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Decolorization and Degradation of azo dyes in textile wastewater effluent by *Aspergillus Niger* and *Aspergillus ochraceous*

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Abstract

In this study, fungi were isolated from contaminated soil collected from textile wool factories in Thi Qar Governorate. The results showed a high potential for removing Methylene Blue dye by *Aspergillus niger* and *Aspergillus ochraceous*. The removal process was conducted under optimized conditions, including different concentrations of the azo dye, pH levels, carbon sources, and nitrogen sources. The results indicated that the fungi *Aspergillus niger* and *Aspergillus. Ochraceus* were the most common isolates. Both fungi were treated with Methylene Blue dye in solid and liquid media, and both showed the ability to degrade this dye. However, *Aspergillus niger* demonstrated higher activity in color removal compared to Aspergillus *ochraceous*, with a diameter of (9) cm in solid medium for *Aspergillus niger* and (8) cm for *Aspergillus. ochraceus*. These results were confirmed by statistical analysis, which showed significant differences between these fungi and concentrations. The decolorization percentage was confirmed by Fourier Transform Infrared (FTIR) spectroscopy of which showed complete disappearance of peaks at 500 nm and at 1500 nm, indicates the degradation of dyes due to fungal activity, The technology also confirmed that a pH of 7 was the optimal pH for dye biodegradation compared to pH 4. This was observed through the dry weight of the fungi, with *Aspergillus niger* reaching to (2.35) and *Aspergillus ochraceous* reaching to (2.15) at pH 7. Also, in pH 4, the dry weight of *Aspergillus niger* and *Aspergillus. ochraceus* reached (2.17) and (2.05) respectively. Furthermore, the study confirmed the potential use of filamentous fungi in treating dye-contaminated water, regardless of their pH values.

Keywords: Azo dye; Decolorization; Methylene blue; Fungi; FTIR

1. Introduction

Industrial wastewaters are a major source of water pollution, contaminating rivers, lakes, and oceans. Various industries, including textile, paper, dyestuffs and pulp, distillery, tannery, oil mill, and metal industries, release these wastewaters (El-Gamal *et al.*, 2018; Hamza *et al.*, 2018). Textile wastewaters contains a wide range of chemical additives, and dyes used in the dyeing process, such as soda ash, heavy metals, acetic acid, and caustic soda. The presence of these dyes poses a significant environmental challenge for the textile industry (Bansal and Kanwar, 2013). The waste discharged from the textile dyeing industry contains hazardous compounds that are difficult to degrade, including azo dyes, which are a major source of environmental pollution (Hassan *et al*., 2015; Fouda *et al*., 2016;). Textile industries produce a large volume of wastewater due to the extensive use of water in finishing, and dyeing processes (Saratale et al.,2011).

Some studies have explored technological solutions to improve textile processes, and reduce environmental pollutants (Emam and Abdelhameed, 2017; Emam, 2019). It is estimated that around 10-15% of dyes are released into the treated water (Omar, 2019).

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However, the excessive use of dyes leads to environmental pollution, particularly by increasing the concentration of dyes in soil and water. The concentration of dyes in textile dyeing effluents can range from 10 to 200 mg/L (Kadam et al., 2011). Even low concentrations of azo dyes (10-50 mg/L) in wastewater from dyeing processes are toxic, and carcinogenic to organisms (Wong and Yu, 1999). Additionally, the release of dyeing wastewater into the environment negatively impacts the process of photosynthesis, and reduces oxygen content (Aksu et al., 2007). Azo dyes are persistent and difficult to degrade, leading to their accumulation in soil, and water at toxic levels, with mutagenic effects on microorganisms, plants, and humans (Lade *et al*., 2015).

Furthermore, the presence of aromatics, metals, chloride, and other substances in dyeing wastewater can have a significant impact on the photosynthetic activity of aquatic life by reducing light penetration. These substances may also be toxic to certain aquatic organisms (Daneshvar *et al*., 2007).

Methylene blue dye is a type of chemical compound with an aromatic structure that is not uniform throughout. It exists as a solid substance in the form of a dark green, odorless powder at room temperature. When mixed with water, it forms a blue solution. This dye is widely used in various industries such as textiles, paper, leather, cosmetics, plastics, printing, and food (Yagub et al., 2014; Singh et al., 2015). It has multiple applications in chemical and biological water formulas (Rafatullah et al., 2010; Singh et al., 2017).

Bioremediation is a process that utilizes biological mechanisms to transform pollutants into a harmless state by either detoxifying or degrading them (Azubuike *et al*., 2016). One advantage of bioremediation over traditional methods is its lower cost. It also has the potential to completely break down pollutants, offering a long-term solution. Additionally, bioremediation is a non-disruptive technique that helps maintain the resilience of ecosystems (Perelo, 2010). In cases where physico-chemical methods would be insufficient to clean contaminants at low concentrations, bioremediation can be effective. For successful bioremediation, microorganisms need to enzymatically attack pollutants and convert them into harmless byproducts. The effectiveness of bioremediation is influenced by environmental conditions that support microbial growth and vitality. Therefore, optimizing environmental parameters is often necessary to promote microbial growth, and enhance the degradation rate (Vidali, 2001). In recent years there has been intense research on fungal decolorization of dye wastewater. It is thus turning into a promising alternative for replacement or substitute to present treatment processes. Here, this present work is focused on the isolation, and characterization of fungal strains, which would efficiently decolorize the textile dye, reactive blue MR. the aims of the present study were to investigate the ability of *A. niger*, *A. ochraceous*, to removal methylene blue.

2. Material and Methods2-

2.1. Azo dyes and samples collection

Wastewater samples and sediments were collected from textile wool factories in Thi Qar Governorate. Fungi were isolated from three stations and transfer to the laboratory, and three samples were collected from each station as replicates. A type of azo dye, methyl blue (MB), was used.

2.2. Isolation of fungi

The samples collected for fungal isolation were examined, and one of the techniques was applied to the fungi isolated from the sediment, and water samples using the dilution method. Specifically, 1 ml of wastewater or 1 gram of soil was sequentially transferred to 9 ml of distilled water and serially diluted water up to 10^{-4} (Al-Nasrawi, 2012). After homogenization, the pour-plate method was used, where 1 ml of each previous dilution was withdrawn and poured into sterilized petri dishes, to which the culture medium, Potato Dextrose Agar (PDA) was added after adding the antibacterial agent Chloramphenicol (250 mg). The plates were incubated at 25°C for7days.

