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Monitoring 2,4-D removal by filamentous fungi using electrochemical methods

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Abstract

Pesticides that spread into the environment from residues not only cause environmental pollution but also have negative effects on living organisms. Studies in recent years have focused on removing pesticides from aquatic environments. This study investigated the biological treatment of 2,4-D (2,4-Dichlorophenoxy Acetic Acid), one of the pesticides widely used in Turkey, by *Aspergillus versicolor* and *Rhizopus arrhizus*. The 2,4-D is used as a herbicide, and its water solubility allows it to pass from soil to water and spread easily. Our research aims to monitor 2,4-D removal from aqueous environments with different fungal species using electrochemical methods. Molasses was used as a carbon source to reduce the cost of the medium used for fungal growth. The bioremoval and biosorption mechanisms were examined for 2,4-D removal. The maximum 2,4-D bioremoval rates by *R. arrhizus* and *A. versicolor* species growing on molasses medium were 78.58% and 85.78%, respectively. Also, *R. arrhizus* and *A. versicolor* achieved 62.7% and 78.1% biosorption of 2,4-D at optimal conditions (pH 2 and 15 mg/L 2,4-D concentration), respectively. This study showed that filamentous fungi can be used in the bioremediation of pesticide-contaminated environments with a cheap and environmentally friendly approach.

Introduction

Pesticides are substances or mixtures used to prevent, control, or reduce the damage of harmful organisms (Gupta et al., 2011). Modern agricultural practices have led to the widespread use and development of synthetic pesticides (Tudi et al., 2021). Considering the amount of pesticide use in Turkey, while a total of 38.6 thousand tons of pesticides were used in 2010, this amount reached 57.8 thousand tons in 2023 (Ministry of Agriculture and Forestry, 2023). Pesticides can be found in air, water, soil, rain, snow, ice, and surface water (Boonupara et al., 2023). When pesticides are applied to soil, plants, or seeds, they reach water, air, and soil by various transports depending on the chemical properties of the substance and cause seriousenvironmental problems (Ahmad et al., 2024). While some of the pesticides used evaporate and cause environmental problems in the atmosphere, some of them break down by photochemical means and turn into toxic or non-toxic substances (Pathak et al., 2022). Another part is retained in the soil, pollutes the soil, and breaks down as a result of chemical and microbiological activities in the soil. Pesticide residues are also transferred from the soil surface by rain, flood, and snow, polluting rivers, lakes, and groundwater (Kim et al., 2023). The detection of dichlorodiphenyltrichloroethane (DDT) even in creatures living at the poles where no pesticides have been applied shows the transport of pesticides around

the world (Mitra et al., 2024). The damage to the environment is increasing, especially as a result of unconscious and excessive use and storage of pesticides and not paying enough attention to the disposal of excess pesticides (Pathak et al., 2022).

The term pesticide covers all chemical substances classified as insecticides (insect killer), herbicides (weed killer), fungicides (mold killer), rodenticides (rodent killer) etc. (Tudi et al., 2021). Pesticides, despite their harm, are compounds that produce a wide range of toxic effects that pose potential pollution in the environment (Kaur et al., 2024). Pesticides have high solubility in water, low absorption in soil, and high persistence in nature. Therefore, the pesticide-contaminated water reaches the surface and groundwater and pollutes these environments (Lousada et al., 2023). Studies conducted in recent years have detected the presence of high concentrations of toxic and carcinogenic pesticides in groundwater, surface waters, and living creatures in these environments, and it has been stated that these substances are carried into groundwater and pollute water resources (Mishra et al., 2023). In addition, if pesticides are not degraded by bacteria, fungi, sunlight, or chemical means, they can accumulate in the soil over time and be taken up by plants (Swathy et al., 2024). Pesticide-exposed plants have the potential to penetrate human and animal bodies.

In addition to environmental problems such as contamination of soil, groundwater, and surface water, the chemicals used also pose some risks to human health, as they cause direct poisoning and food/drinking water residues (Mishra et al., 2023).

