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Production of Organic Biofertilizer from Olive Mill Waste Water

Amal M. Omer

Soil Fertility and Microbiology Department, Desert Research Center, Cairo, Egypt

Abstract: Despite the phytotoxicity of olive mill waste water (OMWW), OMWW have fertilizer characteristics, which make it a potential source for bio-fertilization. Aerobic OMWW bio-treatment processes were conducted to eliminate the phyotoxicity by removal of phenolic compounds. For all biotreatments, the phenol content of OMWW decreased gradually with time reaching the maximum phenol removal (71.9 and 71.4%) by a bacterial mixture grown at 50 and 30 % dilution, respectively after 25 days of fermentation. *Pseudomonas putida* and *Pseudomonas flouresence* individually were more efficient than *Azotobacter vinelandii* in degrading phenols. The microbial counts of OMWW increased slowly during fermentation reaching up to about 10^{10} CFU ml^{-1} after 25 days. The increase in the bacterial counts during the fermentation process, would indicate a potent reduction in the polyphenol level in the OMWW waters which are responsible for the inhibition. At the end of the fermentation process, there was an increase in total nitrogen and pH of OMWW samples .Biotreatment process increased the germination percentage to significant values. Treatment of 30% OMWW with *Pseudomonas flouresence* recorded the highest germination index followed by *A.vinelandii* and a mixture of bacterial strains while *Ps. putida* recorded the lowest values. The physicochemical characteristics of fermented OMWW showed that it is slightly acidic with high electric conductivity and significant amounts of nutrients especially potassium and calcium. The microbiological analysis revealed that the product is very rich with microbial population as *Pseudomonas spp.* HPLC analysis showed that the most abundant phenolic compounds in raw OMWW were catechol and tyrosol followed by catchin, Qurectin, Caffiec acid and Gallic acid. After bioremediation, remarkable degradation of phenol compounds ranged from 7.2 to 98.27 % compared to raw OMWW was detected. These mean that the possibility of using the fermented product produced from OMWW as a natural biofertilizer.

Key words: Olive mill waste water, phenol degrading bacteria, biotreatment, *Azotobacter vinelandii Pseudomonas putida*, *Pseudomonas flouresence*

INTRODUCTION

Olive mill waste water constitutes a major environmental problem in Mediterranean countries as aqueous by-products of olive oil separation, the annual olive oil mill waste water production in Mediterranean countries is estimated to be over 30 millions m^3 per year (Borja *et al*.,1993). The volume of olive black water produced by the traditional press process is 0.5-0.8 m3/ton of olive (Boari *et al.,*1984).

Currently, there is no appropriate method applied for treating OMWW in Egypt; it is usually discharged in to open environment, thus producing disruption of biological activities and pollution to the soil surface and underground water. The main toxicity of this effluent is related to its phenolic and aromatic compounds, with a concentration range that may vary from 1.5 g up to 10 g/l (Rodriquez *et al.*,2005). Because of the high organic and polyphenol content of OMWW, its direct disposal may pollute both land and aquatic environments (Hamdi *et al.,*1991 ; Moreno et *al.*,1990).The phenolic content of this waste causes phytotoxic and antimicrobial effects (Rodriquez *et al*., 1988). It affects the soil quality, toxic to plants and soil micro flora when disposed into the soil. Therefore, direct discharge of olive mill wastewater into receiving media is not permissible and certain measures must be taken before disposal of the OMWW into the environment (Capasso *et al*.,1992 ;Azbar *et al*.,2004).

The large proportion of organic matter and valuable nutrients, especially potassium, make OMWW a valuable resource for beneficial utilization, particularly in degraded agricultural soils. Also, OMWW contains no xenobiotics or heavy metal contaminants .In fact, it has been proven that this waste may potentially act as good sources of plant nutrients (Ergu *et al.,* 2008). OMWW also has been successfully assayed as a foliar fertilizer (Piperidou *et al*.,2000) and as a soil-less substrate in combination with peat (García *et al.,*2002). and its application to soil of low organic matter content, abundant in the Mediterranean basin, would be a sustainable recycling option, if its toxicity to microorganisms and plants was first eliminated or reduced. High OMWW organic concentration and content of antimicrobial compounds, such as phenols, should be subjected to pretreatment before being discharged in the environment (Ehaliotis *et al.,* 1999).

