Al-Kitab Journal for Pure Sciences (2024); 8(2):48-60.
https://doi.org/10.32441/kjps.08.02.p5

Al-Kitab Journal for Pure Sciences
ISSN: 2617-1260 (print), 2617-8141(online)

https://isnra.net/index.php/kjps

Bioremediation of Liquid Waste of Olive Presses (Al-Jeft water) by Using the Biomass of the Local Isolate of The Fungus Helvella bachu

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Citation: Al-rawi JM, Abdul-hadi SY. Bioremediation of Liquid Waste of Olive Presses (Al-Jeft water) by Using the Biomass of the Local Isolate of The Fungus Helvella bachu. Al-Kitab J. Pure Sci. [Internet]. 2024 June. 10 [cited 2024 June. 10];8(2):48-60. Available from: https://doi.org/10.32441/kjps.08.02.p5.

Keywords: Helvellaceae, COD, Total phenol concentration, Bioadsorption.

Article History
Received 18 Apr. 2024
Accepted 21 May 2024
Available online 10 June 2024

Abstract:

The study included investigating the potential of the biomass of the local isolate of the fungus Helvella bachu in bioremediation of the liquid waste of olive presses (Jeft water) at two concentrations of 10%-20%. The mycelium was inoculated on solid media treated with olive press water, with concentrations of 10% and 20%, for several incubation days 2, 4, and 6, respectively. We observed that the diameter of the fungal colony is directly proportional to the increase in the incubation period, as the largest diameter of the fungal colony was 13.56 mm and 11.46 mm after 6 days of incubation at a concentration of 10% and 20% of Jeft water, respectively, compared to the diameter of the fungal colony growing in untreated medium, which it reached 15 mm, while the least diameter of the fungal colony was 6.00 and 5.36 mm at a concentration of 10% and 20% of Jeft water, respectively, after two days of incubation compared to the control sample. As for using liquid media, the results revealed a decrease in the total phenol concentration, reaching 2.14 mg / 100 g after 30 days of treatment with a concentration of 20% of Jeft water, compared to the control treatment of 23.58 mg / 100. The percentage of black color removal was 64.39% at a concentration of 10% and an incubation period of 30 days, while at a concentration of 20%, the percentage of color removal was 49.53% and 42.10% after 30 days of incubation. The biomass of the fungus isolate was able to reduce

the chemical oxygen demand in both concentrations of Jeft water used with different responses. The percentage of chemical oxygen requirement was 65.83% at a concentration of 10% of Jeft water after 30 days of treatment, while the percentage of COD was 8.08% at a concentration of 20% for the same incubation period above.

Keywords: Helvellaceae, COD, Total phenol concentration, Bioadsorption.

المعالجة الحيوية للنفايات السائلة لمعاصر الزيتون (مياه الجفت) باستخدام الكتلة الحيوية للعزلة المحلية للفطر *Helvella bachu*

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الخلاصة:

