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FUNGAL BIOTREATMENT OF OLIVE MILL WASTE WATER

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ABSTRACT

The current study was aim to remediate olive mill waste water (OMWW) to reduce its phenol content and Chemical Oxygen Demand (COD) using fungal isolates. OMWW samples were drawn from the outlet of olive presser at Agriculture Research center, Egypt and characterized. Inoculating 25% diluted waste sample on Potato dextrose agar plates, incubated for 2 weeks at 25^{o}C, resulted in 8 fungal isolates, of which isolate 5 was selected based on its capabilities to degrade phenol and reduce COD, compared to the rest of the obtained isolates. Comparison was conducted between the selected isolate and the fungus *Pleurotus columbinus* as a reference to test their potencies to degrade phenol and reduce COD in OMWW at concentrations from 100 to 10% over 4 weeks and results showed low degradability and weak tolerance of the two organisms at concentrations from 50 to 100%, while at 40, 30, 20 and 10%, phenol degradation and COD reduction over the 4 weeks treatment were more obvious. At all concentrations *P. columbinus* showed better competency for phenol degradation and COD reduction than isolate 5. Decolorization and growth of the two organisms were investigated in OMWW at 40 to 10% concentrations. *P. columbinus,* again, showed better competency over isolate 5 where it 79 and 49% of the color were removed after 4 weeks by *P. columbinus* and isolate 5, respectively. Total carbohydrate was also determined in the treated OMWW over 4 weeks and results showed it decreased from 6.05 to 5.2g/L in 40% OMWW and from 4.27 to 3.6 g/L in 30% OMWW, while it increased from 3.1 to 4.37g/L in 20% OMWW and from 1.46 to 3.9 g/L in 10% OMWW. Finally, the presence of indol acetic acid and gibberellins in 20 and 10% OMWW was tested as affect by the treatment with *P. columbinus* over the period of 4 weeks. Results showed that, after 4 weeks, IAA content decreased from 29.4 to 23.25 µg/ml in 20% OMWW, and slightly from 15.6 to 13,15 µg/ml in 10% OMWW. For gibberellins, the change after 4 weeks in 20% OMWW was not significant, that it decreased from 1.36 to 1.25mg, while it increased from 0.667 to 1.58 mg/ml in 10% waste in the same period. It can be concluded that remediating OMWW with *P. columbinus* can achieve a better results and the treated waste may be suitable for irrigation of crops.

INTRODUCTION

Olive oil extraction is a process that is conducted by mechanical procedures in olive mills. During this process, large amounts of liquid effluents and solid residues are produced, with a high organic load, the nature of which depends on the technology of the extraction process and system employed **(Duarte et al 2011)**. These wastes usually have harmful effects on the environment due to its high organic content and phytotoxicity **(Bhatnagar, et al 2014).** Moreover, the management of olive oil residues is an economic burden to the producers **(Esteve et al 2015).**

The phytotoxicity of the olive mill wastewaters can be attributed to the phenolic compounds **(Lanciotti et al 2005**). In fact, the olive pulp is very rich in phenolic compounds but only 2% of the total phenolic content of the olive fruit remains in the oil phase, while the remaining amount is lost in the OMW (approximately 53%) and in the pomace (approximately 45%) **(Rodis et al 2002).**

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There are many biological, physical, thermal, and physico-chemical treatment of OMWW. Biological processes use microorganisms to break down the chemicals present in OMWW. They are divided into aerobic and anaerobic processes according to the type of the microflora used. Proposed physical, thermal, and physico-chemical processes have not been efficient in decreasing the high COD، toxicity of OMWW and scaling up for this methodologies with serious practical difficulties, long term economic and complexity and high costs. As an alternative to these processes, and to reduce the ecological impact of OMWW, biological remediation is environmentally compatible, feasible and more economic **(Mantzavinos and Kalogerakis 2005)**. It also enable recovering primary components and forming valuable products such as fertilizers and soil conditioners, substrates for the production of biomass (algae and yeasts), protein, biogas, biopharmaceuticals, biopolymers, feed additives and edible mushrooms **(Morillo et al 2009).**

