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Biovalorization of Olive Mill Wastewater Using Phenol Degrading Bacteria to Produce Biofertilizer

Amal M. Omer* and Ghada A. Z. Ibrahim

Soil fertility and Microbiology Department, Desert Research Center, Cairo - Egypt

Abstract

Biological treatments for olive mill waste cleanup using bacterial strains have proven to be effective and environmentally friendly. Three phenol-degrading bacterial strains identified as *Lysinibacillus macroides, Lysinibacillus boronitolerans*, and *Brevundimonas olei* were isolated from olive mill wastewater (OMWW) have high efficacy in degrading polyphenols. These strains exhibited both enzymes, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase, responsible for degradation of phenolic compounds to varying degrees. *Lysinibacillus macroides*, rather than *Lysinibacillus boronitolerans* or *Brevundimonas olei*, have the highest catechol-dioxygenase enzyme activities. Various factors were optimized during the OMWW fermentation process to maximize the phenol degradation process. Maximum degradation rates were reached after 35 days at 20% dilution and pH ranged from 6 to 7 with starch and yeast reaching 1 g/l when added as carbon and nitrogen sources. In greenhouse experiments, the final fermentation product was evaluated as an organic biofertilizer. Bioassay data showed that the application of fermented OMWW significantly increases barley height, fresh and dry weight, nitrogen, potassium, and phosphorus contents compared to non-fermented barley, regardless of the type of bacteria used. The results of this study demonstrate that biologically fermented OMWW is proved to be economically beneficial to be used as an organic biofertilizer.

Keywords: biofertilizers, bacteria, Di-oxygenases enzyme, olive mill wastewater (OMWW)

1. Introduction

The process of manufacturing of olive oil released high amounts of olive mill effluent from the mill as byproduct, which, if not treated properly can have adverse impacts on the territory and the environment. Olive oil extraction produces two types of waste: solid waste and wastewater from an oil mill (OMWW), highly polluted and phytotoxic waste (1).

The majority of these wastes are created by small businesses with little financial capabilities to appropriately treat them before releasing them into the environment. As a result, these naturally formed products were thrown away without being valued. Polyphenols were the most prevalent molecules in olive mill wastewater, and when discarded at high concentrations, they had hazardous impacts on ecosystems (2). During olive oil extraction processes, polyphenols are divided unequally between waste water and produced oil; only 2% of phenolic compounds is passed in the oil, while the remaining 53% and 45 % passed in the olive mill wastewater and pomace, respectively (3). Phenol pollutants are often soluble in water and so, accumulate in the soil due to their high toxicity, resulting in massive pollutants of surface water, groundwater, and soil (4), those compounds have excessive toxicity to maximum microorganisms, plants, fish, and animals, which can cause extensive damage to the surroundings (5) which meant that the OMWW can provide a significant risk of organic contamination to the water table and deep subsurface waters (6).

The phenolic compounds in polluted wastes have been eliminated using a variety of techniques (7). Biological treatments for waste cleanup have proven to be an effective method for extracting phenol and

*Corresponding author e-mail: amal_omaram@yahoo.com.; (Amal Mohamed Omer).
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its compounds from wastewater, environmentally friendly, and cost-effective when compared to standard physical and chemical approaches (8). A wide range of microorganisms can either metabolize or mineralize phenolic compounds in environments either aerobic or anaerobic. They either break down phenol directly as a solo carbon source or indirectly in the presence of any other growth substrate through a process known as co-metabolism. Crude cellular extract of many microorganisms confirmed the presence of catechol 1,2-dioxygenase as ring cleavage enzyme (9). Different bacterial strains used to remediate phenol biodegradation were identified as *Rhodococcus, Stenotrophomonas*, *Lysinibacillus, Comamonas, Microbacterium, Pseudomonas,* and *Halomonas*. Phenol-degrading bacterial strain, named *Lysinibacillus cresolivorans*, isolated aerobically from a coking wastewater treatment plant can utilize phenolic compound as its sole carbon source and energy (10). It is important to identify novel non-pathogenic biochemically active bacteria that are phenol destroyers.The phenol-destructive activity of the *Brevibacillus sp.* F14 strain was confirmed through experimentation. After being exposed to Brevibacillus sp. F14 cells for 15 days, phenolic compounds concentration in the treated water dropped from 200.0 12.0 mg/l to 6.8 0.8 mg/l (11). A common process for metabolizing aromatic substances like phenol is the dihydroxylation of the aromatic ring to form derivative of catechol, followed by ortho- or meta-oxidation. The enzymes catechol 2,3-dioxygenase, and catechol 1,2-dioxygenase, convert catechol derivatives to -ketoadipate or semialdehyde either the ortho-cleavage route or the meta pathway (12).

These enzymes that can break down phenol and its derivatives are abundant in many microorganisms. The enzymes that bacteria produce to break down phenol are known as intracellular and extracellular oxidases (13). Among different microbial strains, bacteria have a well-established aerobic breakdown process for phenolic compounds (14). As many of recent researches deal with isolating microbial species with potential ecological significance, many microbial species have enzyme systems that can break down a variety of hazardous aromatic chemicals. Some of the best successes in bacterial and yeast phenolic compound degradation was the high efficacy of strains of *Pseudomonas, Candida tropicalis, Trichosporon cutaneum,* etc.(15). By removing these phenolic compounds from OMWW using aerobic bio-treatment methods, their phyotoxicity was eliminated. It was also discovered that the fermented product made from OMWW acted as a natural biofertilizer due to the remarkable degradation of phenol compounds, which reached 98.27% when compared to raw OMWW (16). The main aim of the article is to enrich the olive mill wastewater using highly efficient bacterial strains for the production of organic bio-fertilizer.

Methods

1. Olive mill wastewater (OMWW) collection

A sample of olive mill effluent was provided from the olive mill of Horticultural Research Institute, Giza, Egypt. The sample was collected in a clean container and filtered through a narrow pore cloth (pore size 80 µm). The olive mill waste sample was stored at 4°C.