تضمنت الدراسة التحري عن امكانية الكتلة الحيوية للعزلة المحلية للفطر *Helvlella bachu* في معالجة النفايات السائلة لمعاصر الزيتون (مياه الجفت) حيويا وبالتركيزين ٢٠-١٠ %. زرع الغزل الفطري على الاوساط الصلبة المعاملة بمياه معاصر الزيتون وبالتركيزين ١٠ و ٢٠٪ ولعدة ايام تحضين ٦,٤,٢ على التوالي , لوحظ ان قطر المستعمرة الفطرية يتناسب طرديا مع زيادة مدة التحضين اذ بلغ اكبر قطر لمستعمرة الفطر ١٣,٥٦ ملم و١١,٤٦ ملم بعد مرور ٦ ايام تحضين عند التركيز ١٠ % و ٢٠٪ من ماء الجفت على التوالي مقارنة بقطر مستعمرة الفطر النامية في وسط غير معامل والتي بلغت ١٥ ملم بينما بلغ اقل قطر لمستعمرة الفطر ٦,٠٠ و٥,٣٦ ملم عند التركيز ١٠ % و ٢٠٪ من ماء الجفت على التوالي بعد مرور يومين تحضين مقارنة بعينة السيطرة. اما باستخدام الاوساط السائلة فقد اسفرت النتائج عن انخفاض في تركيز الفينول الكلي إذ بلغ ٢,١٤ ملغم / ١٠٠ غم بعد مضي ٣٠ يوماً على المعاملة بالتركيز ٢٠ % من مياه الجفت مقارنة بمعاملة السيطرة ٢٣,٥٨ ملغم/١٠٠ . النسبة المئوية لإزالة اللون الأسود بلغت ٦٤,٣٩ % عند التركيز ١٠ % وبمدة تحضين ٣٠ يوماً, بينما عند التركيز ٢٠ % بلغت النسبة المئوية لإزالة اللون ٤٩,٥٣ و٤٢,١٠ % بعد مرور ٣٠ يوم تحضين. تمكنت الكتلة الحيوية لعزلة الفطر من خفض متطلب الأوكسجين الكيميائي بكلا التركيزين لماء الجفت المستخدم مع اختلاف الاستجابات. إذ بلغت النسبة المئوية لمتطلب الاوكسجين الكيميائي ٦٥,٨٣٪ عند التركيز ١٠٪ من ماء الجفت بعد مرور ٣٠ يوماً من المعاملة, بينما بلغت النسبة المئوية لـ *COD 8.08* % عند التركيز ٢٠ % لنفس مدة التحضين اعلاه.

الكلمات المفتاحية: Helvellaceae، المتطلب الكيميائي الاوكسجين، تركيز الفينول الكلي، الادمصاص الحيوي.

1. Introduction:

The problem of environmental pollution was and still is at the top of the problems that concern specialists, That is on the rise with an increase in population in population growth and the nature of modern life. Due to the increasing requirements to develop in different parts of life like industrial and welfare of the human race which become wider. These requirements led to the accumulation of huge amounts of toxic compounds which were thrown into the environment without thinking of its dire consequences [1]. The accumulation of toxic compounds casts a shadow over the ecosystem, such as the atmosphere, soil, and water bodies, that has become a dumping ground for factory waste such as industrial dyes, olive presses, hydrocarbon compounds, heavy metals, and pharmaceutical residues, that maybe interact with other compounds and become even more toxic [2]. Therefore, it was necessary to deliberate plans to get rid of these toxic pollutants, because the treatment of these residues by traditional methods (physical and chemical) is not always effective in addition to its high cost, alternative means were searched for, Bioremediation was found as the best option for managing environmental pollutants [3].

Moreover, biological remediation is an innovative, promising, environmentally friendly, and economical technology for managing a polluted environment. It is an attractive and successful cleaning technique for removing toxic waste from a polluted environment by decomposing or eliminating various hazardous chemical and physical materials or detoxifying the surrounding environment through the vital activities of microorganisms at a low cost and without secondary pollution [4]. Therefore, fungi, with their characteristics and survival instinct, have become of pioneering importance in environmental biotechnology because they can metabolize many pollutants of great diversity [5].

Recent research has proven that fungi play important roles in the bioremediation of pollutants, especially in persistent organic pollutants, such as dyes, coal, leather tanning residues, medicines and personal care products, polycyclic aromatic compounds (gasoline and diesel), and liquid wastes of the sugar industry and olive presses. Fungi take all these pollutants substances (substrates) and effectively decompose them to obtain carbon, nitrogen, or the energy it needs for metabolism [6], thus, a wide range of contaminants could be completely removed or at least transformed into less dangerous compounds [3]. The wastewater resulting from the production of olive oil (Jeft) was thrown randomly without regard to its negative impacts on the environmental system. This Jeft water drains directly into river water without developing any strategies to treat these pollutants, which reflect negatively on the environment because of containing a large percentage of complex phenolic compounds (tannins) that are

difficult to biodisintegrate, not to mention the transformation of phenolic dyes, compounds that give the dark color to Jeft water, into phosphorous hydrogen that is highly toxic [19]. In this regard, the ability of the biomass of the local isolate of the fungus *H.bachu* to treat the wastewater of Jeft was investigated using liquid and solid media and in several axes.