Many bacteria and fungi were shown to have the capabilities to treat OMWW, such as *Penicillium* spp., **(Robles et al 2000)** which helped reducing COD and phenolic content (45%) and decrease its antimicrobial effect, and *Bacillus pumilis* was also reported to reduce phenol content of OMWW by up to 50% **(Ramos et al 1996)**. The N_2-fixing bacterium *Azotobacter vinelandi* was able to reduce the phytotoxicity of OMWW (90%) **(Piperidou et al 2000)**

Fountoulakis et al (2002) reported that the use of *Pleurotus ostreatus* in pre-treatment of OMWW was found effective since use a subsequent anaerobic digestion.

On the other hand, applying fungal enzymes (such as laccases) for OMWW treatment significantly affected germinability and mean germination times of maize seeds and caused phenol reduction (81%) in soil treated with laccase and the white rot fungus *Panus tigrinus* compared with soil irrigated with tap water as control **(Quaratino et al 2007)**.

Of all fungi, five white rot fungi and two brown rot fungi, were reported to highly reduce COD value, total phenol and remove color in OMWW. For instance, liquid cultures of *Pleurotus sajor-caju* (white rot fungi) showed great ability to reduce COD level by 60%, phenol by 81% and decolorize by 60% **(Yesilada et al1999).**

Therefore, the present study was designed to evaluate the efficacy of using fungal strains in remediating OMWW to be used later in crops irrigation.

MATERIALS AND METHODS

1. Sampling

Samples (~60L) of Olive Mill Waste Water (OMWW) were collected from the outlet of olive presser at Agriculture Research center (ARC), Giza, Egypt. Samples were immediately transported in plastic containers to the laboratory and stored at 4^{o}C until being analyzed or used in the experiments described later. Chemical composition of OMWW was determined at Soil, Water and Environment Research Institute, Giza, Egypt.

2. Microorganisms

Pleurotus columbinus was obtained from Unit of Mushroom production, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

3. Microbial media

Potato Dextros Agar (PDA) **(Oxoid, 1979)** was used for the isolation of fungi.

4. Isolation of fungi from OMWW.

Fungi were isolated from OMWW by adding 10ml of OMWW in 90ml of sterilized water. Diluted samples were inoculated onto Potato Dextrose Agar (PDA) medium and incubated at 25^{o}C for weeks. Each individual colony (isolate) was picked up and restreaked onto slant PDA tube for preservation. An inoculum of each isolate was prepared by inoculating 50ml of liquid Potato dextrose broth medium, incubated for 1 week at 25^{o}C. These inocula were used for the following experiments.

5. Screening for isolates efficient in phenol degradation and COD reduction

To test the obtained isolates for their efficacy in phenol degradation and COD reduction, 5ml of one-week old inoculum of each isolate were used to inoculate 250 ml flasks containing 100 ml of sterilized 25% diluted OMWW and incubated at 25^{o}C on rotary shaker (150 rpm) for two weeks. Biomass was separated by filtration through by Whatman filter paper no.1 and the filtrate was used for determination of residual phenols and COD (described later).

6. Comparison of the most potent isolate with *Pleurotus columbinus* as a reference

The best isolate was compared to *Pleurotus columbinus* in their efficacy in remediating the

OMWW by inoculating 5 ml of 1-week culture of either organism in 500 ml flasks containing 200 ml OMWW at 10 to 100% concentration and incubating the flasks at 25^{o}C on rotary shaker (150 rpm) for 4 weeks. Total phenols, COD reduction, total carbohydrate, decolorization, Indole Acetic Acid (IAA) and Gibberellins were determined (described later) one a week over the incubation period.