Corresponding Author: Amal M. Omer, Soil Fertility and Microbiology Department, Desert Research Center, Cairo, Egypt
E-mail: amal_omaram@yahoo.com

Several studies carried out to reduce the phenolic compounds of this waste by using different treatment methods. The biological treatment of OMWW can be use as an alternative to the conventional treatment processes. Biological treatments can be conducted by different microorganisms as fungi such as *Aspergillus niger* (Hamdi *et al*.,1991; Borja *et al.* 1995), *Phanerochaete chrysosporium* (Ahmadi *et al.,* 2006)**,** *Lentinula edodes* (Annibale *et al.,* 2004), yeasts such as *Yarrowia lipolytica* and *Candida tropicalis* (Ettayebi *et al.,* 2003), bacteria such as *Azotobacter vinelandii* (Piperidou *et al.,*2000) and *Pseudomonas spp.* which are widely applied for the degradation of phenolic compounds (Gonzalez *et al.*,2001; Prpich and Douglis,2005).

Different microbial strains can use the phenolic compounds present in this waste as carbon source for the production of different organic compounds useful in agricultural processes .The two strains *Azotobacter vinelandii* and *Azotobacter chroococcum* produced aspartic acid, serine, glutamic acid,glycine, hystidine, threonine, arginine, alanine, proline, cysteine, tyrosine, valine, methionine, lysine, isoleucine, leucine and phenylalanine after 72 h of growth in chemically defined media with 2mmol=L of phenolic compounds (Gonza *et al.,*2005). *Pseudomonas Putida* is known to be capable of using aromatic compounds such as phenol as a sole source of carbon and energy (Movahedyan *et al.,*2009) . The fermented OMWW can be used as organic liquid bio-fertilizer instead of the traditionally chemical fertilizers which is effectively a replacement to the chemical material for any kind of crops, and serves similar functions as traditional chemical fertilizers. Also, it improves the soil quality and therefore the farmers can cut down the cost of soil maintenance tremendously. The organic biofertilizers are not a good source for plant nutrition only but also they improve soil microbial activity and promote the activities of critical soil enzymes and plant growth hormones. So, they play a fundamental role in ecosystem .

Based on these assumptions, the principal objective of the proposed study is the productive utilization of natural resource (OMWW) to produce liquid organic bio-fertilizer assist in improving the living conditions of the community.

Methods:

Isolation, Selection and Identification of Phenol Degrading Bacteria:

Phenol–utilizing bacteria were isolated from olive mill waste water samples from the olive mill of Agriculture Research Center . Ten ml of water sample was mixed with 100 ml of Ramsay phenol broth media (Ramsay *et al.,* 1983) and incubated at 30°C with aeration for one week. Then 1 ml of this media was inoculated to 100 ml of a new Ramsay phenol broth media and aerated (by shaking) in 30°C for another one week. Again, 1 ml of the second passage was inoculated into the new phenol broth media and incubated in the above mentioned situation. These passages were repeated until turbidity was obtained from bacteria growth . After the last passage, it was cultured on Ramsay phenol agar media as an isolate and the bacterium was isolated as a colony alone (Koutny *et al*.,2003). The ability of isolated bacteria to degrade the phenolic compounds in OMWW was examined as the following: Isolated bacteria were inoculated into 250 ml conical flasks containing 50 ml of OMWW . The flasks were shaken (150 rpm) for 1 week at 25°C. Samples were taken both in the beginning and at the end of experiments for phenol assay as described above. The strains which can degrade phenol to a greater extent within a relatively short time was selected as efficient phenol degrader among the isolates. Three isolates were selected as highly effective phenol degrading bacteria and identified using the Biolog system ,Microlog Version 3·20 (Bochner,1989) as: *Azotobacter vinelandii* , *Pseudomonas putida* or *Pseudomonas flouresence* **.**

Aerobic Biological Treatments of Olive Mill Waste Water:

Twelve small-scale biotreatment experiments were carried out. Each of the tested bacteria (*Azotobacter vinelandii , Pseudomonas putida* or *Pseudomonas flouresence*) and a mixture of the three strains was inoculated into 500-ml Erlenmeyer flasks containing 200 ml of OMWW at three dilutions (100%,50% and 30%) .The flasks were shaken at (150 rpm) for 21 days at 25°C. Samples were collected every 5 days to analyze total phenol content and total microbial counts using Nutrient agar medium. All the results were obtained using triplicate cultures .At the end of fermentation, pH, total nitrogen and phenol content were determined.