2.3. Ability of isolated fungi to grow on solid medium supplemented with Methylen blue Dye

The medium was prepared Potato Dextrose Agar (PDA) in fifteen 250 ml conical flasks, and then sterilized with an autoclave device at a temperature of 121 °C and a pressure of 15 pounds / square inch for 20 minutes. After that, the aromatic dye (Methylene Blue) was added to the culture medium after the temperature of the medium dropped to an appropriate level at three concentrations for each compound (50, 150, 250) ppm with three flasks. One flask was left without adding any compound for comparison purposes (Control). Then, the medium was poured into sterilized Petri dishes with a diameter of (9.0) cm and left to dry for 30 minutes.

These dishes were inoculated with the fungal inoculum by transferring a disc from the pure fungi with a diameter of 4 mm, and 7-day age of *Aspergillus niger* and *Aspergillus ochraceous* were used by a sterile cork piercing to the middle of each plate. Then the dishes were incubated at 25 °C for 7 days. The experiment was carried out with three replications for each treatment, and the growth rates of these fungi were calculated by the measure of the diameter of the colony.

2.4. Ability of isolated fungi to grow in mineral salts medium supplemented with methylene blue

The mineral salts solution was prepared for the growth of fungi, This medium consists of the following ingredients per liter: K_2HPO_4, 1.71 g; KH_2PO_4, 1.32 g; $NaNO_3$, 0.42 g; $MgSO_4.7H_2O$, 0.42 g; $CaCl_2$, 0.02 g. Prepare fifteen 250 ml conical flasks, and then sterilize the medium using an autoclave at a temperature of 121°C and a pressure of 15 pounds / square inch for 20 minutes. after the temperature has decreased. Then, add the selected aromatic dyes to the culture medium at reduced temperature. The selected dye is methylene blue, and it is added at concentrations (50, 150, 250) ppm to the medium. Repeat the experiment using different pH values (4, 7), and glucose as a carbon source. A 0.1 gm per litterer for NH_4CL and $NaNO_2$ as nitrogen sources were addition, and the flasks were left without the addition of the material for comparison, then the flasks were inoculated by transferring a disc. 4 mm of 7-day-old fungal cultures for fungi *Aspergillus niger.* and *Aspergillus ochraceous* by used a sterile Cork Borer. The flasks were incubated at a temperature of 25°C for 7 days, and this experiment was carried out with three replications for each treatment. Filter paper and then dried in an oven at a temperature of 50°C for half an hour, after that the mycelium was weighed using a sensitive balance.

2.5. Biodegradation of Methylene blue in liquid medium

After incubation (14 days) of one disc (5mm) from *Aspergillus niger*, *Aspergillus ochraceous* in mineral salts medium (MSM), the contents of the flasks were individually filtered, and the filtrate was collected in a 250 mL flask.

The process of (AL-Jawhari and AL- Mansor,2017) to extract the metabolic products. The filtrate was transferred to the water bath and evaporated until the filtrate reached a volume of 1 ml, then the samples were transferred to sterile test tubes with added 1 ml of ethanol and 1 ml of distilled water, which were separated by a centrifuge (Gallenkamp) at 10,000 rpm per minute for 5 minutes and then transferred the filtrate to the water bath again until the sample volume was concentrated to 1 ml. The samples were transferred to sterile opaque glass test tubes on which all required details were recorded and kept in the refrigerator until analyzed by Fourier-transform infrared spectroscopy (FTIR).

Mycelia were harvested from the cultivation liquid medium by filtration using whattman No.1 Filter paper and then dried in an oven at a temperature of 50°C for half an hour, after that the mycelium was weighed using a sensitive balance.

2.6. Statistical analysis

The present study conducted an Anova (analysis of variance) which was performed on all the treatments and done using the SPSS (version 23.0) package to determine whether significance difference.

3. Result and discussion

3.1. Ability of isolated fungi to grow on solid medium supplemented with Methylene blue

The results mentioned in Table (1) indicate that methylene blue dye with a concentration of 50 ppm, which was grown on the PDA solid medium (Potato Dextrose Agar), had a slight effect on the colony of the fungus *A. niger*, while it did not affect the fungus *A. ochraceus.*

We also observe a decrease in colony diameters for the fungi *A. niger* and *A. ochraceus* treated with the methylene blue dye at a different concentration. The colony diameter for *A. niger* reached to (9.0, 7.9, 6.5) cm at concentrations of (50, 150, 250) ppm, respectively, compared to the control treatment (9.0) cm. Similarly, the colony diameter for *A. ochraceous* reached to (8.0, 7.3,6.5) cm at the same concentrations, compared to the control treatment (9.0) cm Figure (1,2).

From the results, that all concentrations to which the fungi were exposed resulted in a reduction in colony diameter compared to the control. Furthermore, increasing the concentration of the dye led to a decrease in the colony diameter for the fungus, this was confirmed by the results of statistical analysis, with a significant difference between the fungi and the concentrations.

The decrease in the colony diameter rates of the fungi treated with the dye daily indicates that the fungi *A. niger* and *A. ochraceous* were affected by the dye treatment at all concentrations. This can be attributed to the complex composition

of the dye, as it contains active complex groups that are difficult for the fungi to biodegrade (Carolin *et al.*, 2021). These results are consistent with the findings of AL-Jawhari (2015), who concluded that the blue methylene dye did not inhibit the growth of the fungus *A. niger*. However, this fungus was not able to effectively remove the dye color. The same results were obtained by (Chandana *et al*.,2008) in this study show that when the white rot fungi *Carioles versicolor* was good mycelia growth on solid media contained MB, but the efficiency of decolorization was very low, and at the same time the decolorization index with this fungus reached to 0.11. (Novotny et al., 2007) reported that when the white rot fungus*, Irpex lacteus,* was grown on different media containing MB it did not show any considerable decolorization. In addition, the results of a study conducted by (Abo-State et al ., 2011) also showed that the ability of *Pleurotus ostratus* to decolorize MB also increased, so the removal % increased for a wide range of concentrations(25-700 mg/L)MB, and in the same time (Abo-State et al ., 2011) refer that this result due to may attributed to the increasing in production of ligninolytic enzymes as the concentration of MB increased due to their stress on the mycelia cells of *P.ostratus*. However (Boer et al., 2006) found that *Lentinula edodes* decolorized media that contain 200ppm MB by 60%.