2. Materials and methods:

2.1. Test organism: Research experiments were conducted using the biomass of the local fungal isolate *Helvella bachu*, which was morphological and molecular identified by (Al-Rawi, 2022) [7], fruiting bodies obtained from Nimrud district located about 30 km south of the city of Mosul – Iraq. The fruiting bodies were transported to the laboratory and washed with tap water several times to remove all dust and dirt stuck to them, the pollination booth was sterilized with 70% ethanol. The fruiting bodies were cut from different areas, including the stem, the cap, and the heart of the fruiting body, using a sterile scalpel into small parts. The pieces were immersed in 70% ethyl alcohol for 3 minutes, then they were transferred using sterilized forceps by alcohol irradiation to a volumetric bottle containing sterile distilled water. For 5 minutes to remove traces of alcohol, then the pieces of fruiting bodies were placed on sterile Whatman No.1 filter papers and left to dry completely. Pieces of different fruiting bodies were inoculated into sterile Petri dishes containing the Potato Dextrose Agar (PDA) culture medium. The dishes were incubated after covering them with foil in the incubator at a temperature of 28 ± 2º C for a period of 5-10 days while monitoring the growth of fungal hyphae during them to obtain a pure, densely growing fungal colony [21].

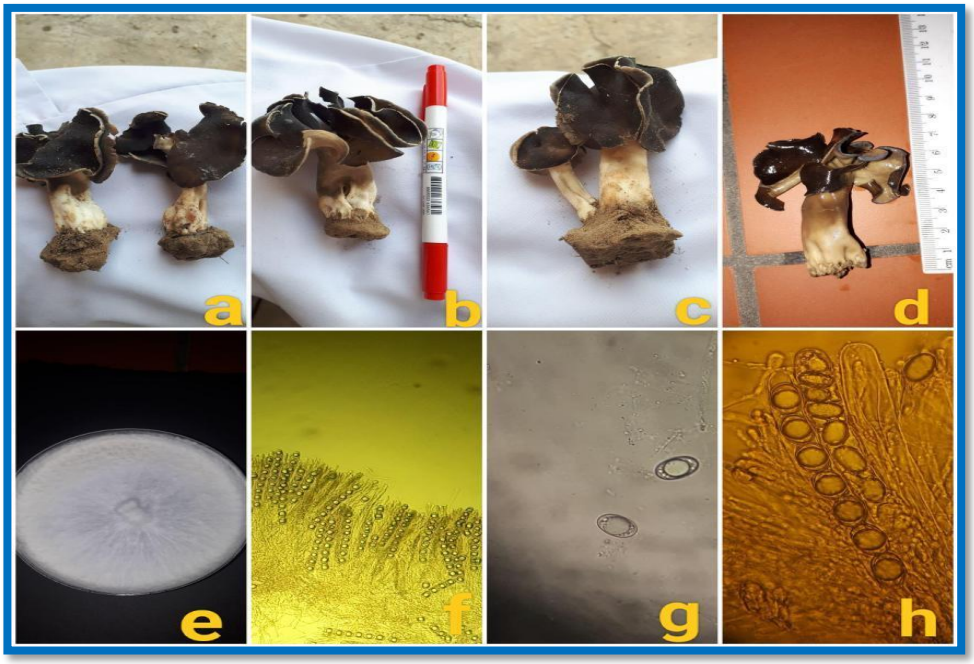

Figure (1): Phenotypic and microscopic identification of the selected local fungal isolate, a-b-c-d: fruiting body, e: fungal colony on PDA nutrient medium, f: asci cysts and paraphysis at 40 X magnification, g-h: ascospores under an optical microscope after staining with lactophenol.

2.2. Olive Presses Water (Jeft Water): Jeft water was obtained from one of the olive presses (the black type) in the Al Fadhiliya district. As a first step, Jeft water was left in a glass flask in the refrigerator for 24 hours so that the oil floated above the surface of the water and was manually disposed of. Then, Jeft water was filtered by passing it through several layers of gauze to remove impurities. The process was repeated three times. The water was kept in dark glass volumetric tubes with a temperature of - 20 ºC until use [15].