2. Characterization of OMWW's physicochemical and microbiological components

Phenolic compounds in OMWW were extracted using ethyl acetate which is evaporated at the end and the obtained residue was dissolved in methanol (3 ml) for determination of phenolic compounds **(17)**. Total phenols were determined spectrophotometrically at an absorbance of 725 nm. Under the same circumstances, a calibration curve was created using gallic acid as standard **(18)**. Total organic carbon was analyzed in OMWW samples with potassium dichromate and sulphuric acid digestion methods **(19).**

For measuring nitrogen, phosphorus, potassium, and cations, OMWW samples were digested using a 1:1 combination of pure $HClO_4$ and H_2SO_4 **(20)**. In digested OMWW samples, total nitrogen, phosphorus, potassium, and soluble cations (Mg, Na,

Zn, Fe, Cu, Mn, Ca , and B) were quantified. In this concern, nitrogen content was determined by the modified Kjeldahl method (**21**), and phosphorus was determined using an acidified solution of ammonium molybdate containing ascorbic acid which was added to a digested sample. Blue color was developed and its intensity was measured spectrophotometrically at 660nm **(22)** while potassium content was measured by using a flame photometer **(23)**. Soluble nutritional cations of OMWW were then analyzed using atomic absorption spectrophotometry with a Perkin-Elmer **(24).** Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) were assayed at the analytical central lab, Desert Research Center.

For microbiological analysis: Total microbial count (TMC) was assayed using serial dilution pour plate method **(25)** using the nutrient media for TMC **(26),** Potato Dextrose Agar (PDA) medium for fungi counts **(27),** acidified Potato Dextrose Agar medium for yeast counts, King medium for Pseudomonas counts **(28),** and Modified Ashby's medium for nitrogen fixers counts **(29).**

These physicochemical and microbiological parameters are assessed before and after treatment of OMWW to track changes in each parameter and, as a result, the efficiency of the bio-treatment applied.

3. Isolation of phenolic degrading microbes

Phenol–degrading bacteria were isolated from the olive mill wastewater sample **(30)**. Briefly: Ten ml of waste water was mixed with Ramsay phenol broth medium (100 ml) and incubated at 30°C with aeration for one week. 1 ml of this medium was then reinoculated into 100 ml of fresh Ramsay Phenol Broth medium and shaked for another week at 30°C. After that, 1 ml of second passage was inoculated into fresh phenol broth medium and incubated under the same conditions described before. Those passages had repeated until turbidity appeared because of the bacterial growth. After the final subculture, it was cultured as an isolate on Ramsey phenol agar medium, and only the cells were isolated as single colony (**31**). Isolated bacteria were injected into 50 ml of diluted OMWW at 20% concentration. At the end of the experiment, the inoculated OMWW were shaked at 25°C for 1 week, and samples were tested for phenol degradation **(18).** In the same conditions, a gallic acid used for creating a calibration curve to be used as the standard. As effective phenol degrader isolates, isolates that can degrade phenol to a extra level in a minimum period were chosen for further examination by assessing their enzymatic activity. This assay was carried out at a concentration of 20% dilution, which is appropriate for enzyme assays.

4. Preliminary investigation of catechol-dioxygenase enzymes in phenol- degrading isolates

The activity of catechol 1,2-dioxygenase was evaluated using procedures **(32)** and **(33).** Briefly, 50 ml of each phenol-degrading bacterium was added to 150 ml of the solution composed of 0.004 % phenol red, 1 mM EDTA, and 10 mM catechol (pH = 7.5, corrected with ammonium hydroxide). After 10 minutes in the dark at room temperature, the color of the tested samples turned to yellow/orange, confirming the activity of catechol 1, 2-dioxygenase. Also, catechol 2,3-dioxygenase activity was measured using the methods described by **(34)** and **(33).** To every 50 ml of bacterial liquid culture, 150 ml of 90 mM catechol dissolved in 50 mM tris-acetate buffer solution (pH 7.5) was added. When the analyzed samples were placed in the dark at room temperature for 2 hours, they developed a green-brownish color, which related to catechol 2,3-dioxygenase activity. From these investigations, three bacterial isolates which recorded the highest activities were selected for identification processes.

5- Identification of bacteria used

The three selected bacterial isolates grown on nutrient agar plates were evaluated for their physiological and morphological parameters as illustrated in Bergey's Manual of Determinative Bacteriology (**35**). Furthermore, selected isolates in degrading phenols were identified at the molecular level using a partial 16S rRNA gene sequence technique in Sigma Scientific Services Co. **(36)**. Bacterial isolates were identified using direct extraction of genomic DNA from colonies grown on NA medium. PCR was used to amplify the bacterial

16S rRNA gene sequence using forward and reverse primers:
F (5′AGA GTTTGA TCC TGG CTC AG-3′)
R (5′-GGT TACCTT GTT ACG ACTT-3′).

A total volume of 50 µl containing 10 µl Taq-&GO™ master mix 5x concentrated (MP Biomedicals, Eschwege, Germany) was added to the 40 µl volume containing primers and template DNA (95°C, 95°C 30 cycles for 5 min, 30 sec; 57 °C, 30 sec; 72 °C, 90 sec; 72 °C, extended for 5 min). PCR products were purified using the Gene JET™ PCR Purification Kit (Thermo K0701). PCR products were sequenced using an ABI 3730xl DNA sequencer and forward and reverse primers (37). The obtained sequences of bacterial isolates were analyzed using the BLAST tool from the National Center for Biotechnology Information Database (NCBI) Genbank database to identify the most similar 16S rRNA sequences recorded in the Genbank. **(38).**

6- Enzymatic Assay (Extracellular and intracellular)

For extracellular enzyme assay: Bacterial cells were cultivated for 20 days on only 20 % OMWW, then centrifuged for 10 minutes at 6000 rpm to extract crude enzyme for the extracellular test.

For intracellular enzyme assay: Bacterial cells were cultivated for 20 days on only 20 % OMWW, centrifuged for 10 min. at 6000 rpm, harvested, and washed two times with solution of 0.33 M Tris-HCl (pH 7.6). For intracellular assay, the cells were sonicated for 4 minutes (30 seconds on, 30 seconds off) and then centrifuged for 20 minutes at 12000 rpm.

Activities of two catechol dioxygenase enzymes (Catechol 1,2-Dioxygenase and Catechol 2,3-Dioxygenase) were measured **(39).** To evaluate the enzyme activity of catechol 1,2-dioxygenase, the production of cis,cis-muconic acid is recorded as the end product. Enzyme assay should be used in a quartz cuvette, 2ml of 50 mM Tris-HCl buffer (pH 8.0), 0.7ml of distilled water, 0.1 ml of 100 mM 2-mercaptoethanol, and 0.1ml of cell-free extract were inverted to mix component well, and then 0.1ml of catechol (1mM) was added and the contents mixed again. For 10 minutes, the absorbance was measured at 260 nm, and the increase in absorbance showed the synthesis of cis,cis-muconic acid. For Catechol 2,3-Dioxygenase activity, the production of 2-hydroxymuconic semi-aldehyde, catechol's meta cleavage product, was used to measure catechol 2,3-dioxygenase activity. The following reagents were placed in a plastic cuvette: Inversion was used to mix the components, which included 2 ml of 50 ml of Tris-HCl buffer (pH 7.5), 0.6 ml of distilled water, and 0.2 ml of cell-free extract. The production of 2-Hydroxymuconic semi aldehyde was detected by a rise in the absorbance over a 10-minute period at 375 nm.