Table 1 Ability of *A. ngier* and *A. ochraceous* to grow on solid medium

Fungi	**Concentration(ppm)**				**Mean**
	Control	**50**	**150**	**250**	
A. niger	9.0± 0.0	9.0± 0.0	7.93±0.11*	6.5±0.00	7.70
A. ochraceus	9.0± 0.00	8.0± 1.0	7.33±0.28	6.5±0.0	8.10
L.S.D fungi =0.029 L.S.D = 0.023					

*Colony diameter: cm

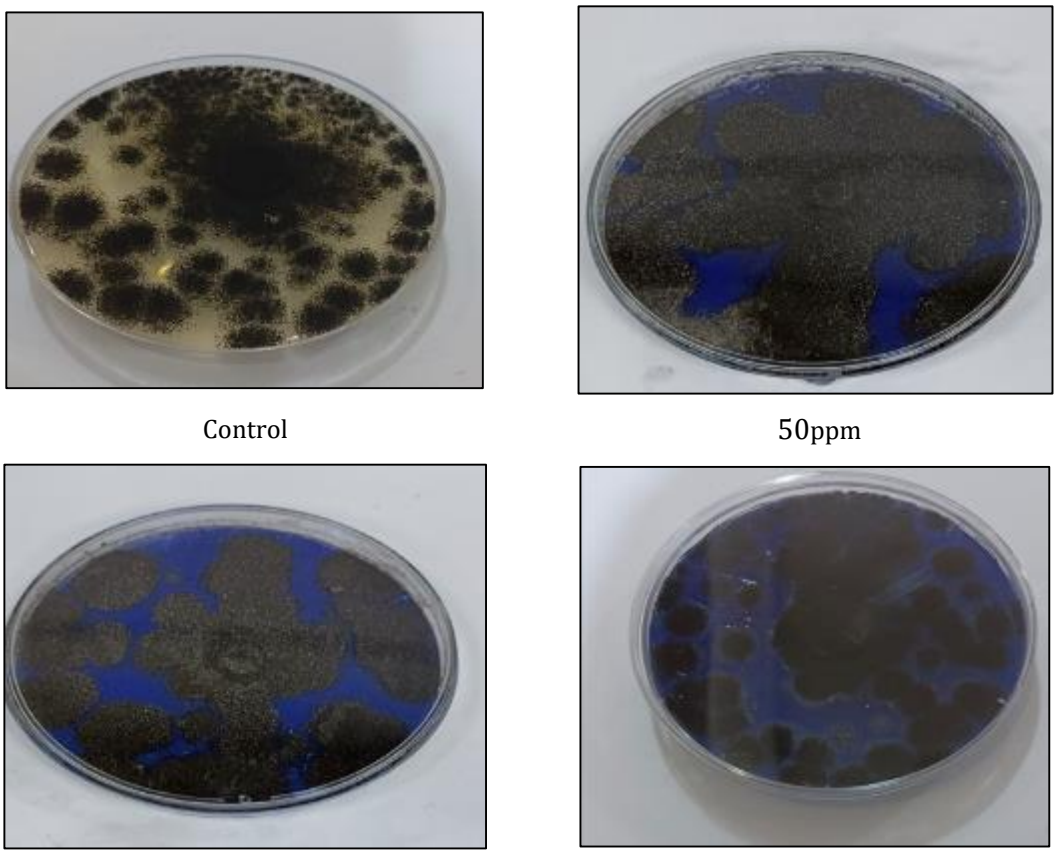

Figure 1 Decolorization of Methylene blue by *Aspergillus niger* on solid medium

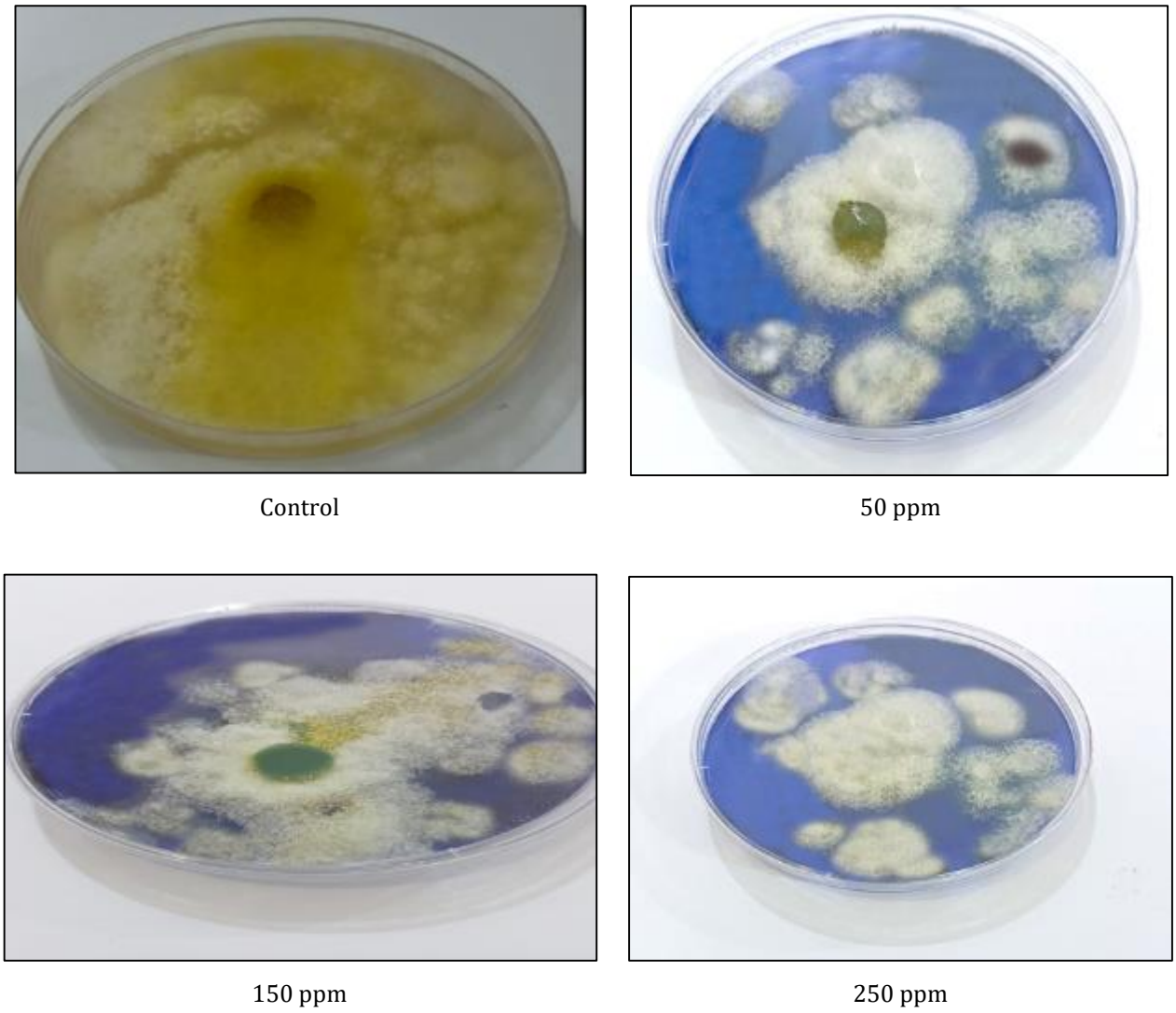

Figure 2 Decolorization of Methylene blue by *Aspergillus ochraceous* on solid medium

3.2. The ability of isolated fungi to grow in Mineral salts medium supplemented with Methylen blue

The results obtained in the current study indicate good fungal growth in the mineral salts medium (SMS), supplemented with methylene blue dye, glucose as a carbon source, and NH_4Cl as a nitrogen source, at pH 7. This suggests that these fungi could grow in a liquid medium in the presence of the dye, at various concentrations, and to varying degrees among the isolated fungal species. This was evident from the statistical analysis results, which showed no significant difference between the fungi and the concentration levels, Table (2,3). It was found that as the concentration increased, the dry weight of the fungus increased. The results showed that the dry weight of the fungus *A. niger* reached to 2.09 gm, 2.15 gm, and 2.35 gm, respectively, at concentrations of 50, 150, and 250 ppm, compared with the control where the dry weight was (1.85) gm.

The results showed that the dry weight of *A. ochraceous* was lower compared *A. niger*. The dry weight reached to (2.00, 2.13, 2.15) gm at concentrations of (50, 150, 250) ppm, respectively, compared to the control where the dry weight was (1.60) gm with pH=7 (Table.3). The results showed an increase in the dry weight of fungal mycelium after adding glucose and NH_4CL. This is because glucose is the most effective and readily available carbon source for microbial metabolism. Additionally, many microorganisms require an additional source of carbon, and nitrogen to enhance growth, cellular production, primary metabolites, and enzyme secretion for biodegradation. Microorganisms cannot use azo dyes as a sole source of energy and carbon. Therefore, microorganisms generally rely on the presence, and type of carbon source used for for the degradation of azo dyes (Chakraborty et al., 2013; Varjani et al., 2020; Carolin et al., 2021).