Pesticides mixed into the soil as a result of agricultural activities mix with surface and ground water (Rasool et al., 2022). New technologies that support the efficient elimination of these types of pollutant compounds need to be developed (Coccia & Bontempi, 2023). Biological treatment of wastewater, groundwater and liquid toxic waste provides a more economical alternative compared to other treatment methods (Gül et al., 2022). Biological treatment is preferred because it is a cheap and easily applicable method for the mineralization of organic substances such as pesticides. Recently, studies on biological treatment of pesticides have gained importance (Gül & Silah, 2023; Pathak et al., 2022).

There are studies in the literature showing that pesticides can be removed from liquid media by methods such as biodegradation and biosorption using different fungal species (Bhatt et al., 2020; Legorreta-Castañeda et al., 2020; Ruomeng et al., 2023; Swathy et al., 2024). Recent studies have focused on the removal of 2,4-D herbicide, a widely used pesticide, and removal studies with filamentous fungi have not yet been presented in the literature. The aim of this study was to investigate the removal capacity of the herbicide named 2,4-D (2,4-Dichlorophenoxy acetic acid), which is widely used in our country, by *A. versicolor* and *R. arrhizus* fungi growing on cheap media. In order to reduce the cost in the study, molasses, which is sugar factory waste, was used as a carbon source in the medium for microbial growth.

Materials and Methods

Microorganism culture and medium

In the project study, pure cultures of *A. versicolor* and *R. arrhizus*, which were in the culture collection of Ankara University, Faculty of Science, Department of Biology, Biotechnology laboratory, were used. In the project study, molasses, which is sugar factory waste, was used to reduce costs and to utilize another waste material that causes environmental pollution. The composition of the molasses medium is 1.0 g/L $(NH_4)_2SO_4$, 0.5 g/L KH_2PO_4, and 80 ml molasses (Dönmez, 2002).

Determination of the pesticide toxicity on microbial growth

Microorganisms were inoculated into media containing 2,4-D at different concentrations (5, 10, 15, 30, and 50 mg/L) to determine the growth ability of microorganisms. The 250 ml flasks containing 100 ml of molasses medium with pesticides were used. Also, molasses medium without pesticide was used as a control group. At the end of the incubation period, microbial growth was determined by the dry weight method (Sadettin & Dönmez, 2007).

Bioremoval experiments

To determine the optimal 2,4-D pesticide removal, 100 ml of liquid molasses media were prepared in 250 ml conical flasks, and its pH was adjusted to 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11. The optimal pH value was determined among these values. Media containing 2,4-D pesticide at different concentrations (3, 5, 7, 10, 15, 30, and 50 mg/L) were prepared to examine the effect of 2,4-D concentration.

Biosorption experiments

In the study, 3 ml of the *A. versicolor* culture, which was grown at 25 °C for 7 days in 100 ml molasses medium (pH: 5) in 250 ml flasks, was inoculated into molasses medium at pH 5 and incubated for 10 days. The entire biomass of *R. arrhizus*, developed in tubes in 5 ml molasses medium (pH: 5) in the incubator at 30 °C for 2 days, was transferred to 100 ml molasses medium in a 250 ml conical flask with pH adjusted to 5 and incubated for 10 days. Both fungal species' biomass was harvested from the medium at the end of the incubation period, treated with 1% formaldehyde, and dried in an oven at 80 °C for 24 hours. Dried samples were ground in a mixer and then sieved and used. Pesticide removal experiments were carried out by adding the obtained biomass to 250 ml conical flasks containing pesticide and distilled water at a rate of 1 g/L. The pH values of 2, 3, 4, and 5 were used to determine the effect of pH on biosorption. In the experiments on the effects of 2,4-D

concentration on biosorption, the effect of 5, 10, 15, 30, and 50 mg/L pesticide concentrations were investigated.

Pesticide Determination Method

In this study, it was also aimed to develop a suitable method for the analysis of 2,4-D pesticides using electrochemical methods and electrodes. For this purpose, it was examined with carbon electrodes, and the electrochemical method and conditions were determined for quantitative determination (Gül & Silah, 2023). Firstly, the most appropriate parameters for voltammetric determinations of the 2,4-D pesticide used in the study were determined. For this purpose, the reduction or oxidation behavior of each active substance and the character of the electrode reaction (whether it is adsorption or diffusion-controlled) were first examined by cyclic voltammetry.

