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Amoxicillin and Favipiravir Bio-Degradation by Aspergillus Flavus Fungus

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Abstract

The aim of this study is to isolate and characterize Amoxicillin and Favipiravir biodegrading fungi as well as determine their characteristics and degradation pathways. The antibiotic-degrading fungus *A. flavus* was isolated from polluted wastewater samples using standard Potato Dextrose agar and Czapek–Dox medium. The biodegradation method was investigated in previous mediums with (Amoxicillin and Favipiravir), as the sole carbon sources. Main degradation intermediates were analyzed by high-performance liquid chromatography (HPLC) and used to deduce the antibiotic degradation pathway of strain *A. flavus* fungal hyphae by Scanning Electron Microscopy before and after 7 days of treatment to find out the accumulation of the antibiotic and morphological changes in fugal hyphae. Fungi can use antibiotics as their sole carbon source. Maximum biodegradation was observed at 91% at the lowest concentration of Amoxcillin. According to HPLC results, the *A. flavus* retention time of standard Amoxicillin was 8.12 minutes after biodegradation, when it broke down into other compounds with retention times of 3.75 minutes by 73.25%, 5.81 minutes by 16.44%, and 8.5 minutes by 8.59. According to standard Favipiravir retention time, it appeared in 3.97 minutes, but after treatment, two materials appeared in 2.20 minutes by area: 62.58% and in 6.16 minutes by area: 20.15%. Scan Electron microscopy images regarding treatment with Favipiravir by *A. flavus* showed an accumulation of particles on the fungal hyphae. This investigation gives insights into the improvement of bio-remediation methods to remove antibiotics from wastewater before they are discharged into rivers.

Keywords: Antibiotics, Aqueous solution, bio- remediation, fungi, fungal pellets.

Introduction

In recent decades, the widespread emergence of matters such as pharmaceuticals, pesticides, and other organic pollutants in the aquatic environment has been recognized as an emerging environmental issue because it can have negative impacts on the ecosystem and human health [1] .The main source of these compounds is municipal wastewater treatment

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plants. A conventional wastewater treatment plant is generally designed to remove high concentrations of mainly biodegradable organic compounds [2]. A major source of pharmaceuticals in the environment is the drug manufacturing factories. Previous studies recorded high concentrations of pharmaceuticals reported downstream from a pharmaceutical company of up to 1 mg/L of carbamazepine in the aquatic environment below a pharmaceutical factory [3]. mbrane processes, and activated carbon is considered a promising alternative to remove pollutants from wastewater, but these techniques are expensive and produce by-products, which are still limiting factors in implementing the advanced treatment technologies as an alternative to an effective wastewater treatment process[4]. Wherefore sewage treatment plants may use biological treatment to treat pharmaceuticals that are no longer metabolized [5,6]. In nature, fungi serve as decomposers, mutualists, and pathogens, which belong to the group of eukaryotic multicellular organisms. They have hyphal filamentous structures, and fungal cell walls contain chitin, which is a carbohydrate polymer consisting of chains. In addition, fungi are heterotrophic, and saprophytes produce analytic enzymes named laccases and ligninolytic peroxidases [7,8]. The cell wall structure of fungi is of great importance because of the natural chemistry of cell walls. As a result, fungi have developed diverse strategies to combat toxic compounds, such as antibiotics in Basidiomycetes involved in the oxidation of a wide range of pollutant compounds with aromatic structures, including polycyclic aromatic hydrocarbons [9]. These strategies include the use of enzymes for enzymatic processes such as adsorption, mineralization (bio-precipitation), and biotransformation. [10]. Biosorption into fungal mycelium plays a key role in the removal of bisphenol A and 17-ethinylestradiol until equilibrium is reached [11,12]. The aim of this study is to evaluate the biodegradation of Amoxicillin and Favipiravir by isolated *A. flavus* fungi.