According to our study, we observed that the dry weight of fungi at pH = 4 decreased compared to the dry weight of fungi at pH =7. The dry weight of *Aspergillus niger* was (1.84, 2.15, 2.17) gm at concentrations (50, 150, 250) ppm, respectively compared to the control weight of (1.85) gm Table.2. As for *Aspergillus ochraceus*, the dry weights at concentrations of (50, 150, 250) ppm were (1.90, 1.97, 2.05) gm, respectively, compared to the control weight of (1.60) gm. These results indicate statistically significant differences between the two fungi and the concentrations. This is because pH is an important factor affecting microbial cells since microorganisms do not have the mechanism to regulate internal acidity. Each microorganism has a range of pH values, and it was found that the optimal pH for the biodegradation of azo dyes is usually between (6-10) (Jamee and Siddique, 2019).

The results also showed an increase in the dry weight of fungal mycelium after adding glucose and NH_4CL. This is because glucose is the most efficient and readily available carbon source for microbial metabolism. The reason for this is that many microorganisms require an additional source of carbon and nitrogen to enhance growth, cell production, primary metabolite synthesis, and enzyme secretion for biodegradation. Additionally, microorganisms cannot use azo dyes as a sole source of energy and carbon since azo dyes do not generally serve as a carbon source. Therefore, microorganisms rely on the presence and type of carbon source used to break down azo dyes in general (Varjani *et al*., 2020; Carolin *et al*., 2021). The results in present study were similar with the results obtained by (Salem et al.,2019) that the decolorization of azo dyes reaches the greatest value at alkaline pH range, but acidic or highly alkalinity was inhibited dye decolorization by fungal strain. As well as other results showed that, maximum decolorization percentage of Reactive yellow (4GL) and Reactive red (4BL) by Aspergillus niger D2-1 was reached to 48.01% and 32.40% respectively at pH 9.0. It is achievable that pH change affects the transport of dye particles crossway the cell membrane of microbes, which is reflected as the rate-limiting stage for the decolorization (Saratale et al,.2011).