To determine pesticide removal, an electrochemical method was developed to quantify pesticides in samples. According to this method, Britton Robinson buffer (BRT) was first prepared for pH screening. For BRT, a stock buffer solution was prepared by dissolving 2.7 mL phosphoric acid, 2.3 mL acetic acid and 2.5 grams of boric acid in 1 L of pure water. Then, taking 100 mL of this stock solution and adding 0.1 M HCl drop by drop to pH 2, and again taking 100 mL and adding 0.1 M NaOH drop by drop to pH 3, 4, 5, 6, 7, 8, 9, 10, and 11 were prepared. Stock 2,4-D solution was prepared by dissolving it in pure water to contain 500 mg/L 2,4-D. Voltammograms obtained for 50 and 100 mg/L 2,4-D standard solutions in different pH environments are given in Figure 1.

As a result of the experiments, it was seen that the most suitable pH value for electrochemical analysis of 2,4-D using a potentiostat was 2 (Figure 1). In all electrochemical analyses, the solution pH was adjusted to 2. The removal percentages by microorganisms were determined using electrochemical analysis.

The removal percentage of 2,4-D was calculated from Equation (1);

$$\text{2,4-D removal (\%)} = (C_o - C_f) / C_o \times 100 \qquad \text{Eq. (1)}$$

Where C_o is the initial 2,4-D concentration (mg/L) and C_f is the final 2,4-D concentration (mg/L) in the 2,4-D removal working solutions.

FT-IR Analysis

FT-IR analyses were performed to determine pesticide and fungal surface interactions. Fungal biomasses grown in the 2,4-D (5 mg/L) medium used in the experiments for 7 days were harvested from the medium, washed with water, dried (12 hours at 800 °C), and FT-IR analyses were performed. Also, samples developed and dried in a pesticide-free medium were used as the control group. Thus, the FT-IR technique was used to identify the functional groups on the surfaces of *A. versicolor* and *R. arrhizus* that 2,4-D interacts with and to observe the changes in the pesticide environment. FT-IR spectra were recorded using a Perkin Elmer (Spectrum 100) brand Fourier transform infrared spectrophotometer.

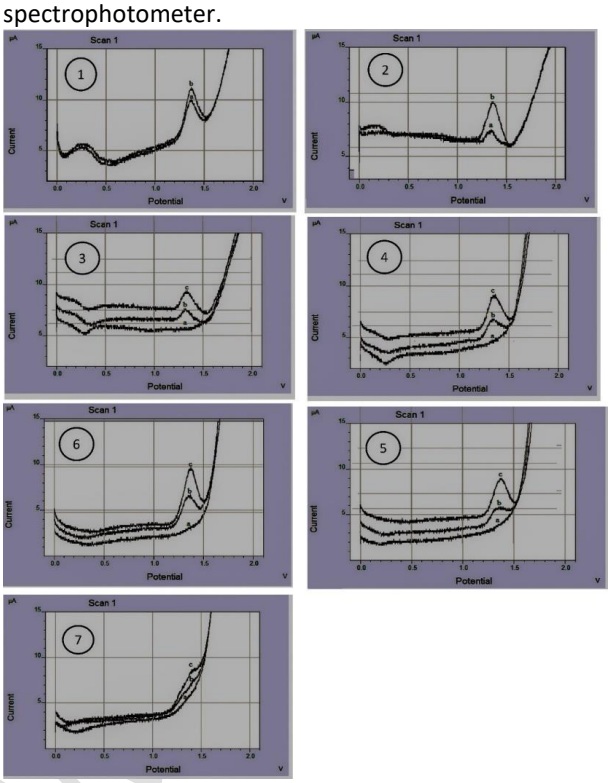

Figure 1. Voltammograms obtained for 50 and 100 mg/L 2,4-D standard solutions in different pH environments. 1: a) 50 mg/L 2,4-D in pH 2 BRT buffer b) 100 mg/L 2,4-D; 2: a) 50 mg/L 2,4-D b) 100 mg/L 2,4-D in pH 3 BRT buffer; 3: in pH 4 BRT buffer a) 10 mL pH 4 BRT buffer b) 50 mg/L 2,4-D c) 100 mg/L 2,4-D; 4: in pH 5 BRT buffer a) 10 mL pH 4 BRT buffer b) 50 mg/L 2,4-D c) 100 mg/L 2,4-D; 5: in pH 6 BRT buffer a) 10 mL pH 4 BRT buffer b) 50 mg/L 2,4-D c) 100 mg/L 2,4-D; 6: in pH 7 BRT buffer a) 10 mL pH 4 BRT buffer b) 50 mg/L 2,4-D c) 100 mg/L 2,4-D; 7: in pH 8 BRT buffer a) 10 mL pH 4 BRT buffer b) 50 mg/L 2,4-D c) 100 mg/L 2,4-D.