Determination of Phenolic Contents of OMWW:

Phenolic compounds were extracted according to the method of (Elena *et al*., 2006). Briefly,10 ml of OMWW was 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 two times successively. Extraction of phenolic compounds was then carried out with 10 ml of ethyl acetate. The phases were separated and the extraction was repeated four times successively. The ethyl acetate was evaporated under vacuum and the dry residue was dissolved in 3 ml of methanol and this solution was used for determination of phenolic compound. Total phenols were determined spectrophotometrecally using a modification of the (Romero *et al*.,2002). A calibration curve was established in the same conditions using gallic acid as standard. The absorbance (OD) at 725 nm was measured in a spectrophotometer against a blank .

Germination Assay:

Crude and fermented OMWW samples were subjected to toxicity test. The tomato (*Lycopersicum esculentum*) seeds (as standard plant) were disinfected in absolute ethanol for 5 min., rinsed with sterile distilled water and placed in Petri dishes lined with filter paper containing 5 ml of the OMWW solutions. Petri dishes were incubated in a growing chamber at 26°C. After 7 days, germinated seeds were counted and an index of germination (expressed as the percentage of germinated seeds) was calculated for each treatment. Three Petri dishes were replicated for each treatment .

Biological Treatments of OMWW at Large Scale for Production of Biofertilizer:

Olive mill waste water (20 liter) was diluted to 50%, inoculated with a bacterial mixture of *Azotobacter vinelandii* , *Pseudomonas putida* and *Pseudomonas flouresence* and shaked at150 rpm for 20 days at 30^0C. At the end of fermentation process all physicochemical (pH, EC, BOD, COD, total phenols, N,C and mineral content) and microbiological constituent of fermented OMWW (biofertilizer) were measured.

Determination of Physicochemical Constituents of OMWW:

Minerals (P, K, Ca, Na, Zn, Mn, Cu, Fe, Mg , and B) were determined according to the method described by Cottenie *et al.* (1982).Biochemical oxygen (BOD) was determined as described by Lenore *et al*. (1992).Chemical oxygen demand (COD) was determined according to the method described by Arnaldo *et al.* (1992). Total nitrogen and total carbon were determined by Kjeldahl method (**(**Bremner and Mulvaney,1982 and Jackson, 1958), respectively.

Determination of Microbiological Constituents of OMW:

Total microbial counts on Nutrient medium, fungi and yeasts counts on Potato Dextrose Agar medium, *Pseudomonas* counts on King B medium, phenol degrading bacteria on Ramsay medium and total coliform, E.coli counts on MacConkey medium were determined. For yeasts, Potato Dextrose Agar medium was acidified to pH 4.5.

HPLC Analysis of Phenolic Compound Before and After Fermentation:

Phenolic extracts were also analysed by High- Performance Liquid Chromatography using a Waters 600E HPLC equipped with a Waters 990 photodiode array detector and Millipore software for data analysis. An efficient gradient of acetonitrile-o-phosphoric acidified bi-distilled water (pH = 2.6) was used with an Interchrom C18, 5 µm reversed phase column. Three wavelengths (280, 320 and 350 nm) were used during the elution, and data collection and integration were performed with the Millipore software. Phenolics were identified on the basis of their retention times and their spectra in comparison with standards. When necessary, co-injection and elution with standards were used to confirm the identity of some compounds. (Siham and Elhadramy, 2007).