Table 2 Ability of *A. niger* and *A. ochraceous* to grow in mineral salts medium with Methylene blue, pH=4

Fungi	**Concentration(ppm)**				**Mean**
	Control	**50**	**150**	**250**	
A. niger	1.85±0.00	1.84±0.02*	2.15±0.01	2.17±0.02	2.00
A. ochraceus	1.60±0.00	1.90 ±0.01	0.02±1.97	0.05±2.05	8.10
L.S.D = 0.104 L.S.D = 0.01					

*Colony diameter: cm

Table 3 Ability the growth of *A. niger* and *A. ochraceous* in mineral salts medium with Methylene blue, pH=7

Fungi	**Concentration(ppm)**				**Mean**
	Control	**50**	**150**	**250**	
A. niger	1.85± 0.00	2.09± 0.02*	2.15±0.05	2.35 ±0.02	2.11
A. ochraceus	1.60±0.00	2.00± 0.03	2.13± 0.02	2.15±0.01	1.97
L.S.D =0.041					

*Colony diameter: cm

3.3. Biodegradation of Methylene blue in liquid medium by *A. niger* and *A. ochraceous*

Figures (3-15) demonstrated the ability of the fungi *A. niger* and *A. ochraceus* to degrade methyl blue dye in a liquid culture medium using (FTIR) technique. These figures showed the capability of *A. niger* to degrade methylene blue dye at concentrations (50, 150, 250) ppm at pH 7 when compared to the composition of the standard methyl blue dye shown in figure (3). It was found that there were changes in the dye's composition, as many peaks in the region of (500-1500) disappeared, as well as a decrease in the peak at 700 cm^{-1}, indicating the loss of aromatic compounds. as there was disappearance of many peaks in the region between 1000-1500 compared to the standard dye, and a new peak appeared in the range of 2500-3000 at a concentration of (50) ppm, and this indicates the formation of carboxylic acid. As for the remaining forms, a new broad and wide peak appeared in the region confined between (3000-3500) at all concentrations. This indicates the presence of the OH group or the formation of carboxylic acid or aldehyde because of

glucose fermentation in the liquid culture medium. This is consistent with (AL-Jawhari *et al*., 2017) regarding the ability of filamentous fungi to degrade azo dyes, especially aromatic compounds, into metabolites.

Figures (7, 8, 9) indicate the degradation of methylene blue dye by *A. ochraceus* at concentrations of (50, 150, 250) ppm, compared to the composition of the standard dye shown in figure (3). We observe a change in the dye's composition and almost complete disappearance of peaks in the region of (500-1000). Additionally, several peaks are reduced at (500, 700, 1500) cm^{-1} indicating the degradation of C=C bonds and aromatic bonds. Three peaks disappear in the region of (1500-2500) and a new peak appears in the same range, indicating the presence of a ketone group. In the region of (3000-3500) a broad and wide new peak appears, indicating the presence of an OH group, carboxylic acid, or a dihydric compound resulting from the fermentation of glucose in the liquid culture medium.

In pH=4, addition of glucose and NH_4CL, we observe, through figures (10-15) that there is also degradation, and reduction of the dye by *A. niger* and *A. Ochraceus*. We notice the disappearance, and reduction of a significant number of peaks in the region of (500-1500) especially around the peaks at 700, 800, and 1500 cm^{-1}indicating the degradation of aromatic compounds.

In the region of (1500-2000) two peaks disappeared, and two new peaks appeared, which suggests the presence of a ketone. In the region of (3000-3500) one peak disappeared, and new peaks appeared, indicating the formation of a carboxylic acid or aldehyde. The results in present study were similar with the results obtained by (Namdhari et al., 2012) showed that the highest decolorization of Reactive Blue MR by *Aspergillus niger* was achieved with glucose supplementation.

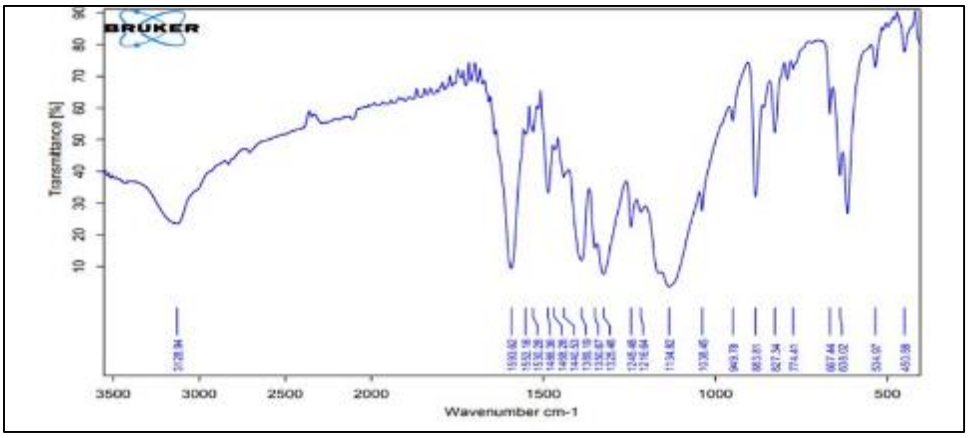

Figure 3 Methylene Blue (standard)

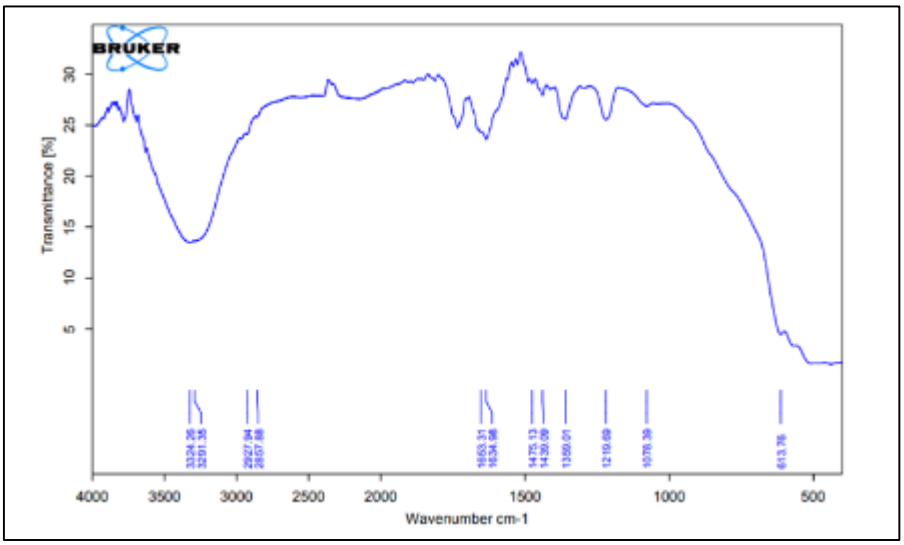

Figure 4 Biodegradation of Methylene Blue by *A. niger* after 7 days incubation by (FTIR) at a concentration of (50) ppm with pH = 7