Materials and Methods

Fungal Isolates and Fungal Pellets Formation

10 soil samples were taken for this study from the Tigris River in Baghdad, which was polluted with pharmaceuticals, Iraq. After growing fungal isolates were purified and identified based on morphological characteristics, these samples were used to isolate fungi on PDA plates. Two different types of media were used for the growth of fungus, including Potato Dextrose Agar: 200 g of unpeeled, sliced potatoes are boiled in 1 liter of water for 30 minutes. The resulting potato infusion is then filtered through cheesecloth, and the effluent is added together with dextrose, agar, and water. The media was sterilized in an autoclave at 121°C with a flow rate of 15 l/min for 15 minutes, then pouring it into a Petri dish where it can harden before being planted. The yeast extract broth was made by dissolving 20 grams of yeast extract powder and 5 grams of peptone in one liter of distilled water. The pH of the broth was then corrected to 5.5 by adding drops of 1 M HCL and sodium hydroxide [13].

Antibiotics Preparation

Amoxicillin 250 mg as trihydrate by SDI in Iraq was used, and Favipiravir 200 mg by Atabay Pharmaceuticals and Fine Chemicals Inc. in Turkey was used in concentrations of 1 mg/ml in commercially available tablet samples containing the active ingredient. The stock standard solution of favipiravir, which has poor solubility in water, was processed by dissolving it in 70% concentrated methanol. Acetic, boric, and orthophosphoric acids of analytical grade, as well as extremely clean deionized water, were used to create (PBS) buffer (0.04 M in each component, pH 5.5) [14].

After the DPA culturing was finished, the petri plate was divided into two groups, one of which was used to cultivate *A. flavus*. The other group, however, was used to work the testes to degrade the antibiotic by fungi pieces. To do this, we made small pits of equal dimensions in the group of petri dishes that did not

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have any fungal cultivation. of *A. flavus* from the first group.

Determination of Antibiotics Concentrations by (HPLC) Analysis

A high-performance liquid chromatography method was used to evaluate the amount of amoxicillin and favipiravir eliminated by the fungus. By adding 5 ml of the stock solution to a 250-mL conical flask containing 200 mL of ordinary saline, a concentration of 500 mg/L of antibiotic was attained. Fungal pellets weighing 1 mg were likewise injected and incubated at 25 °C. A 3 mL sample of the culture's aqueous solution was used for the biodegradation investigation after 3, 5 , and 7 days. The experiment was conducted using three replicate flasks, each containing fungus pellets and an identical amount of antibiotic. Prior to HPLC analysis, the treated samples were filtered using a 0.45-m membrane filter. The high-performance liquid chromatography HPLC technique was used to analyze the antibiotic solution before and after the reaction. The HPLC was performed at a flow rate of 1.0 ml/min for 12 minutes, using a UV detector at 250 nm and a waters model column with symmetry of 250 x 4.6 mm, using methanol as the mobile phase, and a flow rate of 0.1 ml/min for 12 minutes.

Calculating the concentrations of antibiotics extracted by fungus and researching antibiotic removal

$$D = \frac{Ci - Ce}{Ci} * 100$$

where D is the removal percentage, Ci is the initial absorbance, and Ce is the absorbance after incubation time [15].

Statistical Analysis

Statistical analysis was achieved by using a one-way ANOVA followed by a student t-test to find out the significance difference between different values of incubation periods using SPSS version 16.0 software. Statically significant values were taken at $P \leq 0.05$.

Results and Discussion

Identification of Isolated Fungal Genera and I ts A bility of Pellets Formation

Identifying specific isolates in studies of the morphology of chosen fungi in cotton-shaped colonies in slide culture medium, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Penicillum* sp., and *Trichoderma* sp. were the five fungal isolates examined for their ability to form pellets. Table. 1, reveals that only *Aspergillus flavus* pellets remained stable after 14 days (after one week). Previous research demonstrated the Zygomycetes fungi's potential[13]. For this reason, fungal pellet biomass was separated from the culture medium by centrifugation at 4000 rpm for 10 minutes [16]. Antibiotic bio-remediation was assessed by wet biomass of *A. flavus* pellets and absorption by wet and dry biomass at antibiotic concentrations of 250 mg/l and 200 mg/l, respectively. The isolated biomass was then washed three times with normal saline.