7. Measurements

Determination of total phenol

Liquid–liquid extraction with ethyl acetate was carried out on olive mill wastewater samples. OMWW was acidified to reach pH 2 with HCl and washed with hexane in order to remove the lipid fraction. 10 mL of OMWW were mixed with 15 mL of hexane; the mixture was vigorously shaken and centrifuged for 5 min at 3000 rpm. The phases were separated and the washing was repeated successively two times. Extraction of phenolic compounds was then carried out with ethyl acetate: the OMWW samples were carefully washed then mixed with 10 mL of ethyl acetate; the mixture was vigorously shaken and centrifuged for 5 min at 3000 rpm. The phases were separated and the extraction was repeated successively four times. The ethyl acetate was evaporated under vacuum, the dry residue was dissolved in 3 mL of methanol and this solution was used for determination of phenolic compounds **(Elena et al 2006).**

For determination of phenolic compound, total phenol content of OMWW was determined colorimetrically at 730 nm, using the Folin–Ciocalteau reagent according to the method described by **(Swain and Hillis, 1959).**

Chemical oxygen demand (COD) was determined according to the method described by **(APHA, 1992).**

Determination of pH and E.C.

pH was determined using CG 710 Schott Gerate pH meter and Electrical Conductivity (E.C) was determined using **Inolab cond710 conductivity meter**.

Determination of microelements. K and Na were determined using flame photometer model 400, while P was determined using spectophotemeter and Mn, Zn, Cu, Fe, Mg and Ca were determined using atomic absorption spectrophotometer according to the methods of **(Cottenie et al 1982)**.

Determination of total nitrogen

Total nitrogen was determined by Kjeldahl according to the method described by **(Cottenie et al 1982)**.

Decolorization Assay

Decolorization was determined according to the method described by **(Ysilada et al 1995)**. Cultures were harvested and filtered through Whatman filter paper No.1. Filtrates were diluted 50 fold and their absorbencies were recorded at 395 nm. Blank was composed of non-inoculated medium and incubated under the same conditions.

Determination of total carbohydrates

The total carbohydrate content was determined as glucose by the phenol sulphuric acid method **(Dubois et al 1956).** One ml of the sugar solution was mixed with 1 ml of 5% redistilled phenol solution and then 5ml of sulphuric acid (AR). After cooling by standing for 10 minutes at room temperature, each tube was shaken and placed in a water bath at 30^{o}C±2 for 20 minutes. The produced yellow orange color was determined colorimetrically at 490 nm. A standard curve was constructed in the same way using different concentration of glucose (10-100 mg/ml).

Determination of Indole Acetic Acid (IAA)

IAA was measured by the Salkowski colorimetric method according to the method of (**Glickmann et al 1995**).

Determination of Gibberellins. (GA) were measured calorimetrically according to the method described by (**Udagwa and Kinoshita, 1961**).

RESULTS AND DISCUSSION

Chemical Analysis of the collected OMWW samples

Analyses of the OMWW collected from the used source **(Table 1)** showed that wastes had acidic pH, which is normal due to the presence of various organic acids. E.C. around 17ds/ml, COD

of 99g/L and phenolic content of 7g/L**,** which are relatively close to the average OMWW, where OMWWs from various sources had EC between 8-14, COD values from 53 to as high as 191 an phenol content ranging from 8 to 12 **(Gonçalves et al 2009 and Mekki et al 2013).**

Table 1. Chemical composition of OMWW from Agricultural Research Center

pH	E.C d.s/m	Cations (meq/l)			Anions (meq/l)		
		Ca^{++}	Mg^{++}	Na^{++}	HCO_3^-	Cl^-	SO_4^-
5.4	17.2	38.7	35.5	68.9	4.2	115.5	11.3

Macro nutrients (ppm)			Micro Elements (ppm)				Organic materials (g/L)		
N	P	K	Mn	Zn	Fe	Cu	COD	Phenolic compound	Total Carb.
0.257	0.126	1.2	0.266	0.660	0.577	0.052	99	7	14

Isolation and Screening for fungal isolates efficient in phenol degradation and COD reduction from OMWW

Culturing samples onto PDA medium resulted in 8 fungal isolates. Testing the obtained isolates for their abilities to degrade phenol and reduce COD of the OMWW was carried out by inoculating the 25% diluted OMWW with 5 ml of 1-week old culture of each isolate and incubating the flasks at 25^{o}C for 2 weeks. Results showed that all isolates were able to degrade phenol by a range of 7.5 to 38.17% and reduce COD by 20 to 40% **(Table 2)**. The best results of phenol degradation and COD reduction were obtained by isolate 5. Therefore, this isolate was selected for the following experiments.