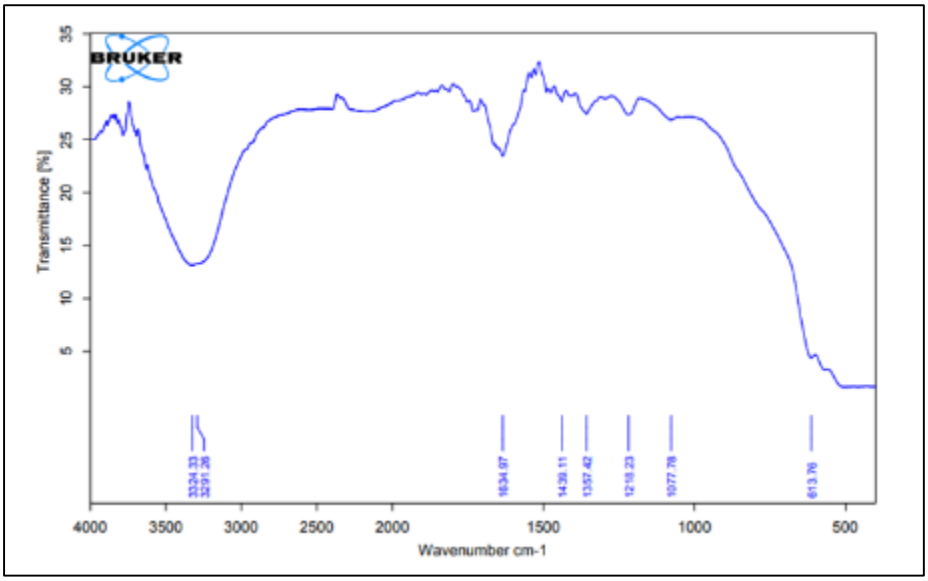

Figure 5 Biodegradation of Methylene Blue by *A. niger* after 7 days incubation by (FTIR) at a concentration of (150) ppm with pH = 7

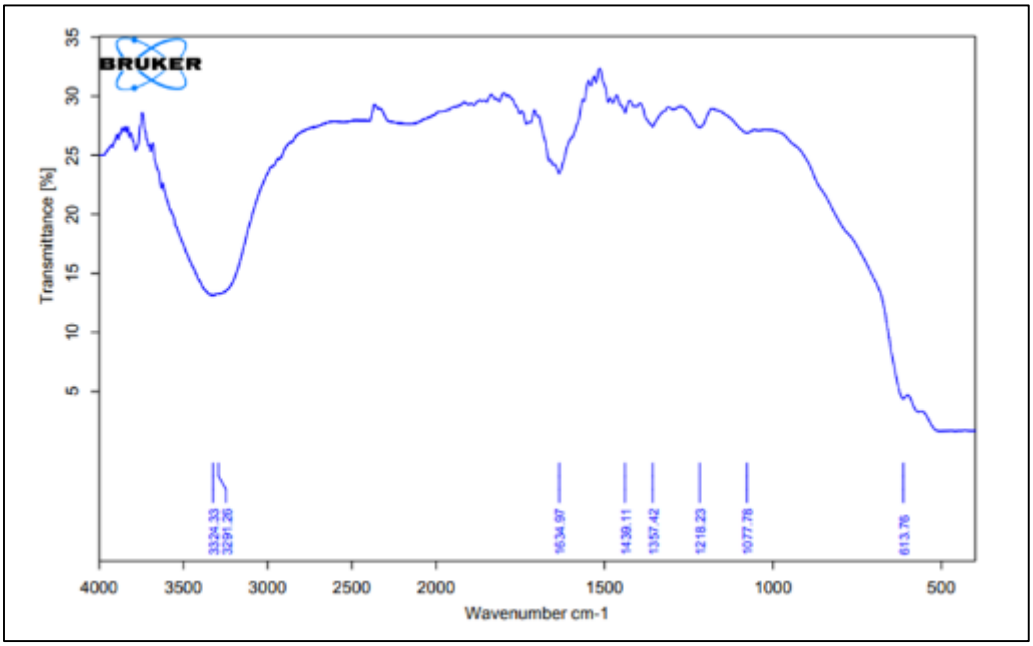

Figure 6 Biodegradation of Methylene Blue by *A. niger* after 7 days incubation by (FTIR) at a concentration of (250) ppm with pH = 7

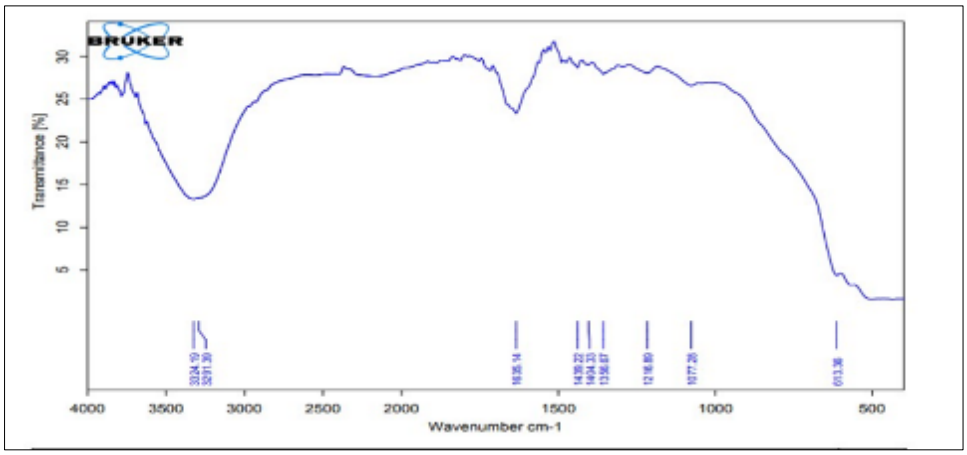

Figure 7 Biodegradation of Methylene Blue by *A. ochraceus* after 7 days incubation by (FTIR) at a concentration of (50) ppm with pH = 7