Table 1.Antibiotics Removal percentage D% and fungal Pellets forming of isolated fungi

Fungi Isolates	Pellet formation ability after 7 days	Mean ± SD with l\mg 250 Of Amoxicillin	l\Mean ± SD with 200 mg Of Favipiravir
Aspergillus niger	Able	66±1.7b	67± 0.7 b
Aspergillus flavus	Able	90 ±0.5a	4.1 ±92 a
Aspergillus fumigatus	able-Non	67±0.3 b	67±0.3 b
.sp Mucor	able-Non	77±b3.1	77 ± 1.1b
.sp Trichoderma	able-Non	87±5.0 b	88±0.3 b

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Different letters represent significant 0.05 ≥difference at P .

Characterization of Antibiotics Bio Degradation by HPLC and SEM

According to the method, the results appeared after fungi activation and antibiotic addition (Amoxicillin, Fig. 1 A: chromatography, Amoxiciilin 10 ppm control). Degradation of antibiotics to two types, Amoxicillin and Favipiravir, in both methods, but to varying degrees according to results. The growth of *A. flavus* was active, which means that antibiotic did not prevent growth, and the fungal enzyme activity is perhaps double the metabolic processes and receiving the degradation. The rationale for using this relatively high concentration range was the possible application of fungal bioremediation technology in the treatment of drug manufacturing effluents. Previous studies indicated that Aspergillus species were able to depredate pollutants in aerobic or anaerobic environmental conditions and can produce a high quality of compost by degradation [15,17].

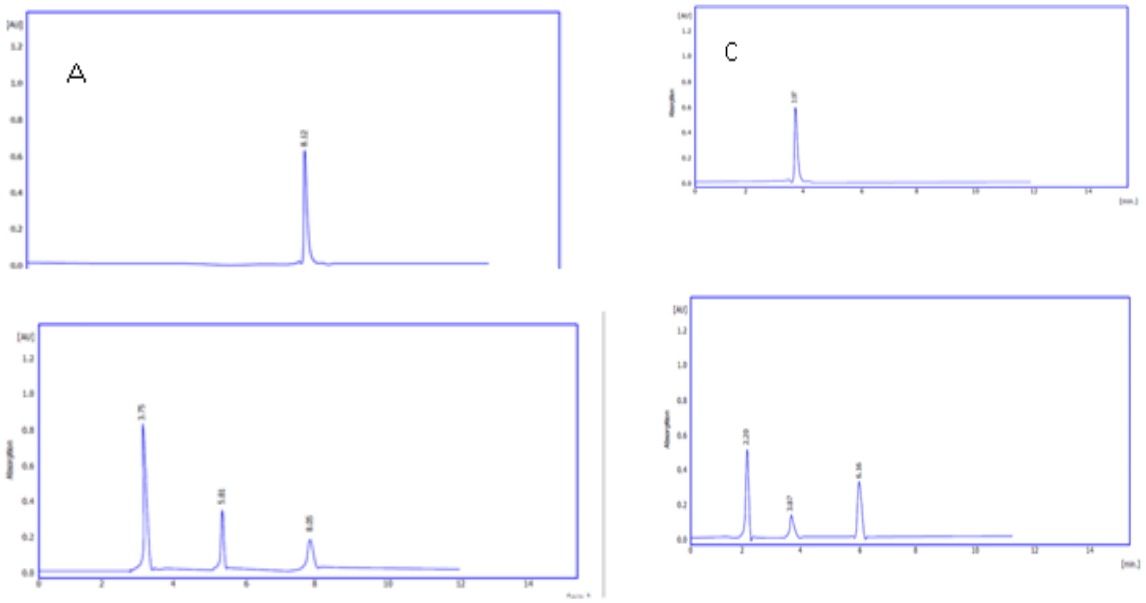

Figure 1. Results of High performance Chromatography A: Amoxcillin control,B: Amoxcillinafter 7 days of treatment,C: Favipiravir control,D: Favipiravir after 7 days of treatment .

