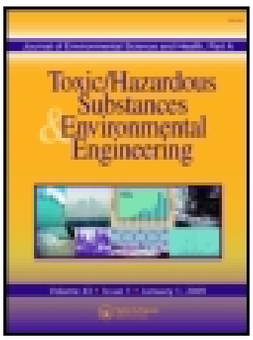

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Isolation and characterization of phenol degrading bacterium strain *Bacillus thuringiensis* J20 from olive waste in Palestine

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ABSTRACT

This study aimed at isolation of phenol degrading bacteria from olive mill wastes in Palestine. The efficiency of phenol removal and factors affecting phenol degradation were investigated. A bacterial strain (J20) was isolated from solid olive mill waste and identified as *Bacillus thuringiensis* based on standard morphological, biochemical characteristics and 16SrRNA sequence analysis. The strain was able to grow in a phenol concentration of 700 mg/L as the sole carbon and energy source. The culture conditions showed a significant impact on the ability of these cells to remove phenol. This strain exhibited optimum phenol degradation performance at pH 6.57 and 30 °C . Under the optimized conditions, this strain could degrade 88.6% of phenol (700 mg/L) within 96 h when the initial cell density was OD_{600} 0.2. However, the degradation efficiency could be improved from about 88% to nearly 99% by increasing the cell density. Immobilization of J20 was carried out using 4% sodium alginate. Phenol degradation efficiency of the immobilized cells of J20 was higher than that of the free cells, 100% versus 88.6% of 700 mg/L of phenol in 120 h, indicating the improved tolerance of the immobilized cells toward phenol toxicity. The J20 was used in detoxifying crude OMWW, phenolic compounds levels were reduced by 61% compared to untreated OMWW after five days of treatment. Hence, *B. thuringiensis*-J20 can be effectively used for bioremediation of phenol-contaminated sites in Palestine. These findings may lead to new biotechnological applications for the degradation of phenol, related to olive oil production.

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Bioremediation; *Bacillus thuringiensis-J20*; olive mill waste; Phenol

Introduction

Olive oil extraction industries are mainly located in Mediterranean countries and seasonally accompanied by certain amounts of waste (by-products) including olive mill wastewater (OMWW) and solid olive husk.[1,2] Palestine dedicates 45% of its land to this valuable crop with growing consumer demand for olive oil, as it is considered an essential food for Palestinians.[3] OMWW has a considerable amount materials that resist biological degradation such as reduced sugars, high phosphorus, organic and phenolic compounds that have a toxic action to some organisms.[4,5] Phenol has been classified as a highly hazardous chemical and a major pollutant reported by the US Environmental Protection Agency (EPA).[6,7] The inadequate and uncontrolled disposal methods of OMWW directly into sewer systems, valleys, rivers and lakes result in contamination of groundwater and is an environmental risk factor to aquatic organisms, including microorganisms, plants and fish.[2,8] Thus, due to the toxicity and persistence of OMWW in the environment, it is necessary to develop highly efficient techniques to reduce the phenol level in waste water to environmentally tolerable limits prior to their being released into the environment.[9] In this regard, different technologies, conventional and advanced, for removal of phenols have been described, such as electrochemical oxidation, photo-oxidation, ozonation, UV/H_2O_2, Fenton reaction, membrane processes and enzymatic treatment.[10,11] These treatments are usually complex, expensive and produce hazardous end-products.[10,12,13] Bioremediation, using microorganisms, has been universally accepted as an effective, low cost method, that reduces environmental pollution.[14] Recently, several fungal and bacterial species known to utilize phenol and other aromatic compounds as their sole carbon and energy sources have been used in phenol biodegradation studies. These include *Candida tropicalis, Acinetobacter calcoaceticus, Alcaligenes eutrophus, Pseudomonas putida, Burkholderia cepacia G4* and *Bacillus stearothermophilus.*[15–18] Furthermore, it has been reported that their phenol biodegradation efficiency could be further enhanced by the process of cell immobilization.[19] Several studies showed that the immobilized cells could tolerate a higher phenol concentration and protect the bacteria against changes in temperature and pH, thus leading to better performance than free suspended cells.[20,21] To isolate and characterize phenol-degrading bacteria, from olive mill wastes in Palestine, is the aim of this study.

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Materials and methods

Sampling and growth media

OMWW and solid olive mill waste (SOMW) samples were collected in October 2015 from an olive mill in Bethlehem, south of Palestine. All samples were collected in sterile containers and delivered to the laboratory within 24 h. Two types of growth media were used in the present study: The minimal salt media (MSM) and Luria-Bertani (LB). The LB, a nutritionally- rich medium, which is primarily used for the growth of bacteria contained 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1,000 mL double-distilled water. The MSM contained Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NaCl (0.5 g), NH_4Cl (1 g), $CaCl_2{\cdot}2H_2O$ (0.02 g) and $MgSO_4 7{\cdot}H_2O$ (0.5 g) in 1,000 mL double distilled water.