Enzyme Activity Calculation: The following equation was used to calculate enzyme activity:

$$\text{Activity } \mu \text{ moles product formed/min} = \frac{E \times C \times L}{V} \times \frac{\Delta OD}{\text{min}}$$

Where ΔOD is defined as the optical density change at different wavelengths. Where E is the product molar extinction coefficient. V is reaction volume and L is path length. Path length is measured in centimeters. Whatever is assumed to be 1cm Molar extinction coefficient:

- **E260 for catechol-1,2-dioxygenase** = 16,800 L/mol/cm
- **E375 for catechol-2,3-dioxygenase** = 14,700 mol/L/cm

One unit (U) of enzyme activity had been defined as the amount of enzyme that produced one mole of a given product per min. under test conditions.

7- Growth factors of bacteria used

Using the three selected bacteria, several factors such as OMWW concentrations, fermentation time, pH, and carbon and nitrogen sources added to fermentation media at (1gm/L) were modified for optimization of the bio-fermentation process.

8- Biological Treatments of OMWW and characteristics of fermented product

Olive process mill water was diluted to 20% and inoculated with the bacterial broth of each of the three bacterial strains for 35 days. In the end, all physicochemical characters (pH, EC, BOD, COD, phenols, N, C, and mineral substance) and microbiological constituents of obtained biofertilizer (fermented OMWW) were measured as described before for unfermented OMWW.

9. Field experiment

Barley **grains** (*Hordeum vulgare*) were purchased by Agricultural Research Center (ARC), Ministry of Agriculture and Land Reclamation, Egypt. **In** November and December (2018), pot **experiments were** conducted under greenhouse condition at Desert Research Center (DRC) to evaluate the three fermented **OMWWs** as **biofertilizers.** Design of the pot experiment was whole block with three replications. Following seed germination, barley grains were planted in pots of 10 Kg capacity, filled with soil provided from the Baloza Station, DRC in South Sinai, with 100 ml of each biofertilizer added to each pot. The pots were watered twice a week. After 60 days, plant heights, fresh and dried weights, and plant heights were measured. Dry plants were digested using a combination of pure $HClO_4$ and H_2SO (1v: 1v) according to **(20)**. In digested barley grains samples, total nitrogen, potassium, and phosphorus were determined **(21,22,** and **23),** respectively as described before in that of OMWW.

9- Statistical analysis

The obtained data were statistically analyzed using the method outlined by **(40)**. Duncan was used to differentiate means according to **(41).**

Results

1. Chemical and microbiological analysis of raw OMWW

The raw OMWW was evaluated for its physical-chemical characteristics, which found that it is highly acidic and has a moderate salinity (EC 2.82 dS/m), as presented in **Table (1).** It also had a high organic load and low nitrogen content, resulting in a high C/N ratio, making the waste exceedingly stable and difficult to degrade. The waste's key trait is its high polyphenol content. The waste has a low microbial count, and even though different types of microbes were recorded, yeasts had the highest number compared to others, which can be explained by the waste's low pH, as in **Table (1).**

Table (1)
Physico-chemical and microbiological assay of unfermented OMWW

Physico-Chemical assay									
EC (dS/m)	**pH**	**Organic Carbon (%)**	**Available Nitrogen (%)**	**C/N**	**Available Phosphorus (%)**	**Available Potassium (%)**	**Total phenols (ppm)**	**COD (ppm)**	**BOD (ppm)**
2.82 (±0.88)	4.62 (±0.89)	20.89 (±2.99)	0.17 (±0.02)	122.8 (±12.1)	0.013 (±0.001)	0.18 (±0.02)	2040 (±8)	8120 (±7.4)	365 (±5.9)

Microbiological assay				
Total count (x 10^3 CFU/ml)	**Fungi (x 10 CFU/ml)**	**Yeast (x 10^3 CFU/ml)**	**Nitrogen fixers (x10^3 MPN/ml)**	***Pseudomonas spp.* (x 10^3 CFU/ml)**
85^{a} (±1)	3^{cd} (±2)	7^{b} (±1.5)	5.6^{bc} (±0.2)	1.6^{d} (±0.9)

Bars show means of three independent replicates (n = 3) ± standard error

2. Isolation and preliminary detection of both Catechol -Dioxygenase enzymes activities in microbial isolates:

Ten isolates (7 bacteria and 3yeasts) capable of degrading phenol to a highest amount within 7 days were chosen as efficient phenol-degrading microbes and employed for further inquiry by evaluating their enzymatic activity, as shown in **Table (2).** Catechol 1, 2-dioxygenase activity was positively detected in all tested phenol-degrading isolates as a yellow or orange color developed in the reaction tubes

containing phenol-degrading bacterial samples combined with the reaction solution. Under the same experimental conditions as catechol 1, 2-dioxygenase, catechol 2, 3-dioxygenase activity was recorded in only **70%** of the bacterial strains **tested, none of which** showed no color change in reaction mixture indicating that the tested enzyme could not be produced. Three bacterial isolates coded OWB4, OWB5 and OWB6 were selected for further investigation and identification as they recorded the highest phenol degrading abilities and enzyme activities.

Table (2)

Selection and preliminary detection of both catechol -dioxygenase activities in microbial isolates (at 20% OMWW)

Isolates code	Type of isolate	Phenolic compounds degradation (%)	Catechol 1,2-Dioxygenase	Catechol 2,3-Dioxygenase
OWB1	Bacteria	15.6^{d} (±1)	+	+
OWB2	Bacteria	11.4^{efg} (±0.4)	+	-
OWY1	Yeast	13.2^{de} (±0.9)	+	+
OWB3	Bacteria	$10f^{g}$ (±05)	+	+
OWB4	Bacteria	33^{a} (±2)	+	+
OWB5	Bacteria	28^{b} (±1.1)	+	+
OWY2	Yeast	12^{ef} (±1)	+	-
OWB6	Bacteria	25^{c} (±2)	+	+
OWY3	Yeast	8.7^{g} (±0.2)	+	+
OWB7	Bacteria	5.5^{h} (±0.8)	+	-

Bars represent the mean of three independent replicates (n = 3) ± standard error. Different letters indicate significant differences between means by LSD test at p <. 0.05

4. Identification of selected bacteria:

The three strains (OWB4, OWB5, and OWB6) were identified based on their biochemical and molecular characterization as in **Table (3)** and **Figs. (1a, 1b, 1c)** to be belonging to the three strains: *Lysinibacillus macroides* strain LMG 18474, *Lysinibacillus boronitolerans* strain SC03, and *Brevundimonas olei* strain (MJ15) using 16sRNA. A phylogenetic tree of each of bacterial strain was established using sequences from each isolate based on the consensus sequence of the 16S rRNA. They were given the accession numbers *Lysinibacillus macroides* (NR 114920.1), *Lysinibacillus boronitolerans* (NR 145635.1), and *Brevundimonas olei* (NR 117268.1) in the Gene Bank, respectively.

