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Original Article

Fungal biodiversity of water and sediments in some aquatic systems in Basrah Province and their capabilities to degrade methyl orange

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Abstract: This study investigates the fungal biodiversity of water and sediment samples from some waterbodies and their ability to degrade Methyl orange (M.O.) on a solid medium. Ten fungal genera were isolated, and the genus *Aspergillus* represented the highest percentage (70%). Sixteen fungal species were isolated that 88% of which (14 species) belonged to anamorphic fungi. *Trichoderma* sp. showed the highest percentage (40%). Out of the 16 isolated fungi, *Aspergillus flavus, A. niger*, and *A. terreus* showed the best result for decolourising M.O. on a solid medium potato dextrose agar. These fungi isolates were selected to test their ability to biodegrade M.O. in a liquid medium supplemented with 50 mg.L^{-1} M.O. as the sole carbon source. Based on the results, after 7 days of incubation, *A. niger* degraded 14% of M.O., while *A. flavus* and *A. terreus* degraded 12% of the dye.

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Introduction

Fungi are the second largest biotic community after insects, with 120,000 species, although the total number is estimated to be 2.2-3.8 million. They are one of the biggest sources of biodiversity with fewer discoveries (Hawksworth and Lücking, 2017). Fungi are important organisms not only due to their vital roles in ecosystem functions but also because of their effect on humans and human-related activities. Fungi have high biomass production and a large surface area and can easily adjust to adverse environments, such as those waterbodies with different urban and industrial effluents and strongly contaminated by hydrocarbons, dyes, etc. (Chatterjee et al., 2020; Buratti et al., 2022).

The continuous growth of the population and increasing industrial activities in different sectors require the development of novel dyes with various natures. Many kinds of synthetic dyes are produced annually by hundreds of thousands of tons worldwide (Varjani et al., 2020; Selvaraj et al., 2021). Among synthetic dyes in different industries, azo dyes are the most commonly used, accounting for approximately 70%, due to their stability, chemical versatility, high fixation, and resistance to light and moisture (Shi et al., 2021). Azo dyes are chemically represented as R-N = N-R', with (-N = N-) being the chromophore group referred to as Azo connected to aromatic rings (Liu et al., 2020).

Methyl orange (M.O.) is a sulfonated monoazo dye, colorful and easily applied in textile coloring and in the laboratory as a pH indicator (Akansha et al., 2019; Purnomo et al., 2020). Disposal of untreated dyes attached to the benzidine group poses major problems, such as tumorigenicity and carcinogenicity, due to their biotransformation to benzidine compound, representing a great risk to humans and the environment (Guo et al., 2020). Thus, methods for the treatment of industrial effluents, especially those containing azo dyes, have received much attention worldwide. Many approaches for treatment and detoxification have been explored using physical, chemical, and biological methods (Varjani et al., 2020; Pinheiro et al., 2022). Decolourisation by microorganisms (bacteria, fungi, yeasts, and algae) or plants has significant advantages, such as eco-friendliness, low processing cost, non-toxicity, and complete mineralisation (Kamal et al., 2022).

Fungi can degrade azo dyes through the synthesis

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of a number of ligninolytic enzymes, including laccase, lignin peroxidase, and manganese peroxidase (Chatterjee et al., 2020; Pinheiro et al., 2022). Previous studies on M.O. degradation using fungi, such as *Aspergillus niger*, *Daedalea dickinsii*, and *Gloeophyllum trabeum* have been done (Khalaf, 2008; Purnomo et al., 2020). In this regard, this research aimed to identify fungal diversity in water and sediment samples of some aquatic systems in Basrah Province, Iraq, and to evaluate their ability to degrade methyl orange.

Materials and Methods

Sampling: Ten water and sediment samples of waterbodies were collected between October and November 2021 from four different waterbodies in Basrah Province, Iraq. Approximately 150-200 g of sediment sample was collected and placed in clean bags, and water samples were taken using sterile bottles (Hashem et al., 2018). All samples were transferred to the laboratory and maintained at 4°C until further use.