Evaluation of Fermented OMWW on Maize Growth:

Pot experiment was conducted in green house in Desert Research Center to evaluate the fertilization with crude and fermented OMWW on the growth of Maize. Maize seeds (Hybrid 3 Giza 31) were planted into a 100 mm pots containing a sandy soil. Each pot was inoculated with 10 ml of crude or fermented OMWW. Pots without olive mill waste water was used as a control. There were three replicates of each treatment. Seedling emergence rates were recorded. After emergence, only 2 seedlings of maize will remain in each pot. Maize seedlings were sprayed to run-off with 100% concentration of crude or fermented OMWW once weekly. After 45 days of cultivation, plant height as well as plant fresh and dry weights were measured.

Statistical Analysis:

Data were subjected to statistical analysis using the method described by (Snedecor, 1966). The least significant difference (L.S.D) was used to differentiate means according to (Waller and Duncan, 1969) .

Results:

It is clear From Fig.(1), that the biodegradation of phenolic compounds increased as OMWW dilution increased . At 100% concentration, the bacterial reduction of phenol ranged from (25 - 55%) which increased to (46.8 - 71.9 %) and (61.9 - 71.4%) at 50% and 30%, respectively after 20 days of fermentation process. This is compatible with that the changing levels of the OMWW concentration from 20-40% increasing the pollutant removal as reported by *(* Ahmadi *et al.,* 2006) . The phenol content decreased gradually with time reaching its minimum after 20 and 25 days of fermentation process. The obtained results showed that the maximum phenol removal from OMWW (71.9 and 71.4%) were obtained by a bacterial mixture grown at 50 and 30% , respectively after 25 days . *Ps. putida* and *Ps. flouresence* individually were more efficient than *A. vinelandii* in degrading phenols at all OMWW concentrations, while a bacterial mixture of three strains gave the best result.

The results were in accordance with that the most efficient phenol degrading bacteria mostly belong to the family of Pseudomonaceae (Farshid,2010) . Growth of *A. vinelandii* in OMWW results in the decline of content of most of the compounds associated with phytotoxicity, and this is confirmed by the assessment of degradation yields (Piperidou *et al*.,2000).

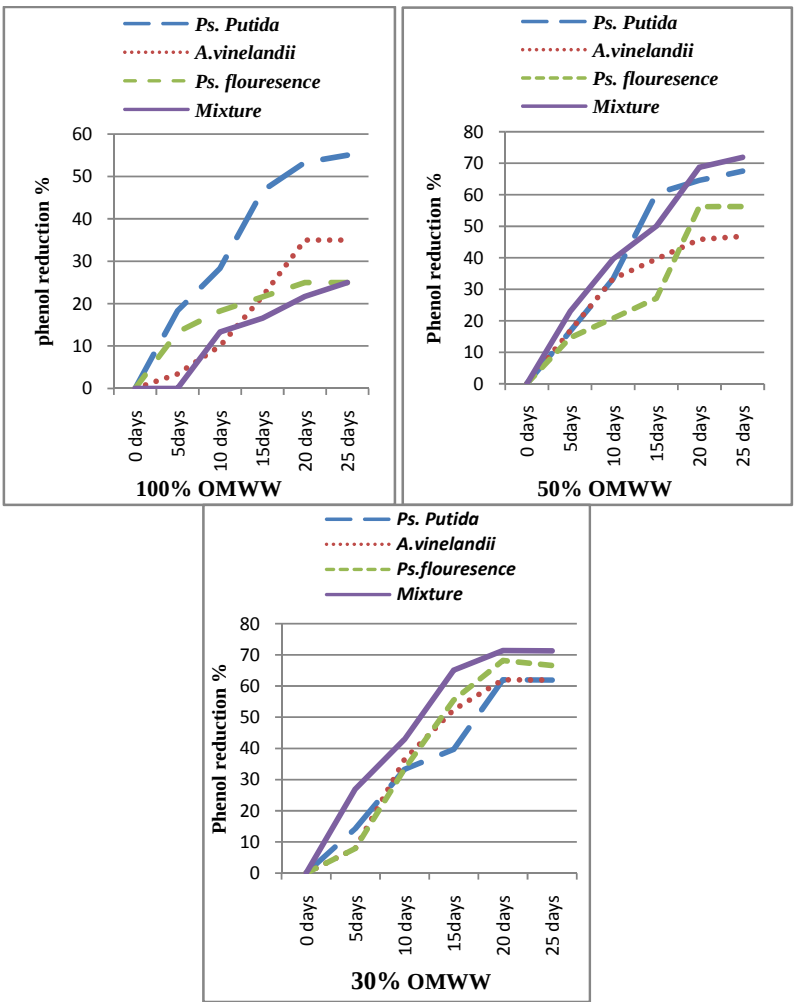


Fig. 1: Phenol reduction pattern at different concentrations of OMWW by phenol degrading bacteria.