2.3. Solid media: Potato Dextrose Agar (PDA)

The nutritional medium was prepared by dissolving 39 gm of the(PDA) powder in a liter of distilled water and mixing well using the magnetic stirrer until complete dissolution. And pH was set at 5.5. The prepared medium was autoclaved at 121 °C at 1 atmosphere pressure for 15 minutes. To avoid contamination of the prepared medium with bacteria, 250 mg/L of chloramphenicol, was sterilized using a 0.45 micron diameter membrane filter. After sterilizing and cooling it in the laboratory atmosphere, it was added to the medium. The medium was distributed in sterile plastic Petri dishes, 15 mL per dish, and the dishes were kept in the refrigerator until the isolate and purifying fungal isolates [8].

2.3. Liquid media: Potato Glucose Broth (PGB)

A 200 gm of peeled potato was cut into cubes were then weighed and put in a glass flask containing 500 ml of distilled water over low heat until cooked. The potatoes were filtered by using two layers of medical gauze then (20) gm of glucose was added to them with continuous stirring until homogeneous on a magnetic stirrer. The volume was filled to 1 L. The pH was adjusted to 5.5. The medium was autoclaved at 121 °C at 1-atmosphere pressure for 15 minutes after distributing it into 250 mL conical flasks [8]. 250 mg/L of chloramphenicol was added to the medium after sterilizing and cooling it in the laboratory atmosphere. Thus to avoid contamination of the prepared medium with bacteria.

3. Agricultural conditions

3.1. solid media: Potato Dextrose Agar (PDA) medium was prepared in glass flasks and peat water was added to it at concentrations of 10% and 20%. The mixture was mixed well and autoclaved at 121ºC and 1-atmosphere pressure for 15 minutes. The medium was left to cool in the laboratory atmosphere, then distributed to sterilized Petri dishes of 15 mL/dish. All plates were inoculated with a disc of a colony of newly growing fungus using a 6 mm diameter cork puncture with three replicates for each concentration leaving a plate untreated to represent the control sample. The plates were incubated after being covered with aluminum foil at a temperature of 28 ± 2 ºC for 6 days. The growth of the mycelium was monitored, as well as,

the growth diameter was measured for periods ranging from 2-4-6 days, and the images were documented using a digital camera [9].

3.2. Liquid media: The researcher indicated [10], that potato glucose medium (PGB) was used. The nutritional medium was distributed in a sterile glass flask with a capacity of 250 mL and at the rate of 100 mL/flask. Jeft water was added to each flask at concentrations of 10% and 20% each separately. Each flask was prepared in duplicate, at the rate of two replications for each treatment. The medium was sterilized under the above-mentioned conditions. After sterilization and cooling, the media were inoculated using sterile alcohol flammable forceps with 5 disks of newly grown fungus culture, taking into account leaving a flask without culturing the fungus for each concentration to represent the control sample. The flasks were placed in the shaking incubator at a shaking speed of 150 cycles/min and a temperature of 28 ± 2 ºC for a period of 15-30 days. After the end of the specified incubation period, the contents of the flasks were filtered with Whatman No.1 filter papers installed on a Buechner funnel connected to the electrovacuum device. The crude filtrate was used in tests for the bioremediation of liquid waste from black olive presses, which included:

- **Degradation of phenolic compounds**

The concentration of phenolic compounds in the filtrate obtained from the previous step was measured based on the method the researcher indicated [11] using the standard Folin-Cioalteu reagent. The reaction mixture consisted of 100 microliters of peat Jeft filtrate treated with the fungal isolate for each concentration, as well as a control sample with 500 microliters of standard reagent and 1.5 ml of 20% sodium

carbonate, which was mixed using an electro mixer. The mixture was diluted with distilled water to reach a volume of 10 ml. The mixture was left for two hours at laboratory temperature, the absorbance was measured with a spectrophotometer at a wavelength of 665 nm and was used to estimate the concentration of phenolic compounds before and after treatment by titration against the standard Gallic acid, where the concentration of phenolic compounds was expressed in Gallic acid equivalent per gram of dry weight of the filtrate.