Table (3)

The morphological and biochemical properties of the selected bacterial isolates

Characteristics	*Lysinibacillus macroides*	*Lysinibacillus boronitolerans*	*Brevundimonas olei*
Cell morphology	Rod shaped	Rod shaped	Rod shaped
Gram staining	Gm +ve	Gm +ve	-ve
Spore staining	Subterminal spore	Subterminal spore	-ve
Motility	Motile	Motile	Motile
Glucose fermentation	-ve	+ve	-
Manitol fermentation	-ve	+ve	-
Lactose fermentation	-ve	-ve	-
Sucrose fermentation	-ve	+ve	+ve
Ureaes test	+ve	+ve	-

Catalse test	+ve	+ve	+
Oxidase tset	+ve	+ve	+
Methyl red test	-ve	-ve	-
Starch hydrolysis	+ve	+ve	+
Gelatin liquification	+ve	+ve	+

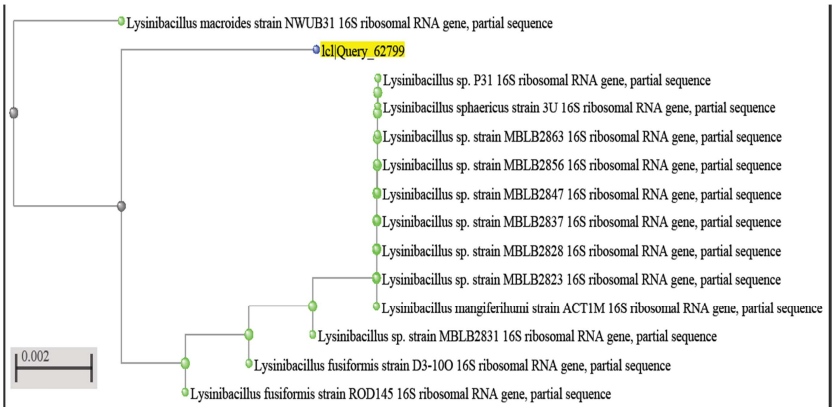


Figure (1a): The **identification** tree of ***Lysinibacillus macroides*** based on 16S rRNA sequences as in **the database** of Gen Bank

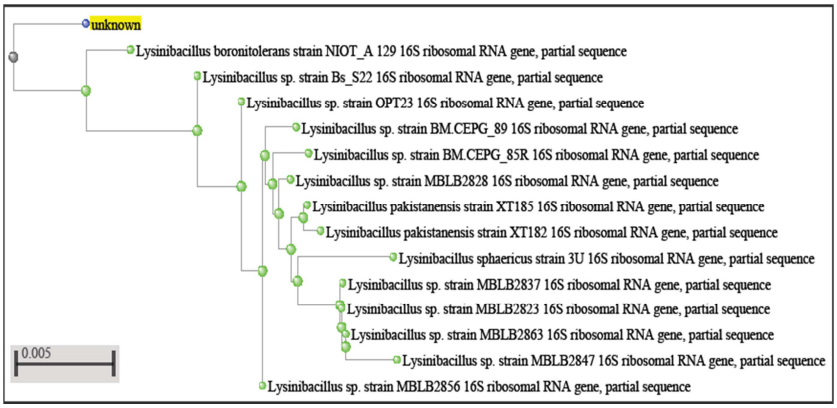


Figure (1b): The **identification** tree of ***Lysinibacillus boronitolerans*** based on 16S rRNA sequences as in **the database** of Gen Bank

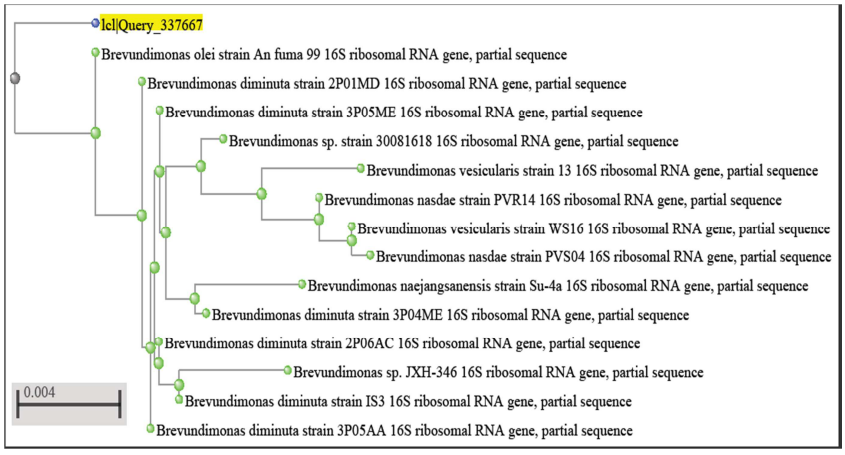


Figure (1c): The **identification** tree of ***Brevundimonas olei*** based on 16S rRNA sequences as in **the database** of Gen Bank

5. Determination of phenolic enzymes

For extracellular enzyme assay, no activity of two bacterial enzymes (catechol-1,2-dioxygenase or catechol 2,3-dioxygenase) was detected in supernatants of the three selected bacterial strains.