Since the OMWW was not sterilized, a relevant amount (10^3-10^4 CFU ml^{-1}) of contaminant bacteria were also present at the beginning of fermentation. The plate count would group all types of microorganisms that would grow on normal media but they are sensitive to the inhibitors like phenols as could be seen from the initial numbers .Their number increased slowly during fermentation reaching up to about 10^{10} CFU ml^{-1} after 25 days (Fig. 2). The increase in the bacterial counts during the fermentation process, would indicate a potent reduction in the polyphenol level in the OMWW which are responsible for the inhibition. This is consistent with data reported by other authors as regards the presence of antimicrobial substances in OMWW (Bressan *et al.* 2004; Capasso *et al.* 1995; Isadori *et al.* 2004).

As no carbon, energy and other sources were added to the OMWW and a significant decrease in phenol was detected, these mean that bacteria used the phenolic compounds present as carbon source. This is compatible with that the phenol removal which was proportional to the growth of fungi indicates its use a primary carbon source (Yesila *et al*., 1995).

The pH increased during the treatment period from 4.5 to 7.2 (Table 1). The increase of pH is may be due to the degradation of some phenolic acids present in the waste. The level of the pH reached at the end of the process (6.9 – 7.2) was suitable for the microbial growth. While there was an increase in total nitrogen in all samples at the end of the fermentation process ,there was a reduction in total phenols as described before . The two strains *Azotobacter vinelandii* and *Azotobacter chroococcum* produced different amino acids after 72 h of growth in chemically defined media with 2mmol=L of phenolic compounds (Gonza *et al*., 2005).

