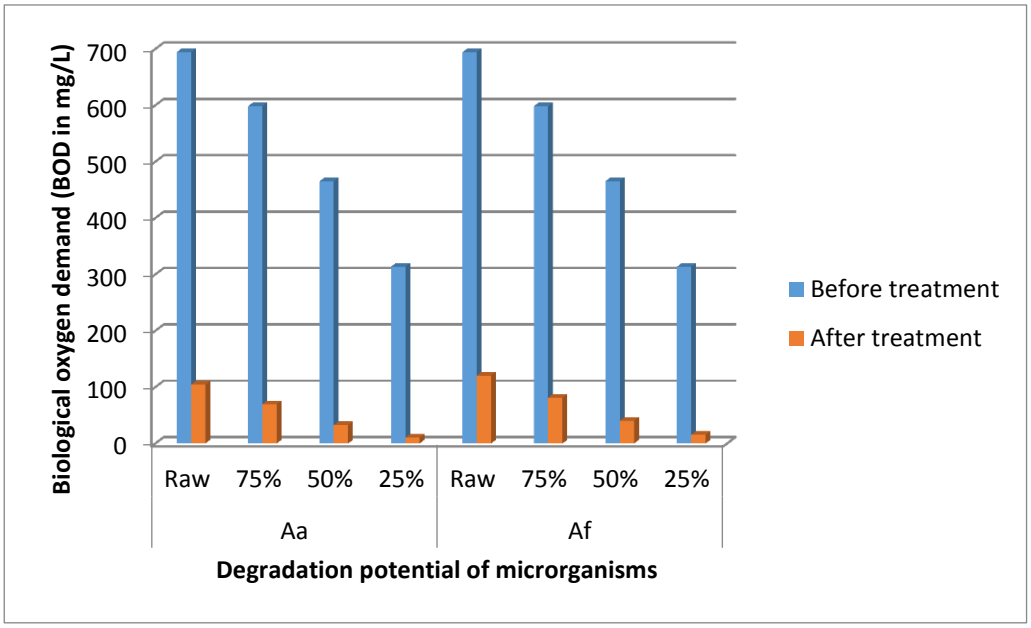
ISSN 2515-8260 Volume 09, Issue 07, 2022

Table 2: BOD (mg/L) at different dilutions before and after treatment with study organisms

Effluent dilution	BOD *Aspergillus flavus*			BOD *Aspergillus aculeatus*		
	Before treatment	After treatment	Percentage reduction (%)	Before treatment	After treatment	Percentage reduction (%)
Control	694.34±0.04	660.30±0.09	4.90	694.34±0.04	660.30±0.09	4.90
Raw	694.48±0.31	120.58±0.10	82.64	694.48±0.31	104.63±0.18	84.93
75%	598.72±0.23	81.44±0.05	86.40	598.72±0.23	69.68±0.31	88.36
50%	465.67±0.25	40.51±0.12	91.30	465.67±0.25	32.63±0.12	92.99
25%	313.85±0.13	15.41±0.07	95.10	313.85±0.13	10.45±0.19	96.67

Values are expressed as mean ± SD (Standard deviation, n= 3)

Figure 3: BOD reduction by study organisms at different effluent dilutions



Note: *Aa- Aspergillus aculeatus* and Af- *Aspergillus flavus*

Conclusion

In the present investigation four concentrations of effluent i.e., 75%, 50%, 25% and raw were studied to compare the reduction efficiency of *Aspergillus flavus and Aspergillus aculeatus*. The result revealed that both isolated fungal strains had a huge potential for decreasing BOD levels in textile industry effluent. However, in lower effluent concentration (25%), maximum reduction was observed because lower effluent concentration enhances the growth of organisms and improves the organism efficiency in effluent treatment. It can be concluded that the isolates have the potential to reduce the BOD of the effluent because of their ability to adapt and grow in such effluent. Hence, *Aspergillus flavus and Aspergillus aculeatus* both are recommended for reduction of BOD in textile industry effluent.

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