Table 2. Effect of fungal isolates on phenol content and COD of 25% diluted OMWW after 2 weeks incubation at 28^{o}C.

no. isolate	Phenol[1]		COD[2]	
	Residual g/L	Degradation %	Residual g/L	Reduction %
1	1.4	24.7	17.9	27.8
2	1.61	13.4	17.3	30.2
3	1.59	14.5	20.5	17.3
4	1.48	20.4	18.6	25
5	1.15	38.1	15.4	37.9
6	1.33	28.4	16	35.4
7	1.58	15.0	16.7	32.6
8	1.72	7.52	16	35.4
LSD	**0.378**		**0.848**	

[1] Phenol content at zero time was 1.86 g/L.

[2] COD value at zero time was 24.8 g/L.

Comparison of the most potent isolate with *Pleurotus columbinus* as a reference

Many species of white rot fungi, such *Pleuortus sp.* are known for their great capabilities in removal of phenolic compounds and biodegradation of polycyclic aromatic hydrocarbons and decolorization of reactive dyestuffs, and that is due to biosynthesis of extracellular enzymes including laccase, manganese-dependent peroxidase and lignin peroxidase produced by these fungi **(Aytar et al 2011)**. Therefore, a species of *Pleurotus*, i.e. *P. columbinus* was used as a reference to compare the selected isolate in their capabilities in degrading phenols, reducing COD, decolorization and their growth in the tested OMWW.

Compared to the rest of isolates, isolate 5 was chosen for its highest potentiality in phenol degradation and COD reduction of 25% OMWW. Isolate 5 was again tested for its abilities to degrade phenol and reduce COD in OMWW at concentrations from 100 to 10% and compared to that of fungus *P. columbinus* (**Tables 3 and 4)**.

Results of phenol degradation, as a function of fungus type and treatment period, showed that the more dilution of OMWW, the better phenol degradation is achieved by both organisms **(Table 3)**. There was no notable increase in phenol degradation in 100, 75 and 50% OMWW after three weeks with either fungus, therefore determination was ceased thereafter. Treatment of in 100, 75 and 50% OMWW, with isolate 5 for 3 weeks resulted in phenol degradation by 9, 13 and 24%, respectively, while *P. columbinus* caused phenol degradation by 15, 21 and 29%, respectively. These results can be explained by the potentiality of the genus *Pleurotus* in degrading phenolic compounds which were previously reported by other investigators

(**Laconi et al 2007, Aytar et al 2011 and Durat et al 2014).**

At lower concentrations (higher dilutions), i.e. 40, 30, 20 and 10%, phenol degradation over the 4 weeks treatment was more obvious. At 40% OMWW, isolate 5 and *P. columbinus* degraded 25 and 28% of phenol after 1 week and 40 and 48% after 4 weeks, respectively. As mentioned above, the more dilution, the easier for the fungus to degrade its phenol content. OMWW at 10%, phenol was reduced by 58 and 86% with isolate 5 and *P. columbinus*, respectively, after 4 weeks. Another study showed that several species of the genus *Pleurotus* were found to be very effective in the degradation of the phenolic substances present in OMWWs **(Morillo et al 2009).**

Same observations were detected in COD determination **(Table 4)**, where both organisms could not tolerate the higher concentration of OMWW. At 100 and 75% OMWW, no detectable, reduction was observed up to 3 weeks with either isolate 5 and *P. columbinus*, while at 50% concentration waste, COD was only reduced by 10.6% and 20.8% after 3 week using isolate 5 and *P. columbinus*, respectively. Going down to 10% OMWW, COD was reduced by 79% and 86% after 4 weeks of treatment with isolate 5 and *P. columbinus*, respectively. Similar observations were mentioned by **Aytar et al (2011)** who reported that biological treatment of OMWW with white rot fungi resulted in significant decreasing of reducing sugar, increasing pH, and consumption of COD.

