



## Effect of Adding Microorganisms on the Degradation of Phenolic Compounds in Olive Mill Wastewater

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### Abstract

Bio-treatment is considered as one of ecologically most efficient methods of wastewater treatment. This study was done in order to reduce the negative effects phenolic compounds included in the olive mill wastewater added to the cultivated soil and to study the individual and collective ability of fungal and bacterial isolates to dismantle them. The experiment, conducted in 2020 with randomization in experimental design, consisted of six treatments and three replications. First four treatments had olive mill wastewater treated with fungal isolates *Penicillium sp*, *Aspergillus flavos* and bacterial isolates *Pseudomonas sp*, *Bacillus sp* individually. The fifth treatment included collective use of both isolates, in addition to the control which was without any treatment. The results of the statistical analysis showed that the phenolic compounds amount remained in the treated water which indicated the superiority of *Penicillium sp* over all treatments, where the apparent superiority of phenolic compounds dismantling was over bacterial isolates. However it was significantly over the collective effects of fungal and bacterial isolates, *Aspergillus flavos* and the control.

**Keywords:** Olive mill wastewater, Phenols, Bacteria, Fungi, Biodegradation.

### تأثير إضافة أحياء دقيقة في تفكيك المركبات الفينولية بماء الجفت

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### الخلاصة

تعتبر المعالجة الحيوية من أكثر الطرائق البيئية فاعلية في معالجة مياه الصرف، درست قدرة العزلات الفطرية والبكتيرية منفردة ومجمعة، على تفكيك المركبات الفينولية الموجودة في ماء الجفت المضاف للتربة الزراعية، بهدف التقليل من الآثار السلبية لتلك المركبات، نفذت التجربة عام 2020 بتصميم إحصائي ذي قطاعات عشوائية ضمت ست معاملات وثلاث مكررات، تم معاملة ماء الجفت الخام بكل من العزلات الفطرية

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عزلة على حدى، ومعاملة العزلات الفطرية والبكتيرية مجتمعة بالإضافة لمعاملة مجموعة السيطرة بماء جفت خام بدون عزلات. لاحقاً وبعد انتهاء فترة الحضان، تم حساب التعداد الحيوي للأحياء الدقيقة ، واختبرت كمية المركبات الفينولية المتبقية في ماء الجفت المعالج، أظهرت نتائج التحليل الاحصائي تفوقمعاملة الفطر *Penicillium sp* على كافة المعاملات، حيث كان التفوق ظاهرياً على المعاملات البكتيرية في تحليل المركبات الفينولية، ومعنوياً على كل من معاملة الفطر *Aspergillus flavos* ومعاملة العزلات الفطرية والبكتيرية مجتمعة (التأزر) ومجموعة السيطرة.

## 1. Introduction

Olive mill wastewater [OMW] is one of the most important by-products of olive oil extraction process. The production of OMW for the year 2019 was about 560,000 m<sup>3</sup> [1]. Due to its organic materials and mineral elements, it can constitute a successful economic alternative to chemical fertilizers by applying it as a natural fertilizer . Its application to agricultural lands has important positive results on olive, grapevine, tomato and maize [2]. On the other hand, some researchers found ([3-6]) that the unstudied application of OMW results in an increase in phenolic compounds concentration in soil, causing major changes in the composition and functions of biological communities, thus affecting the soil fertility [7] through the toxic effects of these compounds on microorganisms and plants grown. Due to their carcinogenic effects on organisms present in contaminated environments, phenolic compounds are classified as high-risk chemicals [8] and have been placed on the list of priority contaminants by the United States Environmental Protection Agency (EPA) [9]. Given the negative health effects of phenol, the World Health Organization (WHO) has set the permissible upper limit for phenol concentration in drinking water at 1 µg/l [10].

Kbeibo *et al.* [11] isolated strains of native anaerobic microorganisms that were capable of degrading phenolic compounds present in both OMW and OMW-saturated soil. The potential of organisms for degrading phenolic compounds was tested by growing them in agricultural media containing caffeic and protocanoic acids separately. The findings demonstrated the potential of isolated microorganisms to degrade the phenolic compounds after five days of incubation at 63%.