Results and Discussion

Determination of the effect of 2,4-D concentration on fungal growth

To determine the effect of increasing 2,4-D concentration on fungal growth, the dry weight of the fungal species that developed at the end of the 15-day incubation period in media containing 3, 5, 7, and 10 mg/L 2,4-D was determined.

As seen in Figure 2, the augment of pesticide concentration decreased the fungal growth. Recently, Gül & Silah (2023) showed that the pesticide called Cyromazine affected the growth of filamentous fungi negatively. Sarhan (2020) also reported that 2,4-D inhibited the growth of different fungal species according to their sensitivity (Sarhan, 2020). As shown in Figure 2, increasing 2,4-D concentration prevented the development of *R. arrhizus*, while there were no significant differences in the development of *A.*

versicolor. Both species showed the least growth in the environment containing 10 mg/L 2,4-D. In the literature, the effects of 0, 1, 10, 50, 100, 200, 300, 500, and 1000 mg/L 2,4-D concentrations on the growth of the fungus *Penicillium chrysogenum*, which is a type of fungus that degrades 2,4-D, were investigated (Mendes & Leitão, 2009). According to the study results, increasing the 2,4-D concentration has been shown to inhibit fungal growth and conidia formation (Tortella et al., 2005). Similarly, the increase of 2,4-D concentration prevented the fungal growth of both fungal species in this study (Figure 2).

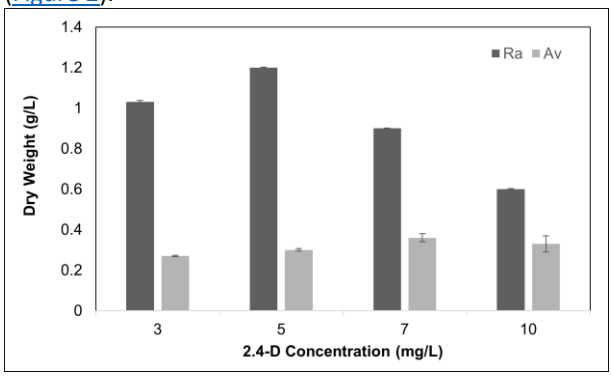

Figure 2. The effect of 2,4-D concentration on fungal growth (Av: *A. versicolor*; Ra: *R. arrhizus*).

Determination of optimum pH for bioremoval

In the studies, 2,4-D was added to molasses media at different pH values (pH: 2,3,4,5,6,7, and 8) and analyzed to determine the pH of the medium at which both fungi provide the best 2,4-D bioremoval. 100 ml of molasses media containing 5 mg/L 2,4-D and pH 2,3,4,5,6,7, and 8 were prepared in 250 ml Erlenmeyer flasks, then *A. versicolor* and *R. arrhizus* were inoculated. During the incubation period, neither fungal species grew at pH 2. While incubation was carried out for 3 days at 25 °C in a 100 rpm shaking incubator, 3 ml samples were taken daily and after centrifugation at 10000 rpm, the supernatants were analyzed electrochemically. The obtained voltammograms were examined, and then the percentage removal calculations were made by following the decrease in peak currents.

Solution pH is the key parameter affecting the electrostatic interactions between surfaces and the contaminant to be removed (Long and Yang, 2001). A study showed that the removal of 2,4-D by activated carbon was best at pH 2 and 3, i.e., low pHs (Sezer and Aksu, 2013). Dehghani et al. (2014) investigated the effect of pH on 2,4-D removal in aqueous solutions and found that the best removal was at pH 3. Since the fungal species used in the study did not grow at pH 2, it was determined that the best removal occurred at pH 3. In another study, it was reported that 2,4-D decreased in the environment as the pH increased, and the reason for this was explained as the acidic structure of 2,4-D may have hydrolyzed at neutral and alkaline pHs (Bazrafshan et al., 2013). It was determined that 2,4-D decreased, i.e. hydrolyzed, in the control groups at neutral and alkaline pH values in this study. Therefore, it was deemed appropriate to try pH 2, 3, 4, and 5 in biosorption studies. The maximum bioremoval of 2,4-D by *R. arrhizus* and *A. versicolor* (growing in the medium) was determined at pH 3 with values of 78.58 and 75.30%, respectively (Figure 3).