Based on the above results where neither organisms was able to tolerate the organic content in OMWW at concentration of 50% and above , the following set of experiments were carried out on OMWW at 40 to 10%. The abilities of isolate 5 and *P. columbinus* to decolorize and grow in OMWW were compared, Change in pH, EC and carbohydrate contents were also monitored as affected by the metabolism of both organisms.

Decolorization of the OMWW was measured as affected by the type of used organism **(Table 5)**.

Treating OMWW with isolate 5 for 4 weeks resulted in color reduction by 37 to 49% when OMWW was at 40 to 10% concentrations, respectively. On the other hand, *P. columbinus* showed to be more efficient than isolate 5 to decolorize the waste, that in 4 weeks, it decolorized the 40% waste by 47% and the 10% waste by 79%.

Growth of both organisms in OMWW at 40 to 10% concentrations was tested and the growth was measured as dry weight g/L. Results **(Table 6)** showed that both organisms could not grow in 40 and 30% wastes. In 20% OMWW, slower change in growth was detected by both organisms, given that after 4 weeks isolate 5 gave about 2g/L while *P. columbinus* gave 5.9g/L. in 10% OMWW, the two organisms produced 6 and 7.6g/L, respectively. These results may explain and confirm why both organisms could not degrade phenol or reduced COD at concentrations above 40% (**Tables 3 and 4)**.

pH and EC were monitored during the treatment course and results showed that there was no significant change in pH, treated with either organism, that it remained around 5.6 in OMWW at 40, 3 and 20% and around 5.8 in OMWW 10%,. For EC values, there was no significant difference in EC values between the organisms used in the treatment. EC values remained constant at 9.8 in 40% OMWW, around 8 in 30% OMWW, between 5.2 to4.8 in 20% OMWW and slightly dropped from 2.4 to 1.95 in 10% OMWW (results not tabulated due to insignificancy).

Table 5. De-colorization of OMWW (measured by O.D at 395nm) at different concentrations by isolate 5 compared to *P. columbinus* as reference

OMWW conc.	O.D	Isolate 5								*P. columbinus*							
		Weeks								Weeks							
		1		2		3		4		1		2		3		4	
		O.D	% Decol	O.D	% Decol	O.D	% Decol	O.D	% Decol	O.D	% Decol	O.D	% Decol	O.D	% Decol	O.D	% Decol
40%	1.73	1.34	22.4	1.25	27.3	1.18	31.5	1.09	37.0	1.25	27.1	1.10	35.9	0.94	45.4	0.90	47.4
30%	1.26	0.91	27.6	0.87	30.5	0.81	35.6	0.78	37.6	0.84	32.6	0.78	37.7	0.68	45.31	0.66	47.2
20%	1.06	0.75	28.9	0.68	35.3	0.62	41.1	0.58	44.4	0.63	40.4	0.53	49.9	0.43	59.4	0.35	66.9
10%	0.73	0.50	31.5	0.43	41.0	0.40	45.2	0.37	49.3	0.39	46.5	0.32	56.0	0.21	71.1	0.15	79.4
LSD	**0.063**	**0.06**		**0.15**		**0.18**		**0.18**		**0.36**		**0.12**		**0.02**		**0.057**	

Decol: de-colorization,change in OMWW color compared to the color of the untreated OMWW as 100%

Table 6. Growth of isolate 5 and *P. columbinus* (dry weight g/L) in OMWW at different concentrations, incubated for 4 weeks at 28^{O}C.