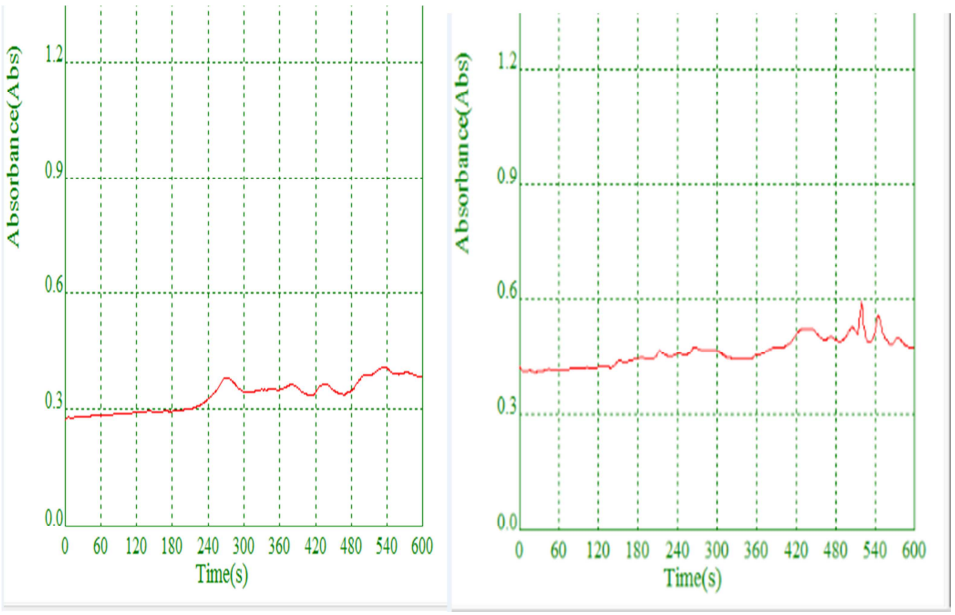
The same tow enzymatic assays were performed for cell extracts of tested bacterial strains in general (Intracellular), and the three bacterial strains recorded both enzyme activities to varying degrees, as shown in Table (4) and three figures (2 a), (2 b), and (2c). Figures indicated the changes of absorbance at 357nm (ΔOD) occurred for the three bacterial isolates concerning catechol 2,3-dioxygenase enzymes, these changes in absorbance were used for the calculation of enzyme activities.

All analyzed bacterial cultures exhibited tow catechol 1,2-dioxygenase activities and catechol 2,3-dioxygenase enzymes to varying degrees. The activities of catechol 1,2-dioxygenases were lower for the three bacterial strains than that of catechol 2,3-dioxygenases. *Lysinibacillus macroides* had the highest enzyme activity of catechol 1,2-dioxygenase rather than any of the bacterial strains tested followed by *Brevundimonas olei*, while *Lysinibacillus boronitolerans* had the lowest activity, with enzyme activities of 11.76 U, 11.2 U, and 10.08 U, respectively.

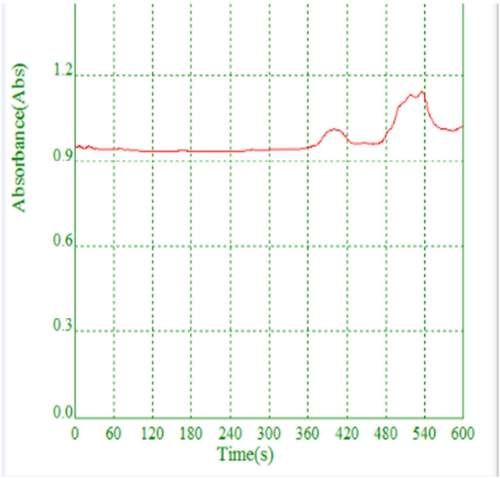
Table (4)
Total enzymatic activities for cell extracts of the three bacterial strains after 20 days of cultivation on 20 % OMWW

Bacterial strains	Catechol 2,3-Dioxygenase activity [U]	Catechol 1,2-Dioxygenase activity [U]
Lysinibacillus boronitolerans	10.08 (±1.01)	0.588 (±0.089)
Lysinibacillus macroids	11.76 (±0.89)	0.735 (±0.104)
Brevurdimonas olei	11.2 (±1.8)	0.56 (±0.1)

Bars **represent the mean** of three independent **replicates** (n = **3) ±** standard error



2 a: ***Lysinibacillus macroides*** **2b:** ***Lysinibacillus boronitolerans***



2C*: Brevundimonas olei*

Figures (2a, 2b and 2c): Changes in the absorbance (ΔOD) at 357 nm indicating the production of 2-Hydroxymuconic semi aldehyde that used for calculation of catechol 2,3-dioxygenase activity

6. Growth factors of bacteria used

Various factors were adjusted to maximize phenolic compound biodegradation during the OMWW fermentation process using the three phenol degrading bacteria individually. The following variables were investigated: OMWW dilution, pH of fermentation media, and carbon and nitrogen sources added to the fermentation media.

6.1: Dilution of OMWW

According to Figure (3), phenolic compound biodegradation increased with increasing OMWW dilution. For undiluted OMWW (100%), phenolic degradation ranged from 0% for *Brevurdimonas olei* to 7.38 % for *Lysinibacillus macroides*. With the dilution of OMWW, increasing in bacterial degradation of phenolic compounds was observed, reaching a 20% concentration to the minimum phenolic compounds contents. After 25 days, the maximum phenol removals from OMWW were obtained by a *Brevurdimonas olei* at 60% concentration reaching 47.5% followed by that of *Lysinibacillus macroides* and *Lysinibacillus boronitolerans*. At 20% dilution, while the degradation rate of total phenolic compounds was recorded at about 36% for all strains, the concentration of phenolic compounds reached its minimum at this dilution making it more suitable to use as fertilizers.

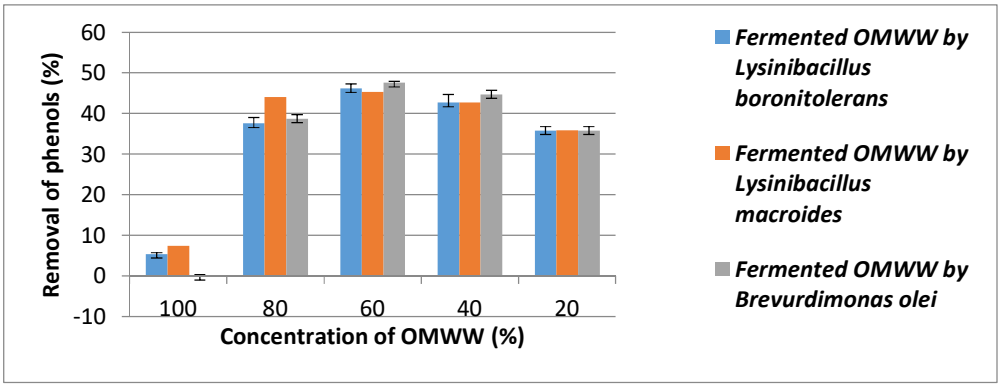


Figure (3): Effect of OMWW dilution and bacterial fermentation on the biodegradation of phenolic compounds after 20 days. Bars represent the mean of three replicates (n = 3), vertical bars indicate (n = 3) ± standard error