- **Bioadsorption using the biomass of the fungus *H.bachu***

A research attempt was made to use the adsorption capacity of the fungus *H.bachu* biomass in removing the black color from the liquid waste of olive presses. The raw filtrate of Jeft water obtained from the paragraph (liquid media) was distributed in test tubes and centrifuged at a speed of 4000 rpm for 20 minutes. 1 mL of the supernatant solution was taken for each concentration and each incubation time in addition to the control sample. The absorbance was

measured with a spectrophotometer at a wavelength of 395nm, and the percentage of black color removal was estimated according to the method [12].

- **Chemical Oxygen Demand (COD)**

To detect the chemical oxygen demand in olive presses' liquid waste follow the protocol described by Pitwell [13]. A special flask called a reflux flask with a volume of 500 mL was used in this experiment. 50 mL of raw filtrate of treated and untreated peat water was taken and 1 g of mercury sulphate was added to it. Carefully add 0.5 mL of Sulfuric acid reagent, mixing gently until the mercury sulphate is completely dissolved, then add 25 mL of 0.041 molar $K_2Cr_2O_7$ solution. The flask containing the above mixture was connected to the condenser submerged in cooled water, then the remainder of the sulfuric acid reagent (70) mL was added. The nozzle of the condenser was closed with a beaker to prevent its contents from scattering and left for two hours. Turn off the device and leave it to cool to laboratory temperature. With distilled water, the mixture was diluted to twice its volume. The excess amount of $K_2Cr_2O_7$ was titrated with graded concentrations (0.15-0.1) mL of FAS indicator (ferrous ammonia sulphate). The titration continued until the solution turned from bluish-green to brownish-red. The COD concentration was calculated based on the following equation:

COD [13] as mg O_2/L = (A - B)× M× 8000/mL sample

A: The amount of FAS used for the control sample.

B: The amount of FAS used for the sample.

M: The molarity of FAS.

8000: Weight of oxygen in milliliters

4. Results and discussion

4.1.Solid media: The results shown in **Table 1** present the possibility of growth of the fungal colony under experiment on the solid nutrient medium (PDA) containing Jeft water, at concentrations of 10% and 20%, in varying degrees, after 6, 4, and 2 days of incubation. In general, it appeared that the diameter of the fungal colony was directly proportional with an increase in the incubation period, the largest diameter of the fungal colony reached 13.56 mm and 11.46 mm after 6 days of incubation at a concentration of 10% and 20% of Jeft water, respectively. This result was compared to the diameter of the fungal colony growing in an untreated medium (control) that reached 15 mm, while the smallest diameter reached 6.00 mm and 5.36 mm at 10% and 20% of Jeft water, respectively, after two days of incubation compared to the control, as shown in **Figure 1**. This result confirms the ability of the fungus to exploit the compounds present in Jeft water as a source of growth [10].

Table 1: Diameters of *Helvella bachu* fungal colonies grown on PDA solid medium supplemented with 10% and 20% concentrations of Jeft water

Jeft water % concentration	Incubation period (days)	The average diameter of the growing colony (mm)
Control		15
10	2	6.00
	4	10.90
	6	13.56
20	2	5.36
	4	9.30
	6	11.46