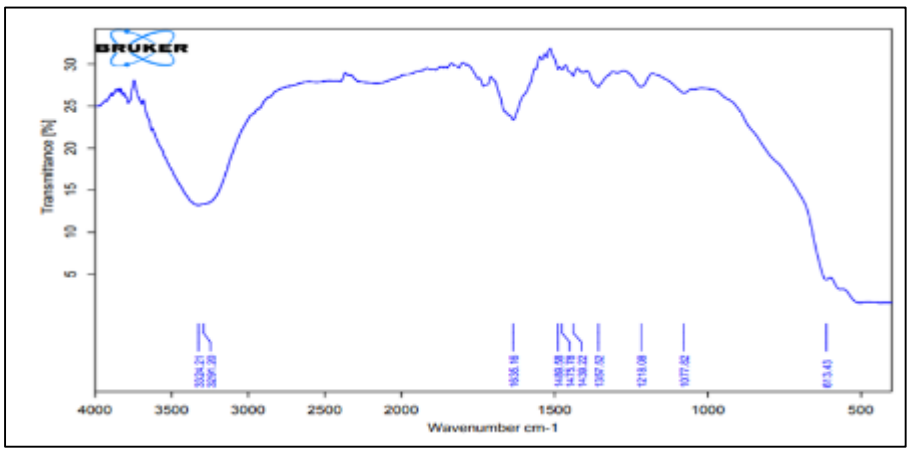

Figure 8 Biodegradation of Methylene Blue by *A. ochraceus* after 7 days incubation by (FTIR) at a concentration of (150) ppm with pH = 7

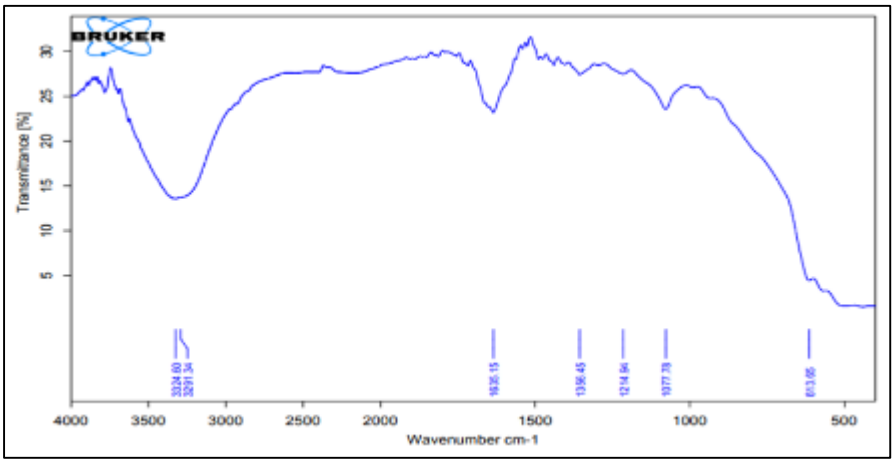

Figure 9 Biodegradation of Methylene Blue by *A. ochraceus* after 7 days incubation by (FTIR) at a concentration of (250) ppm with pH = 7

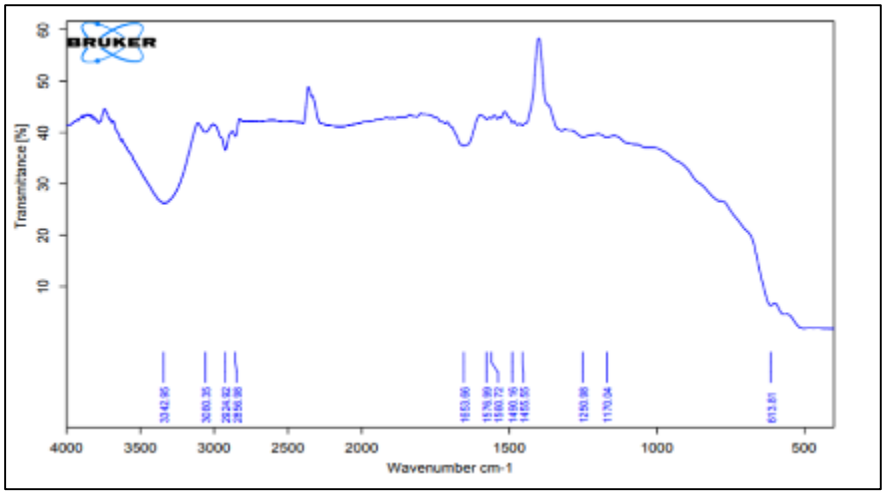

Figure 10 Biodegradation of Methylene Blue by *A. niger* after 7 days incubation by (FTIR) at a concentration of (50) ppm with pH = 4

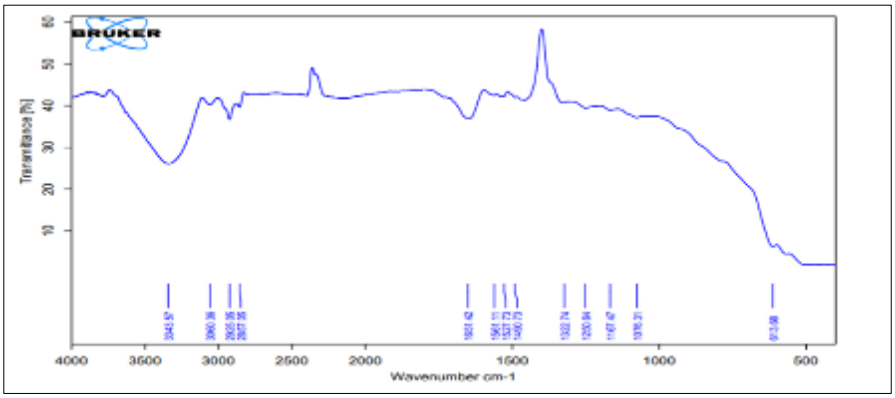

Figure 11 Biodegradation of Methylene Blue by *A. niger* after 7 days incubation by (FTIR) at a concentration of (150) ppm with pH = 4