6.2: Fermentation time

The degradation rate of phenolic compounds increased with time at a 20 % concentration of OMWW, with the highest degradation recorded after 35 days of fermentation for the three bacterial strains. The highest degradation was achieved from using *Brevurdimonas olei* which recorded a removal rate reaching 40.9% of total phenolic compounds, as shown in **Fig (4).**

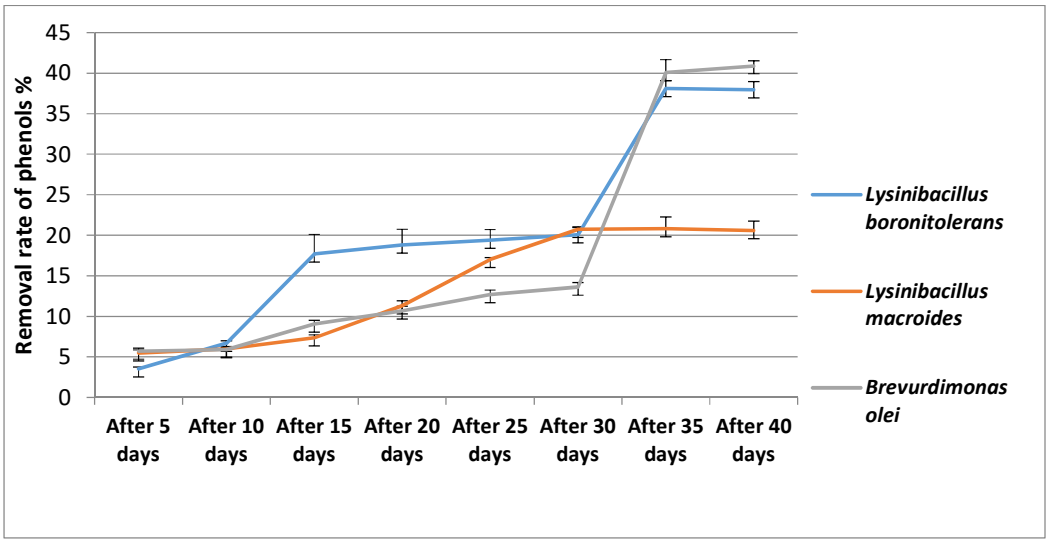


Fig (4): Removal rate of phenolic compounds in OMWW using the three bacterial strains after 35 days of fermentation. Bars represent the mean of three replicates (n = 3), vertical bars indicate (n = 3) ± standard error

6.3: The pH of fermentation media

Change in the pH of the fermentation medium is one of the most effective variables in the breakdown of OMWW phenolic compounds. Both strains *Lysinibacillus boronitolerans* and *Brevurdimonas olei* showed the greatest OMWW degradation at pH 6 as the concentration of phenolic compounds reached 250 and 245 ppm, whereas *Lysinibacillus macroids* showed the greatest degradation at pH 7 where the phenolic concentration reaching 205 ppm only, as represented in **Fig (5).**

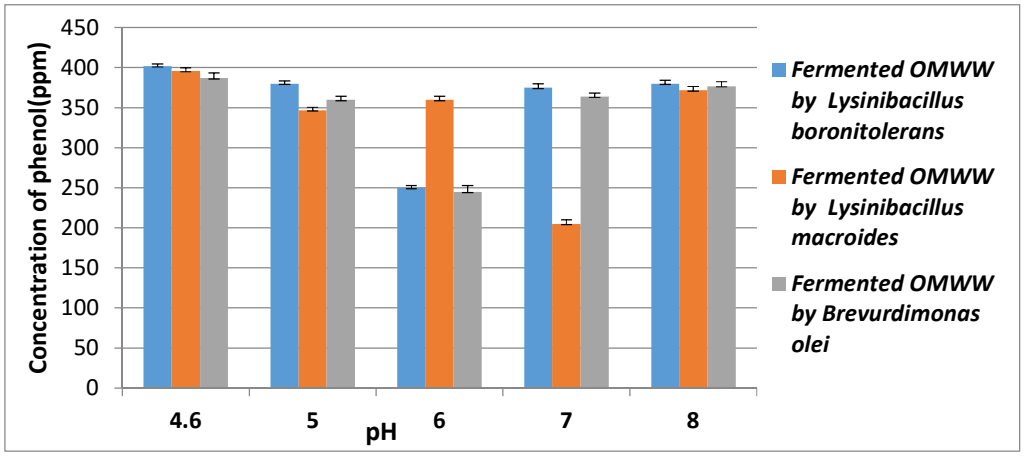


Fig (5): Effect of pH values of the fermentation media (OMWW) on the concentration of phenolic compounds after 35 days of fermentation Bars represent the mean of three replicates (n = 3), vertical bars indicate ± standard error. Where pH4.6 represent the control

6.4: Effect of carbon and nitrogen sources of fermentation media

For both *Lysinibacillus macroides* and *Brevurdimonas olei*, the maximum levels of OMWW degradation were seen in fermentation conditions supplemented with yeast for *Lysinibacillus boronitolerans* and ammonium nitrate for *Lysinibacillus macroides*. In terms of carbon sources, supplementation of starch for *Lysinibacillus macroids* and *Brevurdimonas olei* and glucose for *Lysinibacillus macroides* yielded the highest degradation of phenolic compounds, as shown in **Table (5).**

Table (5)
Effect of carbon and nitrogen sources of the fermentation media on the concentration of phenolic compounds after 35 days of fermentation

Treatments	Phenol concentration (ppm)					
	Nitrogen sources			Carbon sources		
	Ammonium nitrate	Yeast	Peptone	Sucrose	Starch	Glucose
Control (unfermented)	274^{a} (±1)	274^{c} (±1)	274^{a}(±1)	274^{a}(±1)	274^{a}(±1)	274^{a}(±1)
Fermented OMWW by *Lysinibacillus boronitolerans*	256^{b} (±2)	130^{d}(±2)	210^{d}(±2)	224^{b}(±1)	180^{c}(±2)	250^{b}(±2)
Fermented OMWW by *Lysinibacillus macroides*	175^{d} (±1.5)	280^{b}(±0.7)	220^{c}(±3)	217^{c}(±2)	208^{b}(±1.8)	165^{d}(±0.4)
Fermented OMWW by *Brevurdimonas olei*	187^{c} (±0.6)	318^{a}(±1.5)	226^{b}(±0.5)	222^{b}(±3)	173^{d} (±3)	232^{c}(±0.5)

Bars **represent the mean** of three independent **replicates** (n = **3) ±** standard error. Different letters indicate significant differences **between** means **by** LSD test at p **<.** 0.05

Biological fermentation of OMWW and characteristics of obtained products

The pH of the waste increased significantly from 4.62 to around 6.5 after the bio-fermentation process **(Table 6)** which may be attributed to the breakdown of some phenolic acids. At the end of the fermentation phase, total nitrogen content increased, total phenol levels decreased and overall phosphorus and potassium levels only slightly increased in all treatments.