Aspergillus spp. has proven its ability to degrade some chemical materials, such as pesticides and soil compounds. The compounds break down at different rates, depending on their physiochemical properties [18]. By many methods, the removal of chemical compounds can be accomplished by biological or chemical methods [19]. Other researchers confirmed its ability to remove and break down bioremediation of some agricultural and industrial chemicals in soil and show HPLC results, Favipiravir appeared in 3.97 minutes, Fig. 1, but after treating it by *A. flavus* the peak appeared in 2.20 min. by area :62.58% and in 6.16 min. by area: 20.15% Fig. 1. Researchers' observation through the diagram was that Amoxicillin appeared in 8.12 minutes in chromatography HPLC while in treatment by *A. flavus* after the fifth day of treatment HPLC test recorded the peak in 3.75 minutes and 73.25%, in 5.81 minutes by 16.44%, and in 8.5 minutes by 8.59. Fig. 1:A,B. This refers to receiving degradation by fungi because of the formation of another material in less than 8 minutes. According to the results, *A. flavus* was efficient in decomposing Amoxicillin and Favipiravir because of its ability to decompose in environmental conditions (soil, plants) due to its

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production of cell wall degrading enzyme [20]. Despite the differences between the two cases in ratio decomposition, *A. flavus* has endoglucanase (EG) and -glucosidase (BGL). Cell degrading enzyme production is the key to speeding up the rate of decomposition [6].

Fungi can degrade organic compounds when activated with enrichment media, and this ability was confirmed in *A. flavus* when the ratio of nutrition: N, P, and K content in the nutritional medium was [21.] The rate of degradation was different between the tested antibiotics, Favipiravir was more than Amoxicillin depending on its solubility and toxicity to the environment, as previous findings showed that degradation of pharmaceuticals by fungi completed in the seventh day of treatmen[22].

In this study, according to decomposition level, it appears in the results of the HPLC chromatography that according to the structure of the antibiotics (Amoxicillin and Favipiravir) in Fig. 1, fungi cell wall structure can cause antibiotic restriction due to different charge between them, whereas fungi cell wall charge is negative, so the chemical structure of antibiotics contains effective chemical groups, making it easy for fungi to restrict it, and then decomposing it by enzymatic activities, according to the chemical composition of both antibiotics, degradation of it by fungi was easy due to the presence of an effective group and a positive amino group; HPLC refers to that for Favipiravir it is more degradable than other antibiotics[23, 24].

Results of previous studies and research reported that other fungi like *Tricoderma* produce some active enzymes that can convert material to soluble sugar due to their ability to dismantle proteins since the group that was in Amoxicillin and, Favipiravir has amine proteins [23]. Fungi produce different types of analytic enzymes involved in antibiotics degradation which improved by the phylogenetic analysis and gene sequencing of these proteins [24,25].

Other studies confirmed the role of cytochrome P 450 in hydroxylated diclofenac metabolites. Since the presence of hydroxyl group in the chemical structure of both antibiotics, it is expected to receive hydroxylated diclofenac metabolites [26]. In comparison between treating Amoxicillin and Favipiravir, *A. flavus* degradation was better on Favipiravir more than Amoxicillin clearly during HPLC and SEM Fig. 2, resulting in a greater amount of Favipiravir degradation. This may be due to enzymatic activities for fungi that were active in decomposing structural bonds for Favipiravir. As it became clear from SEM Fig. 2, *A. flavus* is able to restrict the antibiotic (Favipiravir) better than Amoxicillin. These findings should encourage sustainable waste management protocols in the agriculture industry and lower environmental pollution[27-29].