Fungal isolation: Potato dextrose agar (PDA) and malt extract agar (MEA) were used for the primary isolation of fungi. The isolation media were prepared according to the protocols of the manufacturing company (Hi Media, India), and added along with chloramphenicol (250 mg/L) to inhibit the growth of bacteria. Fungi were isolated from sediment samples using a dilution method described by Wicklow and Wittingham (1974). A total of 10 g of soil was suspended in 100 ml of sterile distilled water to make a 10^{-1} dilution. For water samples, the dilution method of Khalid et al. (2016) was used, wherein 1 mL of each water sample was added to 100 ml of sterile distilled water to make a 10^{-1} dilution. After exhaustive shaking for 10 min, 1 ml of each dilution was transferred to a sterile Petri dish and added to 15 ml of sterile media. The components of the Petri dish were mixed well before solidification. All the dishes were incubated at 25°C for 7-14 days. Pure cultures from all fungal colonies were maintained on PDA and MEA for morphological and molecular identification.

Identification of isolated fungi: The isolated fungi were identified phenotypically according to Raper and Fennell (1973), de Hoog and Guarro (1995), Watanabe (2010) and Guarro et al. (2012). Pure cultures from the isolated fungal colonies were subcultured on Petri dishes containing PDA medium and allowed to grow for 7 days for molecular identification. DNA extraction and PCR amplification were performed as described by Mirhendi et al. (2006). A forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and a reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (19 and 20 base pairs [bp], respectively) were used. The purification of the PCR products for the recovery of isolates was carried out by Macrogen (Seoul, South Korea). The obtained sequences were aligned and identified by the basic local alignment search tool (BLAST). The percentage of appearance for the fungal isolates was calculated according to the equation of % Percentage of appearance = (No. of samples in which the genus or species appeared/ The total No. of samples) ×100%.

Preliminary screening for fungal isolates to degrade M.O. on solid medium: The ability of the isolated fungal species to grow and degrade M.O on a solid medium was studied according to Dharshini and Sumathy (2014). M.O. solution (50 mg L^{-1}) was added to the PDA medium after autoclaving at 15 lb/inch^2 for 20 min and allowed to solidify. From each fungal isolate, a mycelium disk was made by a cork borer (5 mm) and inoculated into the PDA medium containing M.O. All the dishes were incubated at 25°C for 7 days. Two types of controls were prepared. The first control contains the medium and the fungal isolate without M.O. to compare the fungal growth without M.O. and calculate the percentage of inhibition. The second control containing M.O. without any fungal isolate was used to compare the observable fading of M.O. colour from the inoculated plate. Mycelium diameter growth and colour intensity change were measured at 72 h intervals. The percentage of inhibition of fungal growth during degradation was measured and calculated using the formula of I = C-T/C × 100, where I is the percentage inhibition in fungal growth, C is the growth in terms of colony diameter in control

Table 1. The isolated fungal genera with their percentage of occurrence.

No.	Genera	No. of samples in which the genus appeared	% of occurrence
1	*Acremonium*	1	10
2	*Aspergillus*	7	70
3	*Cladosporium*	1	10
4	*Ectophoma*	1	10
5	*Monascus*	1	10
6	*Mucor*	1	10
7	*Penicillium*	3	30
8	*Phialophora*	1	10
9	*Phoma*	1	10
10	*Trichoderma*	4	40
11	*Sterile mycelia*	3	30

and T is the growth in terms of colony diameter in the sample.

Biodegradation of M.O. in liquid medium: The fungal isolates that lodged the best results in the prior experiment were further tested to evaluate their capability to degrade M.O. in a liquid medium. The isolates were activated by subculturing it on a PDA medium for 7 days. The liquid medium used was mineral salt medium (MSM, g L^{-1}) containing 0.75 g of KH_2PO_4, 0.75 g of K_2HPO_4, 0.05 g of $MgSO_4.7H_2O$, 0.05 g of $CaCl_2.2H_2O$, 0.02 g of $FeSO_4.7H_2O$ and 0.1 g of yeast extract per liter of distilled water (AI-Jawhari and AL-Mansor, 2017).

Conical flasks (250 mL) containing 100 mL of MSM (pH = 6) supplemented with 50 mg L^{-1} M.O. as the sole carbon source were inoculated with three plugs of mycelia (5 mm diameter) obtained from the edge of actively growing mycelia. The culture flasks were incubated at 25°C for 7 days. Control flasks consisting of media with dye were also prepared. All experiments were carried out in triplicate. After 7 days, a 10 mL aliquot was taken from all culture flasks, including the control, and centrifuged for 10 min (5000 rpm). The maximum absorbance at 498 nm was recorded using a UV/VIS spectrophotometer to determine the concentrations of M.O. dye. The biodegradation of M.O. was then calculated using the formula of %Degradation = (initial absorbance-observed absorbance) / (initial absorbance) × 100%, where initial absorbance is the control and the observed absorbance is the treatment absorbance (Hashem et al., 2018).