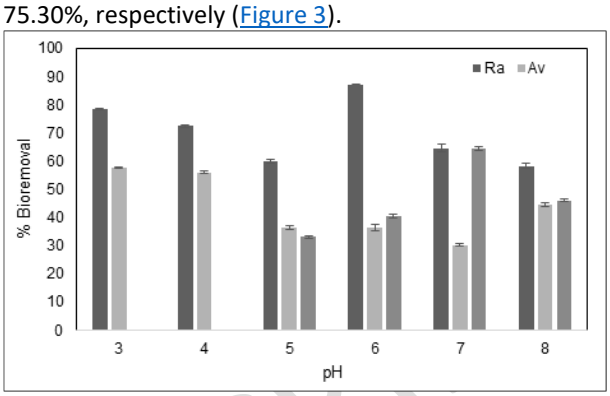

Figure 3. The effect of pH on 2,4-D bioremoval by fungi (Av: *A. versicolor*; Ra: *R. arrhizus*; C: Control).

Determination of optimum 2,4-D concentration for bioremoval

In the studies, experiments were carried out by adding 2,4-D to molasses media at different 2,4-D concentrations (3, 5, 7, and 10 mg/L) to determine the effect of the initial concentration on the best 2,4-D removal of both fungi. The fungal cultures were incubated in pH 3 medium for 6 days and daily samples were analyzed. In this series of experiments, samples were taken and analyzed during the 15-day incubation period and it was observed that there was no significant difference after the 6th day. As seen in Figure 4, different 2,4-D concentrations did not significantly affect the 2,4-D removal performance of both fungi species. The best removal of *R. arrhizus* was 78.58% in the medium containing 5 mg/L 2,4-D. *A. versicolor* species removed 85.78% of 7 mg/L 2,4-D. Boivin et al. (2005) showed that 2,4-D mineralization in soil samples was 48% after 10 days.

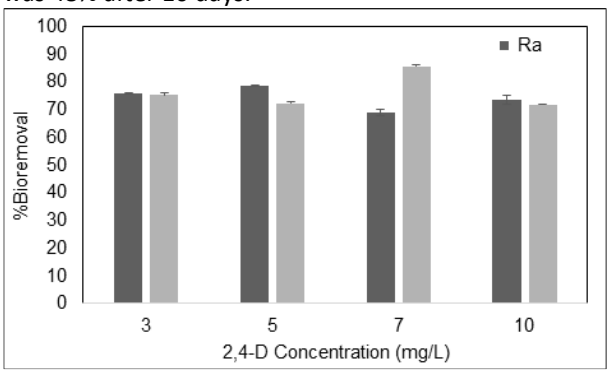

Figure 4. The effect of 2,4-D concentration on 2,4-D bioremoval by fungi (Ra: *R. arrhizus*; Av: *A. versicolor*; pH 3).

Determination of optimum pH for biosorption

In the experiments on the effect of pH on 2,4-D biosorption, low pH values were tried because 2,4-D hydrolyzes at alkaline pH values (Bazrafshan et al., 2013), and as a result of the analysis of the removals in

Table 1. Comparison of the results of this study with the results of 2,4-D removal studies in the literature

Microorganism or adsorbent/biosorbent	pH	2,4-D concentration (mg/L)	R%	Reference
Penicillium sp.	-	100	29.80	Itoh et al. (2000)
Burkholderia cepacia DS-1,	7.2	50	69.00	Cyco´n et al. (2011)
Pseudomonas sp. DS-2	7.2	50	73.00	Cyco´n et al. (2011)
Sphingomonas paucimobilis DS-3	7.2	50	54.00	Cyco´n et al. (2011)
Granular activated-Carbon (Adsorbent)	3	0.5	63.00	Dehghani et al. (2014)
Amine-modified magnetic-Nanoparticles Adsorbent)	6	20	65.00	Jazini et al. (2020)
Aspergillus penicilloides,	4.5	100	52.00	Hayashi et al. (2021)
Umbelopsis isabelina	4.5	100	46.00	Hayashi et al. (2021)
R. arrhizus	3	5	78.58	In this study
A. versicolor	3	7	85.78	In this study
R. arrhizus (Biosorbent)	2	15	62.70	In this study
A. versicolor (Biosorbent)	2	15	78.10	In this study

the medium containing 15 mg/L 2,4-D, it was determined that the optimal pH for the biomass obtained from both fungal species was 2 (Figure 5).