Those interested in using bioremediation continue to discover many microorganisms that have distinct characteristics which enable them to carry out bioremediation (biodegradation) of organic materials. Through their ability to exploit carbon compounds present in organic contaminants as a necessary source of energy, these organisms degrade compounds that are otherwise difficult to be degraded into simpler and less toxic ones [7]. During biodegradation it was found that *Pseudomonas* bacteria were very effective organic analysers by breaking down chemical pollutants (cypermethrin) with a percentage ranging between 80-87.9% [12]. While it was also found also that *Bacillus* bacteria was able to expel salinity and generate energy from wastewater, as the salt concentration decreased from 3500 ppm to 500 ppm after 11 days of treatment [13]. Keeping above research in view, this study was conducted to reduce the negative effects of phenolic compounds present in OMW by treating it biologically with microorganisms that were previously isolated from media rich in OMW.

## 2. Objectives

1. Grow and incubate the fungal and bacterial isolates, separately and combined with OMW.
2. Compare the efficiency of fungal and bacterial isolates, separately and collectively, for degrading phenolic compounds in OMW, and
3. Calculate the total count of microorganisms after incubation.

### 3. Methods and Materials

OMW was brought from a modern olive decanter operating by centrifugal system from the Al-Adliya area in Rural Damascus. Chemical analysis of OMW was conducted at the Laboratories of the Administration of Natural Resources Research in Qarahta. The following analysis were performed:

1. Measuring the pH using 2.5: 1 suspension and pH device.
2. Measuring Ec in OMW using Ec meter.
3. Measuring the available phosphorous using Olsen's method and revealing the colour ammonium molybdate molybides in the presence of ascorbic acid and reading the concentration using spectrophotometer.
4. Estimating the available potassium (dissolved and exchangeable) using 1-ammonium acetate solution and flame spectroscopy.
5. Estimating the organic matter as a percentage, using ashing process.
6. Calculating the viable count of fungi and bacteria at the end of incubation period by direct dilution.

Growth and multiplication of isolates: Fungi and bacteria isolated from OMW and contaminated soil [7] that proved to be significant in degrading the phenolic compounds, namely the fungal isolates (*Penicillium sp* & *Aspergillus flavus*) and the bacterial isolates (*Pseudomonas sp* & *Bacillus sp*) were grown on petri dishes with nutrient agar (NA) for bacteria and potato dextrose agar (PDA) for fungi.

The previous isolates were multiplied in a nutrient broth (NB) within 1000 ml conical flasks, each isolate separately. Another flask was used to combine the fungal and bacterial isolates together. The flasks were incubated for 7 days at  $28\pm 2^{\circ}\text{C}$  for fungi and  $37\pm 2^{\circ}\text{C}$  for bacteria.

Viable count: The viable count of fungi and bacteria was calculated at the end of incubation period by direct dilution. 1 ml of inoculant was added to 9 ml of distilled sterile water to obtain the first dilution, and from it 1 ml was taken to another tube containing 9 ml of distilled water to obtain the second dilution, and so onto get the third dilution of fungi and the sixth dilution of bacteria. Later 1 ml of the third dilution of fungus and sixth dilution of bacteria were added on to petri dishes containing nutrients media before placing these dishes in an incubator to estimate the viable count of bacteria after 24 hours of incubation and after 3 days for fungi.

Addition of inoculants: Both fungal and bacterial isolates (grown in liquid medium, PDA for fungi, NA for bacteria, were added to 1000 ml plastic bottles, and later the homogeneous inoculant was added with 20% inoculant and 80% raw OMW, according to the following treatments:

1. Control: OMW with no biological inoculants.
2. OWBP: OMW with the bacteria *Pseudomonas sp*.
3. OWBB: OMW with the bacteria *Bacillus sp*.
4. OWFP: OMW with the fungus *Penicillium sp*.
5. OWFA: OMW with the fungus *Aspergillus flavus*.
6. OWF2B2: OMW with two fungi isolates + two bacterial isolates.

The inoculated OMW treatments were incubated for 21 days for the added isolates to degrade the phenolic compounds in OMW at  $28\pm 2^{\circ}\text{C}$  for fungi and  $37\pm 2^{\circ}\text{C}$  for bacteria.

Estimation of the remaining phenolic compounds:

The previous treatments, at a rate of three replicates each treatment, were taken to estimate the remaining phenolic compounds in OMW using a polyene reagent and measure the absorptivity with a UV-Vis spectrophotometer, where the measured amount of phenols was attributed to gallic acid. 10 ml of OMW was taken from each treatment + 15 ml of 70%

commercial ethanol, and the sample was then placed in a flask to be separated using an ultrasonic device (ultrasound) to extract phenolic compounds, using ethanol. The amount was left for at least two hours, and then taken and placed in a separation flask. 5 ml was taken from the separation flask and then complemented with up to 100 ml of distilled water. A standard series of gallic acid was prepared in test tubes at 0, 0.2, 0.4, 0.6, 0.8 and 1 mmol/l concentrations using distilled water ,e.g., 0.2 gallic acid and was complemented with up to 1 ml distilled water and so on until the series was completed while avoiding sunlight, until the rest of the samples were ready. Later, 150  $\mu$ L of the sample was placed in a test tube by repeating each sample 3 times to confirm the results. 300  $\mu$ L of Folin-Ciocalteu reagent and 3 ml of distilled water were added to each tube, and then 2.5 ml of 10% sodium carbonate was added after 10 minutes at room temperature. The sample was mixed using the shaker. Half an hour later absorptive reading was taken on a spectrophotometer at 760 nm wavelength and the sample concentrations were calculated as gallic acid equivalents [14].

#### 4. Results and Discussion

**OMW:** Table 1 shows that the pH of OMW is acidic (4.93), Ec is low (1.512 dS/m), content of organic carbon (12.103%) and high content of nutrients (N, P, K) g/l respectively.

**Table 1:** Description of OMW used in the experiment

pH	Ec	Moisture (%)	P (%)	K (%)	OM (%)	C (%)
4.93	1.512	82.79	0.15	0.271	20.86	12.103

For comparison, [7] described the analysis of OMW as following:

pH	Ec	N (Total)	P (%)	K (%)	Fenol (g/l)	C (g/l)
5	8.1	550	367	8601	2.9	17.6

#### *Viable count of microorganisms*

In order to know the amount of microorganisms that were added to OMW and degraded the phenols vehicles in OMW, Table 2 shows the viable count of fungi and bacteria in 1 ml of the sample after incubation.

**Table 2:** Vital count of microorganisms

Treatment	Symbol	Colony Unit in 1 ml of Inoculant
<i>Penicillium sp</i>	OWFP	$1 \times 10^3$
<i>Aspergillus flavos</i>	OWFA	$2 \times 10^3$
<i>Pseudomonas sp</i>	OWBP	$17 \times 10^6$
<i>Bacillus sp</i>	OWBB	$15 \times 10^6$

#### *Degradation of phenolic compounds:*

Table 3 shows the results of the degradation rate of phenolic compounds of mg/l by measuring their residues in OMW in the second column and by comparing the degraded phenolic compounds (the product of subtracting the residues from the control) in the third column. It also shows significance of all treatments over the control in their ability to degrade the phenolic compounds. This implies the effectiveness of isolated microorganisms in degrading the phenolic compounds and this corresponds to what has been reached [7]. *Penicillium sp* treatment was more significant than other microorganism treatments and/or

may be due to the fact that the medium is acidic, allowing fungal activity at the expense of bacteria, followed by *Pseudomonas* sp with no significant differences between it and *Bacillus* sp treatment. *Bacillus* sp was the least in terms of ability to degrade phenolic compounds. The mixed treatment (OWF2B2) had no significance over other individual treatments, and the amount of degraded OMW was the least. which could be due to the presence of antagonism between the added fungal and bacterial strains, and therefore each isolate was not able to survive and degrade the phenolic compounds.

**Table 3:** Degraded phenolic compounds after OMW treatment.

Treatment	Symbol	Residues of Phenolic Compounds (mg/l)	Degraded Phenolic Compounds (mg/l)
Raw OMW	OW	1034.0 <sup>a</sup>	0 <sup>c</sup>
<i>Penicillium</i> sp	OWFP	455.9 <sup>c</sup>	578.1 <sup>a</sup>
<i>Aspergillus flavos</i>	OWFA	751.9 <sup>b</sup>	282.1 <sup>b</sup>
<i>Pseudomonas</i> sp	OWBP	602.5 <sup>bc</sup>	431.5 <sup>ab</sup>
<i>Bacillus</i> sp	OWBB	597.0 <sup>bc</sup>	437 <sup>ab</sup>
Fungi + bacteria	OWF2B2	631.5 <sup>b</sup>	402.5 <sup>b</sup>
C.V.		12.9	24.6
L.S.D.		155.4	155.4

## 5. Conclusion

The application of fungal isolate *Penicillium* sp resulted in a greater ability to degrade phenolic compounds. However, the application of fungal and bacterial isolates together had no positive effects. Therefore, to degrade the phenolic compounds in OMW, it is suggested to apply *Penicillium* sp fungus only and not to mix the fungal and bacterial isolates together to improve its use as a bio-treatment. Whereas, the bacterial isolates apparent superiority clearly outperformed the fungus *Aspergillus flavos* and the synergistic fungi + bacteria as well as the controls.

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