Isolation and identification of bacteria

Two samples of OMWW and 3 samples of SOMW were used for isolation of microorganisms. Two grams of SOMW was suspended in 10 mL of sterile phosphate buffer saline (PBS), incubated for 24 h at room temperature (25 °C). One milliliter was taken from each sample and inoculated samples into 10 mL MSM supplemented with 100 mg/L of Phenol (analytical grade) and then incubated at 30 °C for 48 h. One loopful (0.1 mL) was taken from all samples, inoculated into LB plates and incubated at 30 °C for 24 h. Distinct colonies were sub-cultured repeatedly on new LB plates to obtain pure isolates. A battery of biochemical tests were used to identify bacterial isolates according to Bergey's manual of systematic bacteriology. For further identification, the bacterial broth was taken and centrifuged at 8000*g* for 10 min. The obtained pellet was washed with PBS and the DNA was extracted using the QIAamp DNA extraction kit (QIAGEN GmbH, Hilden, Germany). Amplification of 16SrRNA gene was accomplished using the primers set (pA: AGAGTTTGATCCTGGCTCAG and pH': AAGGAGGTGATCCAGCCGCA) as previously described.[22] The PCR product was purified and sequenced by Hy Laboratories Ltd., Israel. The obtained sequences were assembled using Bioedit software, used in a BLAST search (ncbi.nlm.nih.gov/blast) and aligned with other sequences registered in GenBank.

Phenol tolerance experiment

The toxicity of phenol on bacterial growth was measured using the Alamar blue viability indicator, the cells were grown on LB broth at 30 °C for 24 h, cells were treated with different concentrations of phenol starting by 3% and incubated at 30 °C for an additional 24 h. The same bacterial cells were cultured in the LB medium with no treatment and broth medium devoid of bacterial inocula served as positive and negative controls, respectively. Ten percent of Alamar blue (AB) was added to culture medium and control cells. The redox reaction, in which AB is reduced by the cells, was measured as fluorescence units (ex: 544 nm; em: 590 nm) after 3 and 5 h using Fluoroskan plate reader (Fluoroskan Ascent FL, Finland).

Growth assessment and phenol degradation

The ability of isolated bacteria to utilize phenol as the sole source of carbon was studied by culturing in LB to mid-log phase (Optical density; $OD_{600} \approx 0.50$). The bacterial cells were harvested by centrifugation at 4,000 rpm for 15 min, washed and suspended with MSM medium then adjusted to an OD_{600} of 0.2. Phenol (analytical grade) as a sole source of carbon was added directly to MSM at a concentration of 700 mg/L. The growth behavior of the bacterial isolate was monitored by measuring OD_{600} spectrophotometrically at 24, 48, 72 and 96 h after inoculation. To evaluate phenol elimination by degrading bacteria, the residual phenol concentrations were determined by collecting 1 mL from each sample at 24, 48, 72 and 96 h after inoculation. A similar procedure was carried out for control sample (culture medium without having bacterial cells). The phenol OD_{540} was measured based on condensation of phenol with 4-aminoantipyrine followed by oxidation with alkaline potassium ferricyanide giving a red color,[23] and concentrations determined by comparison to a standard curve.

Evaluation of phenol degradation at different experimental conditions

The effects of pH, temperature and cell density on phenol degradation were investigated. Growth conditions and phenol measurement were conducted as described above. Repeated experiments with different pH values, varied incubation temperature (25 °C, 30 °C, 37 °C and 42 °C) and cell densities (OD_{600} 0.2, 0.5, and 0.6) were carried out using 700 mg/L of phenol.

Immobilization of bacterial cells in alginate

The phenol-degrading bacteria were harvested after 24 h of growth in LB culture medium. The cell pellet was obtained by centrifugation at 4,000 rpm for 10 min and subsequently re-suspended in 10 mL of PBS. A stock of 4% (wt v^{-1}) sodium alginate was prepared in the MSM medium. Ten milliliters of bacterial cell suspension (OD_{600} 0.2) were added to 50 mL of sterile alginate solution and mixed by stirring on a magnetic stirrer. This alginate cell mixture was ejected drop by drop into a cold sterile 0.1M calcium chloride solution ($CaCl_2$) and kept at room temperature for 1 h to complete the gel formation as described previously.[24] The beads were then rinsed with MSM followed by distilled water to remove residual $CaCl_2$. The biodegradation study was done at the same condition described for the free cells.

Stability study

To examine the stability of the immobilized cells, the beads containing the immobilized J20 cells were stored for 35 days at 4 °C and then used for phenol degradation. The immobilized cells were removed from the medium and washed three times with sterile distilled water. The biodegradation experiment was carried out as described above.