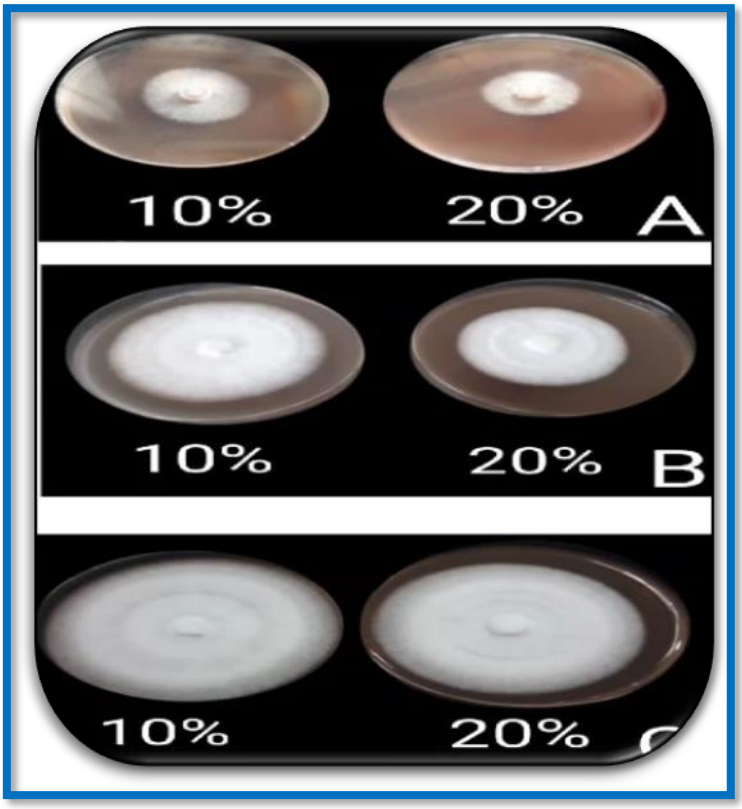

Figure 2: Diameters of *Helvella bachu* colonies grown on PDA solid medium supplemented with concentrations of 10% and 20% of Jeft water. A: after 2 days of incubation, B: after 4 days of incubation, and C: after 6 days of incubation.

4.2. Liquid media:

4.2.1. Degradation of phenolic compounds: Table 2 shows that the concentration of total phenol is inversely proportional to both the concentration of Jeft water and the period of incubation. The concentration of total phenol reached its lowest value 2.14 mg/100 g at the concentration 20% of Jeft water after 30 days of incubation compared to the control treatment 23.58 mg/100g. While at concentration 10% the total phenol concentration reached 10.56 mg/100 g and 6.22 mg/100 g after 15 and 30 days of incubation, respectively. The above results

gave clear evidence of the ability of the biomass of the fungus *H.bachu* to analyze phenolic compounds in Jeft water sample at both concentrations of 10% and 20% by secreting tannase enzyme, which can hydrolyze complex phenolic compounds, most notably Tannic acid. Thus, the fungus has proven its ability to transform highly toxic compounds (phenols) into safe biodegradable compounds [14] by applying the principle of bioremediation through biodegradation and thus recycling the effluent waste of olive presses without affecting the ecosystem.

Table 2: Total phenolic concentration of Jeft water treated with *Helvella bachu* biomass

Jeft water concentrations%	Total phenol concentration mg/100g	
	Incubation period (day)	
	15 day	30 day
10	10.58	6.22
20	8.57	2.14
Control	23.58	

4.2.2. Bioadsorption using the biomass of the fungus *H.bachu*: The results in Table 3 show that the percentage of black color removal from Jeft water reached 52.32% and 64.39 % at a concentration of 10% of black Jeft water after 15-30 days of incubation respectively, while at a concentration of 20%, the percentage of color removal reached 42.10% and 49.53 % after 15-30 days of incubation. It also became clear that the color change depends on the period of incubation, it was observed that as the incubation period increased, the ability of the fungus to absorb color increased until it became yellow to bright brown after 30 days of incubation, as shown in Figure 3. This is the most wonderful form of bioremediation, which is called bio-bleaching. The above can be explained by the ability of fungi to attack phenolic compounds using a very precise enzymatic strategy to consume these compounds as a sole source of energy that provides it with the continuity of life by breaking down complex bonds.

We reviewed a study published in 2005. The authors [15] reported that the tannase enzyme purified from an isolate of the fungus *A. flavus* can remove the black color from the waste of a black olive press in Tunisia. The percentage of color removal reached 46% after 6 days of incubation.

Table 3: Removal of black color from Jeft water after treating it with *Helvella bachu* biomass

Jeft water concentrations%	Color removal percentage%	
	Incubation period (day)	
	15 day	30 day
10	52.32	64.39
20	42.10	49.53