Statistical analysis: All the data were expressed as mean ± SD. One-way ANOVA was conducted using Minitab software (version 20) to determine significant differences among fungal samples.

Results and Discussion

Identification of isolated fungi

Identification of fungal genera: In this study, 10 fungal genera and sterile mycelia were isolated from 10 water and sediment samples (Table 1). About 88% of the isolated fungi were anamorphic fungi belonging to 14 species. The high percentage of anamorphic fungi might be due to the capability of these fungi to produce a vast number of reproductive units that support their widespread distribution in the environment. These fungi can also tolerate adverse environmental conditions and secrete several enzymes, which help them degrade a broad range of organic compounds. In this regard, anamorphic fungi are one of the primary groups of fungi in the environment (Ziaee et al., 2018; Altaee and Al-Dossary, 2021). Two phyla, namely, Zygomycota and Ascomycota, appeared in a low percentage (6% for each one) possibly because they cannot grow under extreme conditions and high temperatures; they also need specific isolation methods and special culture media for isolation or may grow slowly, especially Ascomycota, requiring longer growth periods (Raja et al., 2017; Wu et al., 2019).

The percentage of fungal genera ranged from 10 to 70%, and *Aspergillus* had the highest percentage of 70%. This genus can withstand and adapt to difficult

Table 2. The isolated fungal species with their percentage of appearance.

No.	species	No. of samples in which the species appeared	%of appearance
1	*Acremonium* sp.	1	10
2	*Aspergillus allahabadii*	1	10
3	*A. flavus*	2	20
4	*A. fumigatus*	1	10
5	*A.niger*	2	20
6	*A. terreus*	1	10
7	*Cladosporium* sp.	1	10
8	*Ectophoma multirostrata*	1	10
9	*Monascus pallens*	1	10
10	*Mucor* sp.	1	10
11	*Penicillium* sp.1	1	10
12	*Penicillium* sp.2	1	10
13	*Penicillium* sp.3	1	10
14	*Phialophora* sp.	1	10
15	*Phoma* sp.	1	10
16	*Trichoderma* sp.	4	40

conditions, including a wide range of temperatures, and can release enzymes, such as lignin and manganese peroxides, which help them degrade organic substances as sources of energy and growth. These fungi also have many stress-tolerant asexual conidia (Tian et al., 2017; Ziaee et al., 2018). Other genera isolated ranged from 10 to 40%, and *Trichoderma* recorded the second highest percentage of 40%. This genus, a type of anamorphic fungi, similar to *Aspergillus*, also produces a huge number of conidia and can adapt to different types of environments (Tian et al., 2017). The appearance of some genera in a low percentage does not mean that they do not play an important environmental role and may be effective in their original environment. The method of isolation, the duration of incubation, and the isolation media used in the study may be inappropriate for their growth; they may also grow slowly and need a longer time to grow up or cannot compete with other fungi. Differences in fungal appearance also depend on season and temperature (Saramanda and Kaparapu, 2017; Zhang et al., 2020). The results of the current study are consistent with those reported by Al-Daamy et al. (2018), Minati et al. (2020), and Lima et al. (2021); that is, the genus *Aspergillus* recorded the highest percentage.

Identification of fungal species: Sixteen fungal species were isolated from 10 water and sediment samples (Table 2). The percentage of these species ranged from 10 to 40%. *Trichoderma* sp. showed the highest percentage (40%) and appeared in four different samples. This species has enzymatic capabilities enabling it to degrade a variety of substances in the environment, including toxic substances, and has a high ability to adapt and live in various environmental conditions. It also plays an important environmental role because of its strong competition with other microorganisms in the environment and exhibits hyperparasitism, which enables it to grow and spread (He et al., 2018; Singh et al., 2018). *Aspergillus flavus* and *A. niger* had the second highest percentage of 20%, and the rest of the species, such as *Acremonium* sp. appeared in a low percentage of 10% (Table 2). In general, the number of species isolated was lower than in other studies. Wu et al. (2022) isolated 299 fungal species from 33 different sediment samples. The difference may be due to the number of samples collected, the nature of the environment from which the fungi were isolated, the sample collection period, and the high temperatures during the sample collection because it may adversely affect the growth of microorganisms and reduce their numbers; all these factors affect the fungal diversity (Raja et al., 2017).