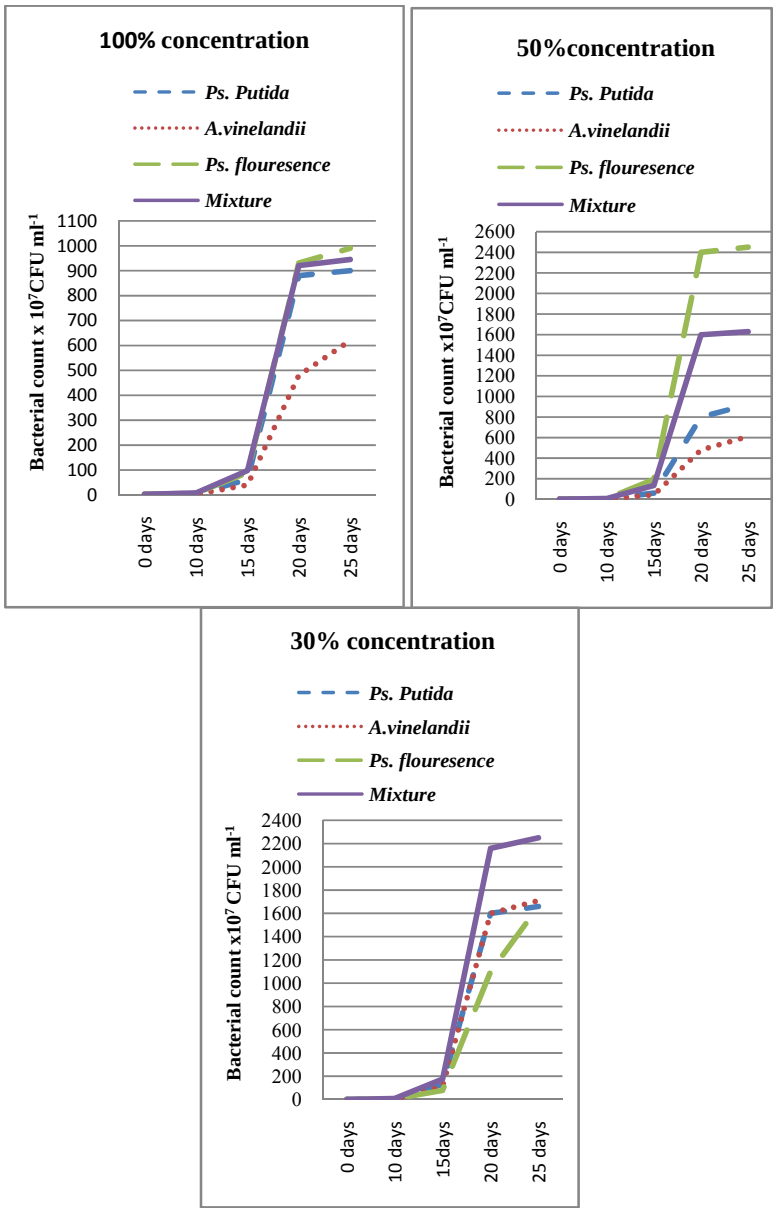


Fig. 2: Bacterial populations of OMWW during fermentation process.

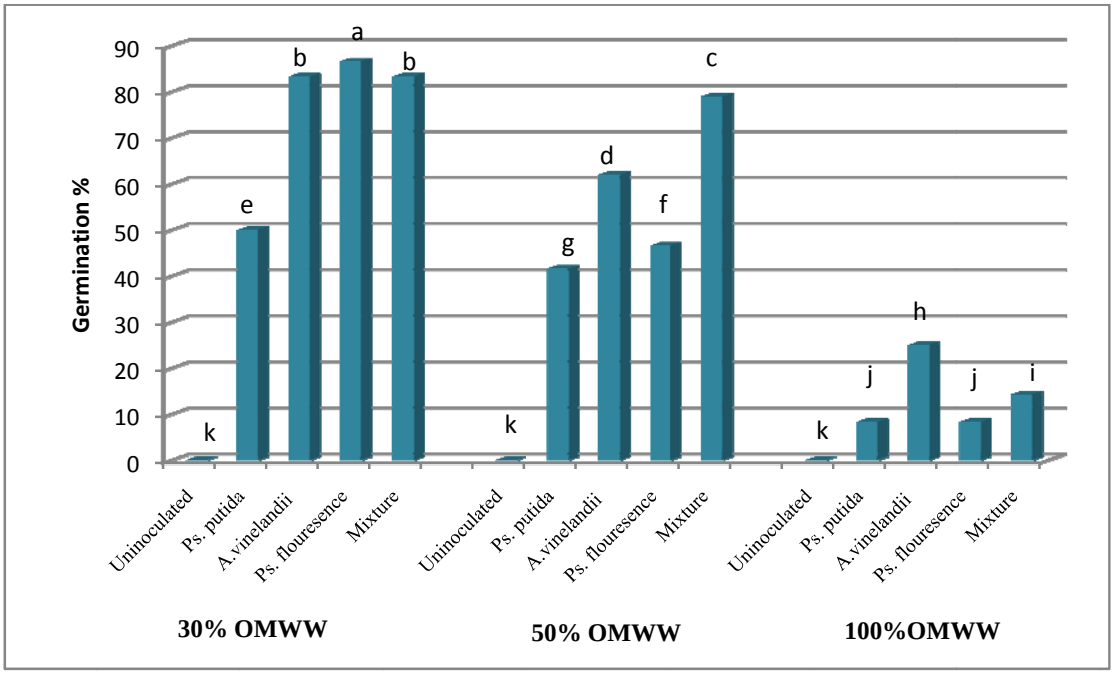
From Fig. (3), no seed germination at the three concentrations of untreated OMWW (100,50 and 30%) were detected and this may be due to the presence of phytotoxic phenolic compounds in raw OMWW. At high concentrations, olive wastes caused inhibition or suppression of mitosis (Pierluigi *et al*.,2011)**.** In fact, several authors attribute OMWW toxicity to their phenolic compounds which inhibits germination of seeds of different plant species (Capasso *et al.,* 1992). Ferulic acid and catechol caused phytotoxic effects toward plants, consisting of the disturbance of their physiological processes even at low concentration (Aliotta *et al.,* 2000; Yang *et al.,* 2002 and An *et al*., 2001). Fermented OMWW at 30% concentration showed the highest germination percentage followed by 50% and 100% the concentration. So, biotreatment process generally increased the percentage of germination to significant values This is in agreement with other results showing the diminution of OMWW toxicity when diluted (Casa *et al.,* 2003; Annibale *et al.,* 2004). Treatment of 30% OMWW with *Ps. flouresence* recorded the highest significant germination index reach 86.6% followed by *A.vinelandii* (83.3%) and mixture of bacterial strains (80.3%) while *Ps. putida* recorded the lowest values. For 50% dilution, using mixture of phenol degrading bacteria showed 79% germination index followed by *A.vinelandii, Ps. flouresence* and *Ps. putida* in descending order. *Pseudomonas flouresence* PU completely degraded up to 1000 ppm of phenol in 72 hours whereas it degrades about 99 percent of phenol in 48 hours (Mahiudddin *et al*., 2012).