OMWW Conc.	Isolate5 [1]				*P. columbinus* [2]			
	Weeks				Weeks			
	1	2	3	4	1	2	3	4
40%	0.94	0.91	0.89	0.85	1.14	1.1	0.91	0.88
30%	1.17	1.04	1	0.9	1.2	1.17	0.9	0.8
20%	1.21	1.74	1.87	1.99	2.14	2.55	4.2	5.92
10%	2.8	4.2	5.5	6	2.97	6.54	7.21	7.58
LSD	**0.15**	**0.14**	**0.40**	**0.14**	**0.06**	**0.38**	**0.43**	**0.79**

[1] Inoculum size was 0.67 g/L, [2] Inoculum size was 0.71 g/L

Determination of total carbohydrates

The goal of treatment of OMWW biologically was to use the treated waste water in irrigation of some crops (results will be shown in a later work). The presence of reasonable amount of carbohydrates, including polysaccharides, in this liquid waste can also be good property, considering that it can be applied as irrigating water, since it can enhance the soil property and function as soil conditioner. Therefore, the carbohydrate content in the OMWW at 40 to 10%, treated with *P. columbinus,* was determined. Results presented in **(Table 7)** showed that total carbohydrate decreased from 6.05 to 5.2g/L in 40% OMWW and from 4.27 to 3.6 g/L 30% OMWW, while it increased from 3.1 to 4.37 g/L in 20% OMWW and from 1.46 to 3.9 g/L in 10% OMWW. These records are compatible rational, since the used organism did not actually grow in 40 and 30% waste, while it showed better adaptation to grow in 20 and 10% waste **(Table 6).**

Table 7. Determination of carbohydrate content (g/L) released from *P. columbinus* in OMWW

OMWW Conc.	Carbohydrate content (g/L)				
	weeks				
	0 time	1	2	3	4
40%	6.05	5.9	5.5	5.3	5.2
30%	4.27	4.2	4.0	3.7	3.6
20%	3.1	3.7	3.76	4.06	4.37
10%	1.46	1.48	2.1	3.7	3.9
LSD	0.23	0.22	0.26	0.16	0.31

Determination of indol acetic acid and gibberellins content in OMWW treated with *P. columbinus*

It was previously reported that IAA is produced by many types of white rot fungi, including *Trametes versicolor*, *Pleurotus ostreatus*, *and Phanerochaete chryosporium* **(Bose et al 2013).** Therefore, the presence of plant growth hormones, namely IAA and gibberellins, was determined in the OMWW at 20 and 10% before and after treatment. Results, shown in **Table 8,** revealed that in 20% OMWW, IAA decreased from 29.4 to 23.25µg/ml after 4 weeks, while a lighter change occurred in 10% OMWW from 15.37 to 13.15 µg/ml. For gibberellins, after weeks, it slightly decreased in 20% OMWW from 1.36 to 1.25 mg/ml while it increased from 0.667 to 1.58 mg/ml

Table 8. Determination of Indol Acetic Acid (IAA) and gibberellins (GA) in OMWW, diluted to 10% and 20% concentrations, treated with *P. columbinus*

OMWW Conc.	IAA (µg ml^{-1})					GA (mg ml^{-1})				
	Weeks					weeks				
	control	1	2	3	4	control	1	2	3	4
20%	29.4	29.11	28.4	28.10	23.25	1.36	1.34	1.310	1.29	1.25
10%	15.63	15.37	14.67	13.51	13.15	0.667	0.675	0.690	1.10	1.58
LSD	**5.05**	**0.65**	**3.01**	**0.12**	**1.74**	**0.22**	**0.16**	**0.27**	**0.46**	**0.18**

CONCLUSIONS

Results on this work showed that the white rot fungi, *Pleurotus columbinus,* possesses great abilities in remediating the phenol-rich waste water of olive mill in four weeks treatment course. In addition to reducing the COD value, it also increased the waste's content of gibberellins, making it more beneficial as irrigating water.

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