Decolourisation of M.O. on solid medium: The ability of the isolated fungi to decolorize M.O. was

Table 3. Growth of fungal mycelium on solid medium.

Fungi	Fungal growth in Control (in cm)	Fungal growth in Sample (in cm)	Inhibition%	Decolourisation degree
Acremonium sp.	6±0.28	6±0.14	14	-
Aspergillus allahabadii	6±1.06	4±1.06	39	+
A. flavus	6±0.70	4±0.14	12	+++
A. fumigatus	8±0.99	6±1.27	20	++
A. niger	8±0.14	7±0.00	7	+++
A. terreus	8±0.14	8±0.00	5	+++
Cladosporium sp.	8±1.41	7±1.06	10	-
Ectophoma multirostrata	7±0.56	5±0.28	23	+
Monascus pallens	6±0.14	5±0.14	14	++
Mucor sp.	8±0.35	6±0.56	21	-
Penicillium sp.1	8±1.06	6±1.63	11	++
Penicillium sp.2	8±0.99	5±0.91	23	+
Penicillium sp.3	8±1.06	5±0.91	23	+++
Phialophora sp.	7±1.06	5±0.42	22	-
Phoma sp.	8±0.70	6±0.35	21	-
Trichoderma sp.	9±0.00	8±0.76	28	-

Abbreviations: −, no color change; +, low color change; ++, mild color change; +++, high color change.

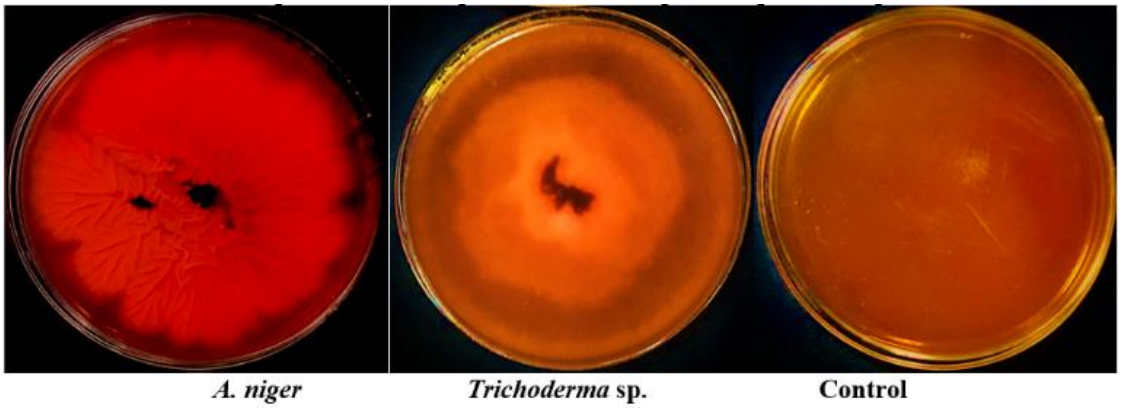

Figure 1. Visual decolourisation of Methyl orange on solid medium.

tested on a PDA medium. The degradation efficiency of the tested fungi was determined based on their growth, inhibition percentage, and decolourisation ability (Table 3). The growth of most fungi on the medium was affected by the M.O. dye but in varying degrees. The inhibition percentage ranged from 5 to 39%. *Aspergillus allahabadii* was significantly affected by M.O. in the medium and showed maximum inhibition (39%), while the minimum inhibition was found in *A. terreus* (5%) (Table 3). Some fungi were sensitive to the toxic effect of M.O., while other fungi did not show a considerable effect, indicating that they can adapt to toxic compounds in the environment; they can tolerate the toxic effects of the dye, degrade and use it to grow (Purnomo et al., 2020). In a similar study, Vasdev (2011) stated that the growth of six species of white-rot fungi was not affected by the existence of dyes in the medium, assuming that some fungi can tolerate and decolorize dyes for growth.