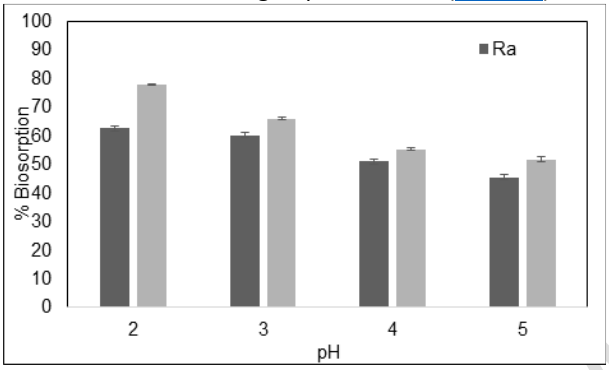

Figure 5. pH effect on 2,4-D biosorption (initial 2,4-D concentration: 15 mg/L, contact time: 480 min.).

Determination of optimum 2,4-D concentration for biosorption

In biosorption experiments using biomass obtained from both mushroom species, different concentrations of 2,4-D (5, 10, 30, and 50 mg/L) were tested to investigate the effect of initial 2,4-D on 2,4-D removal by fungi. Bazrafshan et al. (2013) showed that 2,4-D adsorption onto carbon nanotubes increased with increasing initial concentration (1-5 mg/L). The initial 2,4-D concentration creates the power to overcome the transfer resistance of 2,4-D from the liquid phase to the solid phase; thus, the 2,4-D adsorption capacity increases with increasing 2,4-D concentrations (Bazrafshan et al., 2013). In this study, it was determined that the removal of *A. versicolor* biomass increased with increasing 2.4 D concentrations from 5 mg/L to 30 mg/L, but the removal started to decrease at 50 mg/L (Figure 6). *R. arrhizus* biomass decreased removal at increasing 2,4-D concentrations after 30 mg/L. Aksu and Karabayır (2008) showed that increasing dye concentration increased fungal biosorption because fungal surfaces did not reach adsorption saturation at low dye concentrations. As seen in Figure 6, both *R. arrhizus* and *A. versicolor* species performed the best removal in the environment containing 15 mg/L 2,4-D. Accordingly, the surface of both mushrooms reached saturation in the environment containing 15 mg/L 2,4-D.

Articles related to 2,4-D removal in the literature were examined, and the results are presented in Table 1. Although there is no literature on the 2,4-D removal of the filamentous fungal species used in this study, different adsorbents, bacterial, and fungal species performed 2,4-D removal according to the literature. When the results of this study are compared with the 2,4-D removal performances in some studies in the literature, it is observed that the used organisms perform 2,4-D removal at higher rates (Table 1).

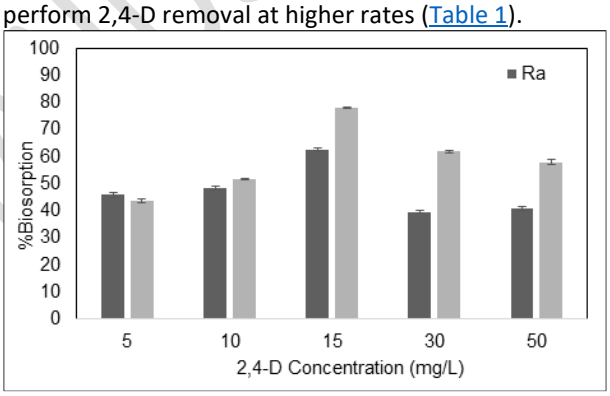

Figure 6. Effect of initial 2,4-D concentration on 2,4-D Biosorption (pH: 2; contact time: 1440 min.).