Table (6)
Physicochemical properties of fermented Olive Mill Wastewater

Physicochemical properties	OMWW Fermented by *Lysinibacillus boronitolerans*	OMWW Fermented by *Lysinibacillus macroides*	OMWW Fermented by *Brevurdimonas olei*
EC (dS/m)	2.76 (±1)	2.55 (±0.05)	2.78 (±0.99)
pH	6.36 (±0.04)	6.8 (±1)	6.58 (±0.89)
COD	4850(±9)	4760 (±8.2)	4820 (±8.7)
BOD	260(±2)	278(±2.3)	255(±2.2)
Organic Carbon (%)	18b (±1)	12.25c (±0.05)	16.57b (±0.98)
Available Nitrogen (%)	0.2 (±0.1)	0.19 (±0.01)	0.23 (±0.08)
Available Phosphorus (%)	0.25 (±0.005)	0.24 (±0.01)	0.26 (±0.02)
Available Potassium (%)	0.36 (±0.05)	0.37 (±0.04)	0.37 (±0.02)
Total phenols (ppm)	248b (±1)	207d (±3)	244c (±2)

Bars represent the mean of three independent replicates (n = 3) ± standard error. Different letters indicate significant differences between means by LSD test at p <. 0.05

7. Pots experiment:

Table (7) illustrated physical and chemical analysis of the soil. Raw OMWW was able to enhance barley height, fresh and dry weight by 6.7 %, 25%, and 15.4 %, respectively, when compared to the control. Regardless of the type of bacteria employed in the fermentation process, there were substantial increases in all traits assessed with fermented OMWW compared to that of control **(Table 8).**

Table (7)

Physical and chemical analysis of the soil

pH (1:1) suspension	EC dS m^{-1} (1:1) extract	Soluble cations (Meq L^{-1})				Soluble anions (Meq L^{-1})			
		Ca^{++}	Mg^{++}	Na^{+}	K^{+}	$CO3^{--}$	HCO_3^{-}	Cl^{-}	SO_4^{--}
8.9 (±0.1)	0.63 (±0.03)	0.88 (±0.02)	0.61 (±0.01)	4.34 (±1.1)	0.23 (±0.03)	-	2.10 (±0.3)	3.12 (±0.98)	0.88 (±0.03)
Texture class	**CEC Meq/100 g soil**	**$CaCO_3$ %**	**OM %**	**Available nutrients (mg kg^{-1})**					
				N	**P**	**K**	**Fe**	**Mn**	**Zn**
Sand	5.20 (±0.8)	0.18 (±0.02)	0.09 (±0.01)	27.4 (±1.9)	3.25 (±0.97)	67.3 (±2.3)	2.63 (±1.03)	1.05 (±0.05)	0.36 (±0.01)

Table (8)

Impact of unfermented and fermented OMWW on the Growth parameters of Barley after 60 days of planting

Treatments	Length (Cm)	Fresh weight (gm)	Dry weight (gm)
Control (Tap water only)	36.16^{e} (±1.04)	1.44^{c} (±1.01)	0.247^{c} (±0.103)
Unfermented OMWW (20%)	38.5^{d} (±1.9)	1.8^{c} (±0.2)	0.285^{c} (±0.101)
Fermented OMWW by *Lysinibacillus boronitolerans*	45^{b} (±1)	3.6^{b} (±0.4)	0.387^{b} (±0.22)
Fermented OMWW by *Lysinibacillus macroides*	54.5^{a} (±1.2)	5.8^{a} (±0.2)	0.785^{a} (±0.203)
Fermented OMWW by *Brevurdimonas olei*	42.7^{c} (±1.8)	2.9^{bc} (±0.5)	0.46^{b} (±0.09)

In terms of nitrogen and potassium content, while all bio-treatments increased nitrogen and potassium content in barley plants, the plants treated with OMWW fermented with *Lysinibacillus macroides* showed the greatest significant increase, as shown in **Table (9).** Concerning to phosphorus content, plants treated with OMWW either fermented or did not show a significant increase. As shown in **Table (9),** *Lysinibacillus boronitolerans* had the greatest increase, while raw OMWW had the least.

Table (9)
Impact of unfermented and fermented OMWW on the NPK content of Barley after 60 days of planting

Treatments	Nitrogen (%)	Phosphorus (%)	Potassium (%)
Control (Tap water only)	0.3^{e} (±0.1)	0.119^{e} (±0.006)	0.23^{e} (±0.04)
Unfermented OMWW	0.43^{d} (±0.09)	0.130^{d} (±0.01)	0.31^{d} (±0.195)
Fermented OMWW by *Lysinibacillus boronitolerans*	0.56^{c} (±0.1)	0.177^{a} (±0.003)	0.42^{c} (±0.02)
Fermented OMWW by Lysinibacillus macroides	0.74^{a} (±0.03)	0.156^{b} (±0.004)	0.57^{a} (±0.11)
Fermented OMWW by *Brevurdimonas olei*	0.65^{b} (±0.9)	0.145^{c} (±0.005)	0.52^{b} (±0.03)

Table (10) showed that using fermented or unfermented OMWW increased the microbial count, nitrogen fixers, and yeast count. The highest increase in yeast counts was observed in the barley rhizosphere treated with OMWW and *Brevurdimonas olei,* whereas OMWW fermented with *Lysinibacillus macroides* produced the highest nitrogen fixers and total microbial count.