Table 1: Change in pH, Total N and phenol content at the end of aerobic fermentation process.

Treatments		pH	Total N (g/L)	Phenol ppm
30% concentration	Uninoculated	4.6 j	0.40i	630e
	Ps. putida	6.7 d	0.45hi	240jk
	A.vinelandii	6.5 e	0.52 hi	240 jk
	Ps. flouresence	6.7 d	0.65 gh	210 k
	Bacterial mixture	6.1 f	0.65 gh	200 k
50% concentration	Uninoculated	4.6 j	0.74 fg	960 b
	Ps. putida	7.2 a	0.91 ef	312 i
	A.vinelandii	6.9 c	1.00 e	510 g
	Ps. flouresence	7.1 b	1.23 cd	420 h
	Bacterial mixture	6.9 c	1.10 de	270 j
100% concentration	Uninoculated	4.8 h	1.30 bc	1200 a
	Ps. putida	5.8 g	1.36 bc	540 f
	A.vinelandii	5.5 h	1.64 a	780 d
	Ps. flouresence	7 b	1.46 ab	900 c
	Bacterial mixture	6.4 e	1.30 bc	900 c
LSD 5%		0.1	0.189	29.55

- Different letters within the same column mean significant difference recorded at LSD 5%.

While 30% OMWW inoculated with bacterial mixture contain the least phenolic content (200 ppm), low amount of nitrogen (0.65g/l) and high germination index reach (83.3 %), 50% OMWW inoculated with bacterial mixture contain the least phenolic content (270 ppm), higher amount of nitrogen (1.10 g/l) and slightly lower germination index reach (79 %). For fertilizer purpose, the product must contain high nitrogen amount and not toxic. So, 50% OMWW inoculated with bacterial mixture (OMWW) was selected to be used as biofertilizer on large scale.



*For every variable, different symbols means there is a significant difference . (LSD (0.05) : 1.88)

Fig. 3: Germination index of raw and fermented OMWW treatments.

The physicochemical characteristics of fermented OMWW showed that the product is neutral (pH = 6.9) with high electric conductivity of 3 mS /cm which could be due to the high level of potassium, sodium and chloride elements (Table 1). The mineral analysis showed that it is very rich in nutrients and the major element is potassium (3206 mg/100ml), followed by calcium and sodium. These results were in agreement with the results obtained by (Paredes *et al*., 1999) .For microbiological analysis, the product is very rich with microbial population, *Pseudomonas spp.* followed by Enterobactereacea and yeasts are the main microorganisms present in the OMWW. Yeasts and lactic bacteria represent the microbiota of OMW waters as was stated by (Mouncif *et*

*al.,*1993) because of the acidity and the high salt concentration .On the other hand, the fermented OMWW free from pathogens.

Table 2: Chemical and microbiological constituents of biofertilizer (Fermented OMWW).

Physicochemical characteristics									
pH	EC mS/cm	T.N. g/L	T.C. g/L	O.M. g/L	Phenol ppm	COD mg/L	BOD_5 mg/L	Germination %	
6.9	3	1.1	6	10.32	270	18800	375	79	
Soluble nutrients, $mg.L^{-1}$									
N	P	K	Ca	Na	Mg	Fe	Mn	Zn	B
175	120	3206	169	102	135	5.22	0.67	2.34	11.4

- COD : Chemical Oxygen Demand - BOD_5: Biological Oxygen Demand

Table 3: Microbiological constituents of biofertilizer (Fermented OMWW).