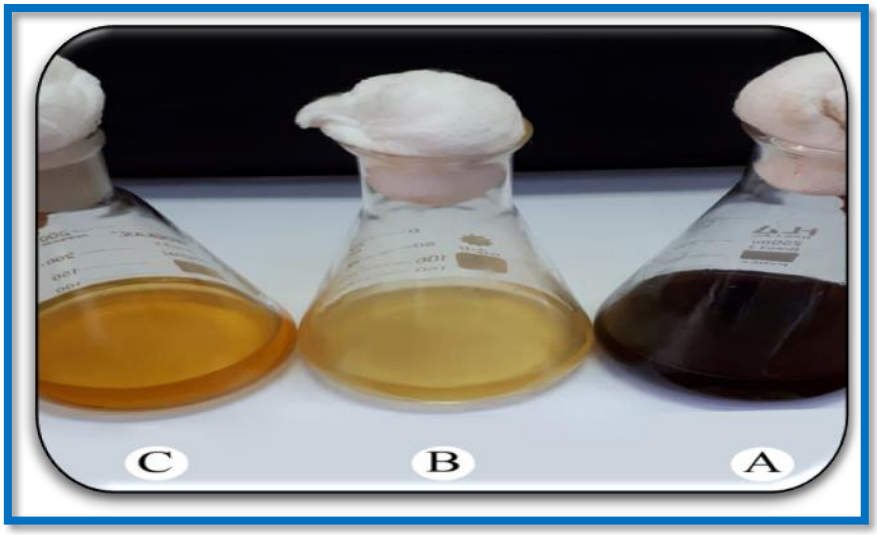

Figure 3: Adsorption of the black color of Jeft water after treatment with *Helvella bachu* biomass, A: Raw, untreated Jeft water (control), B: treated Jeft water 10% after 30 days of incubation, C: treated Jeft water 20% after 30 days of incubation

4.2.3. Chemical oxygen demand (COD): To exploit the biological and chemical properties of *H.bachu* to achieve sustainable development, which often occupies the forefront of headlines in various media, therefore this research was planned to detect the chemical requirement for oxygen, which is a test to measure and determine the amount of organic pollutants present in industrial and domestic wastewater.

The protocol indicated by the researcher **[13]** was followed, as the response came when the experiment reached the end, as in the results of **Table 4**, which indicated the efficiency of the fungus *H.bachu* in reducing the concentration and percentage of COD in liquid media treatments prepared with peat water at concentrations 20 and 10 % after 15-30 days of incubation, as the percentage of chemical oxygen requirement reached 65.83% when concentrating 10% of Jeft water after 30 days of treatment **[16]**, while the percentage of COD reached 8.08% when concentrating 20% for the same incubation period above. This indicates that increasing the incubation period led to an increase in biomass, which was reflected in an increase in the percentage of COD due to the increase in the biomass of the fungus and the increase in the concentration of secreted extracellular enzymes and their ability to decompose toxic phenolic and organic compounds in Jeft water. As for the concentration of 20%, despite the increase in the incubation period, there was an inhibition of the biomass of the fungus due to the high concentration of Jeft water and the high percentage of toxic inhibitory phenolic compounds and thus was reflected in the percentage of chemical oxygen requirement. The researcher indicated **[17]** that the chemical oxygen demand values in these wastes are very high.

Table 4: Percentage of chemical oxygen demand (COD) of Jeft water treated with *Helvella bachu* biomass

COD%	COD concentrations in samples mg/mL	Jeft water concentrations (%) and incubation period
------	25300.0	Raw Jeft water
11.06	22500.0	10 % , 15 Days
65.83	8643.0	10% , 30 Days
2.17	24750.0	20 % , 15 Days
8.08	23255.0	20 % , 30 Days

Many research papers have indicated that the liquid waste of olive presses is affected by many factors that lead to an increase in the risk of toxicity, such as the method of extracting olive oil, the harvesting season of olive fruits (the degree of maturity of the fruits) and the age of the trees producing the fruits [20]. In addition to some chemo physical factors that are affected by the method of storage of these wastes before being disposed of into the environment, they work to oxidize, polymerize, and condense the phenolic compounds in them, making them more toxic. Therefore, those responsible for protecting the environment have begun to follow cleaner production options and appropriate environmental waste management systems in olive presses to reduce their environmental impact [18].

5. Conclusions

H.bachu biomass could be reduced by the phenol concentration of peat water and the chemical oxygen requirement. *H.bachu* biomass also possesses a high adsorption capacity to remove the black color from peat water, which is the most wonderful form of bioremediation, which is called bio-bleaching.

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