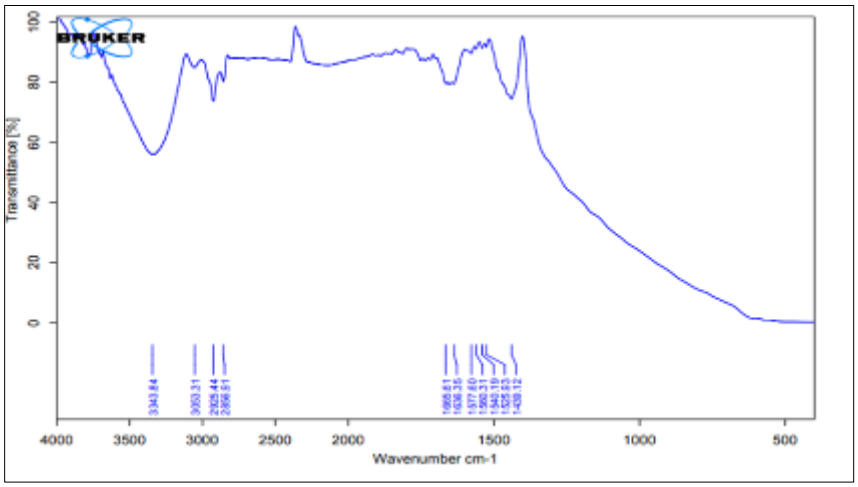

Figure 12 Biodegradation of Methylene Blue by *A. niger* after 7 days incubation by (FTIR) at a concentration of (250) ppm with pH = 4

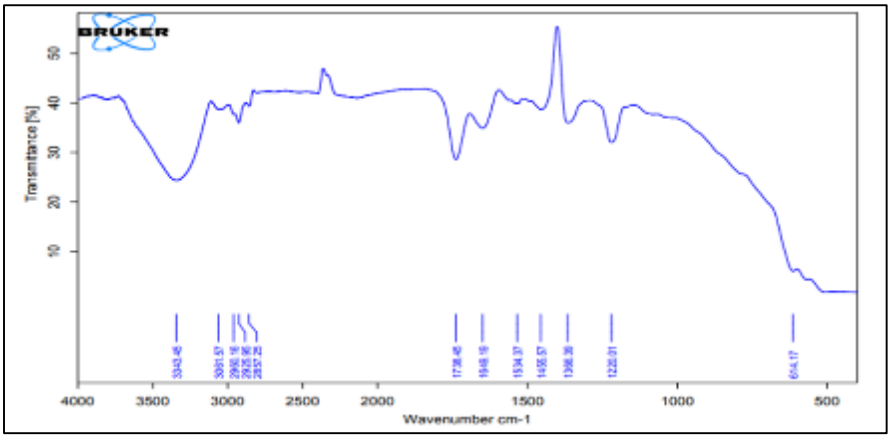

Figure 13 Biodegradation of Methylene Blue by *A. ochraceus* after 7 days incubation by (FTIR) at a concentration of (50) ppm with pH = 4

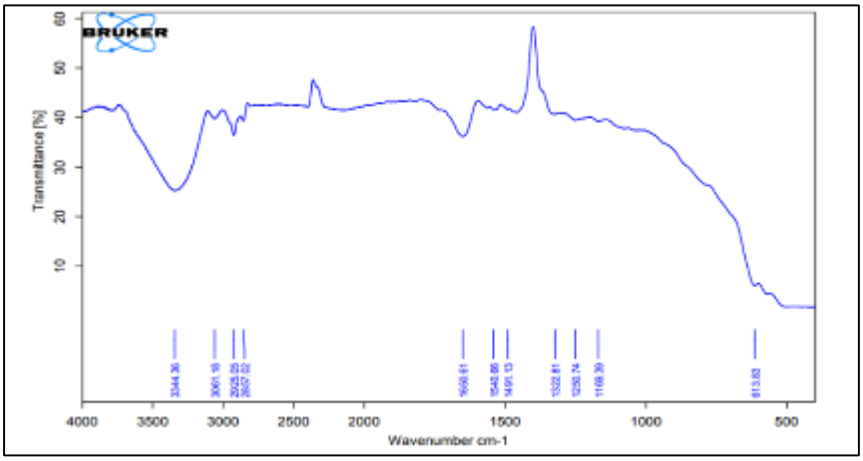

Figure 14 Biodegradation of Methylene Blue by *A. ochraceus* after 7 days incubation by (FTIR) at a concentration of (150) ppm with pH = 4

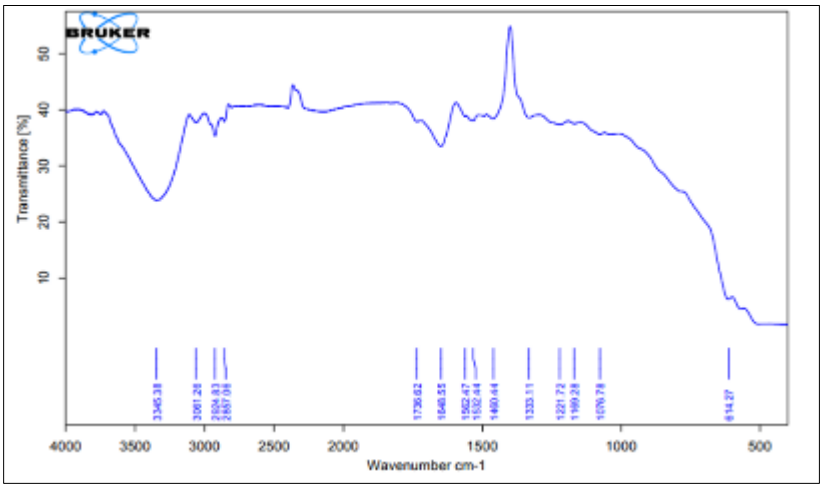

Figure 15 Biodegradation of Methylene Blue by *A. ochraceus* after 7 days incubation by (FTIR) at a concentration of (250) ppm with pH = 4

4. Conclusion

The result of our study we find that these specific types of fungi have the potential to provide an affordable, convenient, and environmentally friendly remedy for dye waste. The use of these fungi in rehabilitating rivers contaminated with MB dye showed promising results in reducing and eliminating dye pollution.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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