Table (10)
Impact of unfermented and fermented OMWW on microbial community of barley rhizosphere after 60 days of planting

Treatments	Nitrogen fixers (10^{3}X MPN /ml)	Yeasts (10^{2}xCFU/ml)	Total count (10^{5}xCFU/ml)
Control (Tap water only)	35^{b} (±2)	12^{d} (±3)	150^{e} (±15)
Unfermented OMWW	43^{c} (±3)	21^{b} (±10)	200^{d} (±9)
Fermented OMWW by *Lysinibacillus boronitolerans*	41^{c} (±1)	8^{e} (±2)	226^{b} (±4)
Fermented OMWW by *Lysinibacillus macroides*	52^{a}(±2)	17^{c} (±1)	240^{a} (±4)
Fermented OMWW by *Brevurdimonas olei*	28^{d}(±4)	24^{a} (±5)	220^{c} (±10)

Discussion

High concentrations of organic components as phenols in olive mill wastewater makes OMWW hazardous and difficult for biological breakdown to be a major polluter. Phenolic water pollution gives off an unpleasant odor and taste and can therefore alter biota **(42).** Phenolic contaminants and their metabolites are mutagenic and carcinogenic in living cells (42, 43). Due to their high toxicity and carcinogenic effects, these pollutants pose threat to human health and environment (44). Because of high chemical and biological oxygen demand of waste water, high content of phenolic compounds, wide C/N ratio, and low pH, OMWW is difficult to biodegrade, posing serious environmental and plant growth risks **(45).** In general, the waste has a low microbial content, which could be attributed to low pH and high harmful chemicals such as polyphenols **(46).** On the other hand, the waste is distinguished by its high nutrient content, which makes it an efficient fertilizer after hazardous substances have been removed. Comparing with other organic wastes, waste of olive mill has high concentrations of potassium, moderate concentrations of nitrogen, phosphorus, calcium, magnesium and iron (46). Many microbial strains with Catechol-Dioxygenase enzymes can be employed to biodegrade phenolic chemicals in wastes. Three OMWW-isolated bacterial strains were chosen for waste fermentation and phenolic compound degradation in this study. Microorganisms that degrade phenol typically produce the enzyme oxidoreductases, responsible for cleavage of phenol at *ortho* or *meta* locations, yielding *cis* and cismuconic acid as intermediates **(47).** In this study, *Lysinibacillus boronitolerans* strain SC03, and *Brevundimonas olei* (MJ15) *Lysinibacillus sp.* were identified as biodegradable agents using 16sRNA. NCCP-313 is one of the most effective strains for phenol biodegradation and can be utilized to bioremediate phenol-contaminated wastewater **(48).** Different phenol-degrading bacteria, *Brevundimonas spp., Sphingomonas spp., and Novosphingobium spp.* were found in olive mill wastewater, can be used for processing microbial fuel cell **(49).** In terms of enzymatic activity, no extracellular enzyme activities of catechol 1, 2-dioxygenases or catechol 2,3-dioxygenases enzymes were detected in the supernatants of the three bacterial strains, whereas the three bacterial cultures showed the two enzymes activities have varying degrees, this is consistent with the idea that the enzymes involved in the process of phenol degradation not to be secreted into the cultural media

and should remain in the microbial cells **(50).** To maximize biodegradation process of phenolic compounds, the study focus on the optimization of the OMWW fermentation process. This can be accomplished by altering several elements such as OMWW dilution, fermentation medium pH, and carbon and nitrogen sources in the fermentation media. We emphasize that waste dilution is a vital factor in the biodegradation of phenolic compounds. Low phenol degradability in OMWW and weak tolerance of the fungus *Pleurotus columbinus* were detected at concentrations ranging from 50% to 100%, on the other hand, phenol degradation and COD reduction were more pronounced at concentrations below 40% after 4 weeks of treatment **(51).** In general, phenolic compound biodegradation increases with time, peaking after 35 days of fermentation. This was due to the fact that the phenol content gradually decreased over time and reached its minimal after 20 and 25 days of bio-fermentation, and that the 50% and 30% diluted bacterial mixtures, respectively, grew and removed phenol. This is consistent with the fact that it reached a maximum (71, 9 or 71.4%) after 25 days of fermentation **(45)**. The pH of growing media for *Lysinibacillus boronitolerans* and *Brevurdimonas olie* reaching its optimum at 6, and7 for *Lysinibacillus macroids*. *Lysinibacillus sp.* strains degraded 750 mg L-1 phenol in 40 to 96 hours at pH 7 **(48)**.The addition of yeast or ammonium sulphate as a nitrogen source of fermentation media and starch or glucose as a C-source could improve phenolic compound degradation. At the end of the fermentation process, the product is distinguished by an increase in the pH of the waste to 6.5, which is suitable for the growth of microbial strains and use as fertilizers. The pH level attained at the end of the process (6.36–6.38) was ideal for microbial growth **(45)**. Total nitrogen, on the other hand, increased, while total phenols decreased dramatically. Biotreatment of organic wastes with *Lysinbacillus boronitolerans* resulted in higher nitrogen, phosphorous, potassium, and total organic carbon levels than treatment without bacteria **(52).** The current study found that using OMWW before or after fermentation boosted barley growth traits (height, fresh weight, and dry weight) considerably. Regardless of the type of bacterial strains utilized in the fermentation process, fermented OMWW was more effective than raw OMWW, which could be owing to its large microbial community and mineral content. The highest growth characteristics were observed with *Lysinibacillus macroides,* whereas the lowest was recorded with *Brevurdimonas olei.* Olive mill wastewater application greatly increased maize growth, reaching 10–11% of control growth **(53).** When the effectiveness of *Lynsibacillus boronitolerans* and *Alcaligens sp.*in producing bio-fertilizer from different organic wastes was compared, it was discovered that *Lynsibacillus macroides* produces more biofertilizer than *A. faecalis* **(54)**. *Brevundimonas* spp-growth-promoting potential was described as it demonstrated nitrogen fixation potential via acetylene reduction assay and phosphate solubilization potential **(55).** The NPK level of the barley plants was measured to see how the fermented OMWW affected the chemical contents. *Lysinibacillus boronitolerans* showed the greatest growth when fermenting OMWW with any of the three tested bacterial strains. In addition, the microbial count, nitrogen fixers, and yeast count all improved. Because of their various plant growth stimulating activities as nitrogenase activity, Indole-3-acetic acid (IAA) synthesis and ammonia production, *Brevundimonas sp*., can be considered a useful plant growth promoters for sustainable agriculture especially in arid regions (55). Microbiological analysis of barley rhizosphere showed increased organic matter content, phenolic compounds and microbial activity in OMWW-treated soil **(56).**

Conclusion

Three naturally surviving phenol-degrading strains in OMWW were identified as *Lysinibacillus macroides, Lysinibacillus boronitolerans,* and *Brevundimonas olei.* Three strains exhibited both catechol-1,2-dioxygenase and catechol-2,3-dioxygenase enzymes. They serve to break down phenolic compounds during the bio-fermentation process. Removal of phenolic compounds from OMWW through a bio-fermentation process is seen as an important method for upgrading the waste and turning it into a valuable organic bio-fertilizer. This bio-product contains agriculturally beneficial microorganisms and compounds that support the growth of barley.

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