Microbiological characteristics					
Total bacteria $X10^8$CFU/ml	*Pseudomonas spp.* $X10^5$CFU/ml	Fungi $X10^3$ CFU/ml	Yeast $X10^3$ CFU/ml	Enterobactereacea $X10^3$ CFU/ml	*Streptococcus* $X10^3$ CFU/ml
30	27	5	18	20	12
Pathogenic microbes					
Coliform group	Faecal coliform	*E.coli*		*Sallmonela*	*Shigella*
- ve	- ve	- ve		- ve	- ve

Qualitatively and quantitatively HPLC profiles of phenolic compounds recorded a remarkable change in OMWW samples before and after biotreatments (Table 4). Indeed, HPLC analysis showed that the most abundant phenolic compounds in raw OMWW were catechol and tyrosol followed by: catchin, qurectin, caffiec acid and gallic acid. A remarkable degradation of phenol compounds in OMWW after bioremediation process was detected. There is a great variations in the ratio of phenol degrradation ,that the phenol reduction ranged from 7.2 to 98.27 % compared to raw OMWW. While qurectin ,caffeic acid and gallic acid showed the highest phenol degradation, catchin showed the lowest degradation value. No complete degradation of any of the phenolic compound was detected. Phenol was degraded by *Pseudomonas flouresence* PU1 via catechol with subsequent meta ring cleavage (Mahiudddin *et al.,* 2012).

Table 4: Main phenolic compounds in OMWW before and after the bioremediation.

Phenol (ppm)	RT (min)	Raw OMWW	Fermented OMWW	Phenol Reduction%
Tyrosol	2.829	194	84.4	56.49485
Gallic acid	3.121	24.3	2.1	91.35802
Qurectin	7.891	63.7	1.1	98.27316
Catchin	3.964	79.8	74	7.26817
Catechol	5.774	266	34	87.21805
Caffeic acid	5.91	33.2	2.2	93.37349

When maize was grown under green house condition, 8.4% decrease in seedling emergence in soil treated with crude OMWW and insignificant increase in soil treated with fermented OMWW (1%) were observed as indicated from Table (5) and this is contrary to that detected in lab condition. The negative effects observed in laboratory assays were not detected under field conditions since pots and field growth were especially contributory to nitrogen availability (Kamara *et al.,* 2000). On the other hand, no negative effect was observed with regard to the growth parameters of the maize. Application of OMWW significantly increased maize height, fresh weight and dry weight comparing with control, recording increments by about 9.3 %, 11 % and 11.4 % for crude OMWW and 31.5, 26.7 and 20.5% for fermented OMWW, respectively. Maize growth was significantly promoted by olive mill wastewater application reaching 10–11% of the growth of the control (Siham and Elhadramy, 2007). The high positive effect of fermented OMWW may be due to its high microbial community and N content.

Table 5: Evaluation of crude and fermented OMWW as biofertilizer on the maize growth.

Treatments	Seedling emergence %	Plant height (cm)	Fresh weight (gm)	Dry weight (gm)
Control	95 a	69.8 c	203 c	31 c
Crude OMWW	87 b	77 b	228 b	35 b
Fermented OMWW	96 a	102 a	277 a	39 a
LSD (0.05)	4.31	4.33	5.88	3.46

- *Different symbols means there is a significant difference*

Conclusion:

Bioremediation system of olive oil mill wastewater (OMWW) was conducted using bacterial mixture of the phenol degrading bacteria, namely: *Azotobacter vinelandii, Pseudomonas flouresence* and *Pseudomonas putida*. Phenol degrading bacteria grow in OMWW at the expense of its constituents and transform it into an organic liquid of high fertilizing value. The system eliminated the phytotoxicity from OMWW, enriched it with an agriculturally beneficial microbes and metabolites, increased the percentage of germination to significant values and improved the maize growth. The end product, "biofertilizer", is used as natural liquid organic fertilizer. Generally, it will be necessary to perform further field studies to know the effect of OMWW as biofertlizer on plants and soil.

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