FT-IR analysis

IR spectra of natural and pesticide-bound *R. arrhizus* biomasses are given in Figure 7a. The IR spectrum of *R. arrhizus* biomass before biosorption shows amino, carboxyl, hydroxyl, and phosphate groups on the biomass surface. The broad band at 3270.8 cm^{-1} indicates the presence of amine groups. 2919.4 and 2853.1 cm^{-1} are characteristic bands indicating the presence of alkyl chains (Gül and Silah, 2023). The strong band seen at approximately 1633.3 cm^{-1} belongs to the vibration of the carboxyl (C=O) group in the amide structure. The band seen in the 1540.5 cm^{-1} region is the stretching vibrations of N-H and C-N bonds, while the band in the 1363.6 cm^{-1} region belongs to more complex amide bonds (Ibrahim et al., 2015). The bands at 1431.8-1409.1 cm^{-1} indicate carboxylate groups (COO-) on the biomass surface. The band at 1032.3 cm^{-1} originates from the C-O bond, which is a characteristic band for polysaccharides. Basic carbonyl and amine groups are also seen in the IR spectrum of *A. versicolor biomass*

(Figure 7b). Shifts in the position of the basic peaks seen in the spectrum and changes in the intensity of the bands indicate the biosorption of the pesticide onto the biomass surface.

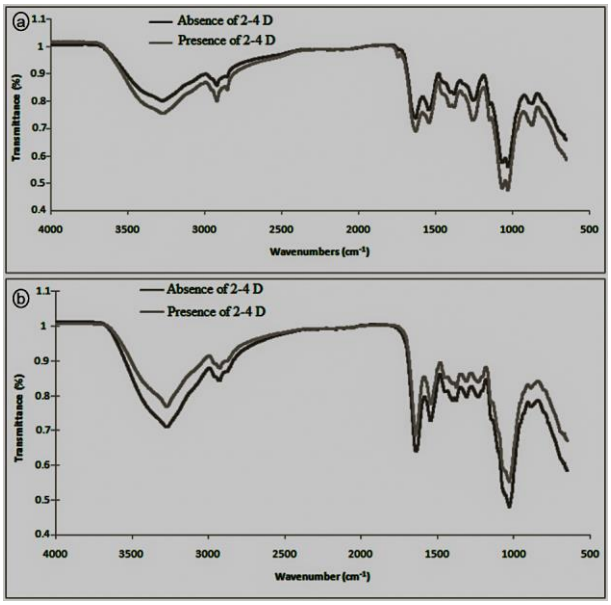

Figure 7. IR spectra of natural and pesticide-bound fungi: a) *R. arrhizus*; b) *A. versicolor.*

Conclusion

In this study, the biological treatment of 2,4-D, one of the pesticides widely used in our country, by *A. versicolor* and *R. arrhizus* species growing in the medium was investigated. According to the results of bioremediation experiments, it was determined that the best removal occurred at pH 3 since the fungal species used in the study did not grow at pH 2. In biosorption studies, the best removal of 2,4-D was achieved at pH 2 with biosorbents obtained from both fungal species. In a study conducted in the literature, it was shown that the removal of 2,4-D by activated carbon was best at pH 2, i.e., at low pHs (8). Dehghani et al. (2014) investigated the effect of pH on 2,4-D removal in aqueous solutions and found that the best removal was at pH 3. In another study, it was reported that 2,4-D decreased in the environment as the pH increased, and the reason for this was explained as the acidic structure of 2,4-D may have hydrolyzed at neutral and alkaline pHs (7). Similarly, in this study, it was determined that 2,4-D decreased, i.e., was hydrolyzed, in the control groups at neutral and alkaline pH values. The results of this study are consistent with the literature findings. According to the results of this study, *A. versicolor* and *R. arrhizus* species successfully removed 2,4-D in a low-cost environment. Since there is no study in the literature on monitoring the 2,4-D removal of the fungal species used in the study using electrochemical methods, the findings of this study are an important source of information.

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Author Contributions

Ü.D. GÜL: Project Administration, Supervision, Conceptualization, Formal Analysis, Investigation, Methodology, Validation Writing – Original Draft Preparation, Writing – Review & Editing; H. SİLAH: Formal Analysis, Methodology, Validation.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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