Of the 16 tested fungi, 11 were found to have the ability to decolorize M.O. on a solid medium. *Aspergillus flavus, A. niger*, and *A. terrue* had the best efficiency of decolourisation (+++), with colour of M.O. changing to red, compared with the control (Table 3, Fig. 1). These fungi have unique enzymes systems for degrading complex organic compounds (Singh and Singh, 2010). The ability of the remaining fungi to decolorize M.O. ranged from (-) with no colour changing, such as *Trichoderma* sp. to (++) with good decolourisation efficiency such as *A. fumigatus.* The results are consistent with the reports of Sharma

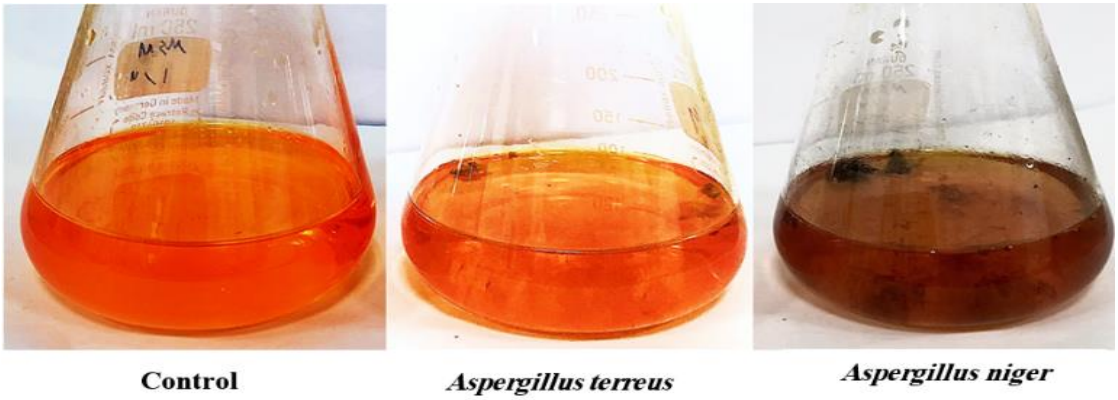

Figure 2. Visual decolourisation of Methyl orange in liquid medium.

et al. (2017) which pointed out that 22 fungal isolates can decolorize M.O. on a solid medium. Jayasinghe et al. (2008) stated that some fungi, such as *Pycnoporus coccineus*, *Fomes fomentarius*, *Stereum ostrea,* and *Pycnoporus cinnabarinus* have good mycelium growth in a medium containing the dye, but they could not decolorize it.

Decolourisation of M.O. in liquid medium: Three fungal isolates, namely, *A. flavus, A. niger,* and *A. terreus,* which showed the best result in the decolourisation of M.O. on the solid medium, were selected to study their ability to degrade M.O. in a liquid medium. *Aspergillus niger* showed the highest degradation percentage of 14% for M.O. within 7 days (Fig. 2), possibly because of its high ability to tolerate the toxicity of the dye and grow faster than the two other species. It also has different extracellular enzymes such as manganese peroxidase which is involved in dye degradation (Balcázar-López et al., 2016; Bagewadi et al., 2017). The result is consistent with the study of Purnomoa et al. (2020), who asserted that *Gloeophyllum trabeum* can degrade 38% of the M.O.. The biodegradation ability of studied fungi appears to be low compared to other studies like Purnomoa *et al.* (2020), who asserted that *G. trabeum* can degrade 38% of the M.O.. This could be because of the complex nature of the functional group in M.O. dye that is difficult to biodegrade by fungi. In addition, the biodegradation of the dye is very slow due to the limited nitrogen source added to the medium and the lack of a carbon source (Zhuang et al., 2020; Carolin et al., 2021).

Conclusions

In this study, 16 fungal species were isolated and their ability to degrade M.O. was investigated. *Aspergillus* had the highest percentage of 70%, followed by *Trichoderma* sp. (40%). Out of 16 fungal isolates, three species, namely, *A. flavus, A. niger,* and *A. terreus* showed the best result for decolourisation of M.O. on the solid medium and were selected for testing on liquid medium. *Aspergillus niger* showed the best result to degrade M.O. in the liquid medium with dye as the sole carbon source, where 14% of the dye was degraded after 7 days of incubation.

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