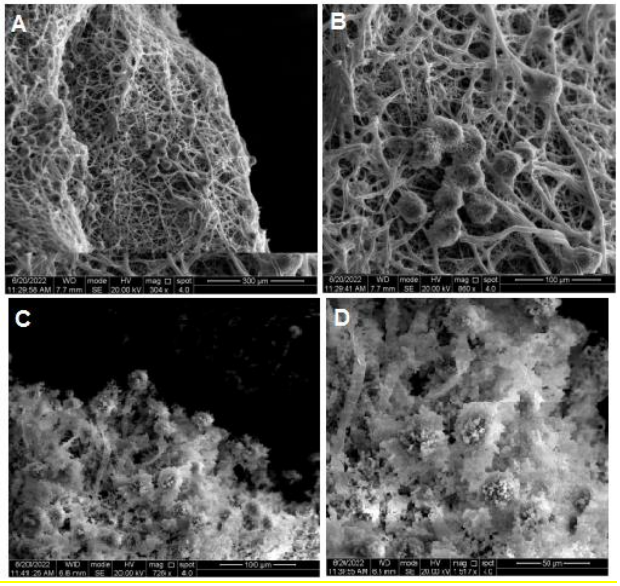

Figure 2. Scanning Electron Microscope A and B: Control without treatment, C and D: after 7 days of treatment with *A. flavus*.

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Conclusion

This study confirms that *A. flavus* is a novel amoxil flavonol-degrading strain. The production of active compounds and degradation rates vary among the tested antibiotics, with Favipiravir showing higher degradation compared to Amoxicillin. This discrepancy can be attributed to its solubility and environmental toxicity. These findings offer a new, environmentally-friendly, and cost-effective approach for treating antibiotic wastewater.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Babylon.

Authors' Contribution Statement

R. H. H. A. designed the study and S. S. M. A. performed the experiments , analyzed data, and wrote the initial draft of the manuscript.A .M . J. A.supervised, reviewed and validated the experiment. All authors revised and approved the manuscript.

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المعالجة الحيوية لمضادات الاموكسيلين والفافيرافير بواسطة الفطريات الخيطية

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الخلاصة

الهدف من هذه الدراسة هو عزل وتوصيف الفطريات القادرة على التحليل الحيوي للأموكسيسيلين و الفافيرافير وكذلك تحديد خصائصها ومسار التحلل. تم عزل الفطر الاسبرجلس من عينات المياه العادمة الملوثة باستخدام أجار البطاطا ووسط الجابك. تم دراسة المعالجة الحيوية في الوسط السابق باستخدام Amoxicillin و Favipiravir كمصدر وحيد للكربون. تم التحري عن المواد الناتجة من التحلل بواسطة كروماتوجرافيا سائلة عالية الأداء (HPLC) واستخدمت لاستنتاج مسار تحلل المضادات الحيوية بواسطة الفطر اسبرجلس. تم فحص الخيوط الفطرية عن طريق المجهر الإلكتروني الماسح قبل وبعد 7 أيام من العلاج لاكتشاف تراكم المضادات الحيوية والتغيرات الشكلية في الخيوط الفطرية. اذ يمكن للفطريات اعتماد المضادات الحيوية كمصدر وحيد للكربون.اذ لوحظ اقصى قدر من التحلل الحيوي هو 91٪ عند اقل تركيز من اموكسيللين. وفقًا لنتائج HPLC كان زمن الاحتجاز للأموكسيسيلين القياسي 8.12 دقيقة بعد التحلل الحيوي إلى مركبات أخرى مع زمن احتجاز 3.75 دقيقة بنسبة 73.25٪ ، وفي 5.81 دقيقة بنسبة 16.44٪ وفي 8.5 دقيقة بنسبة 8.59 ، وفقًا لمعيار احتباس فافيبيرافير. ظهر الوقت في 3.97 دقيقة ولكن بعد العلاج ظهرت مادتان في 2.20 دقيقة. حسب المنطقة: 62.58٪ وفي 6.16 دقيقة. حسب المنطقة: 20.15٪. لاوحظ تراكم جزيئات Favipiravir على الخيوط الفطرية *A. flavus* . تعطي هذه النتائج رؤى في تحسين طريقة المعالجة الحيوية لإزالة المضادات الحيوية من مياه الصرف الصحي قبل تصريفها في الأنهار .

الكلمات المفتاحية: فطريات، كريات الفطريات، مضادات حيوية، معالجة حيوية، وسط مائي .