Microtoxcicity test

Twenty five milliliter (25 mL) of waste water were syringe-filtered (0.2 μm Whatman, Life sciences), inoculated with 0.5 mL of phenol- degrading bacteria (J20) (OD_{600} 0.2) and incubated

at 30 °C for 5 days. Treated and untreated olive waste water samples were diluted with sterile LB broth in a series of gradient dilutions at 0, 20, 40, 60, 80, and 100%. After this, diluted samples were inoculated with 200 μL of an overnight culture of *Escherichia coli* (ATCC25922; $OD_{600} = 1.2$) and incubated for 24 h at 37 °C. Standard plate counts were conducted by plating a volume of 100 μL of each dilution of the J20 treated and untreated olive waste water samples on 4 mm thick and 9 mm diameter MacConkey agar plate, which were incubated aerobically for 24 h at 37 °C. Number of pink colonies indicating *E. coli* was counted on each agar plate, a number of colonies over 300 was considered too numerous to count (TNTC). Numbers of colony-forming units (CFU) per mL were counted based on the formula: No. CFU/mL = No. colonies/(dilution x volume of suspension), and plotted as dilution versus CFU/mL x 10^6.

Statistical analysis

GraphPad Prism software online free service was used for statistical analysis. Data were analyzed assuming Gaussian distribution with tools including Pearson's correlation, repeated measure ANOVA and paired t-test. The level of statistical significance was considered at P-value ≤ 0.05.

Results and discussion

Identification of isolates and phylogenetic analysis

In total, seven strains were isolated and identified based on their morphological and biochemical characteristics as shown in Table 1. The examined seven strains showed different degrees of sensitivity towards different concentrations of phenol. However, only one strain (designated J20) exhibited more growth in phenol-containing media than others and was further examined in this study. Serial exposure to increasing levels of phenol was used to determine phenol tolerance of the J20 isolate. The percentage of bacterial growth inhibition versus phenol concentration is shown in Figure 1, the bacterial growth was significantly reduced with increasing phenol concentrations ($P = 0.03$). A concentration of 0.47% phenol was toxic, killing 50% of cells. The isolate expressed a tolerance to phenol concentration as high as 1,100 mg/L (Fig. 1). This bacterium was a Gram positive bacillus, motile, non-lactose fermenter showing growth under aerobic conditions with the optimum temperature of 30 °C (Table 1). Further identification was done by partial 16SrRNA sequencing and a phylogenetic tree was constructed. BLAST analysis revealed 99% nucleotide sequence homology with the nucleotide sequence of the reference strain of *Bacillus thuringiensis* (CP022345.1) with 100% coverage of 1,393 bp. The obtained sequence was deposited in the GeneBank (accession number MF590746). Based on the phylogenetic analysis, the strain was classified in the *bacillus* genera which belongs to *Firmicutes* phylum. The neighbor-joining methods revealed that the closest relative of strain J20 was *B. thuringiensis* and thus the strain J20 was assigned to *B. thuringiensis* (Fig. 2). Extensive biodegradation studies have described the effectiveness of *B. thuringiensis* in removing many environmental pollutants from contaminated sites; including metals and products of organophosphorus insecticide and petroleum industry,[25–27] another study reported that a strain of *B. thuringiensis*, isolated from mangrove sediments in the Persian Gulf, was able to grow in phenol concentration of 900 mg/L as the sole carbon and energy source.[28] The isolation of native microbial species from local polluted environments has been reported to be more adaptive and efficient than non-indigenous microorganisms as biodegraders. Therefore, the isolation of new phenol-degrading bacteria is recommended for the bioremediation of the phenol-contaminated sites in various regions.[18,29]

Phenol degradation by free cells

Measurements of the phenol concentration as a function of time were performed to monitor the course of biodegradation. Our results show that free cells of the J20 isolate can degrade 88.6% of the phenol within 96 h (Fig. 3). A significant negative correlation between phenol concentration and cell numbers as measured by OD ($P = 0.02$, $r = -0.8$) was observed (Fig. 3). Repeated measures of ANOVA showed significant variation between the initial cell density inoculum and biodegradation of 700 mg/L phenol ($P = 0.0003$, $r^2 = 0.8$). When an initial cell concentration of 0.6 OD_{600} was used; almost complete degradation of phenol (98.8%) was achieved by 96 h compared to 94.2% and 88.6% obtained at 96 h by 0.5 and 0.2 OD_{600}, respectively (Fig. 4). Our study suggested that high cell concentrations enhance the biodegradation of phenol, which is in agreement with other studies.[30]

Table 1. Morphological and biochemical characteristics of seven isolates obtained from an olive mill in Palestine.

Sample code	Gram stain	shape	Catalase	Oxidase	Glucose fermentation	Lactose- sucrose fermentation	Indole	Motility	Hydrogen sulfide	Gas	Simmon's citrate	Urease	MR	VP	ID (%)
J20	+	Bacilli	+	+	+	−	+	+	−	+	−	−	+	+	*Bacilus spp (100)*
J70	−	Bacilli	+	−	+	+	+	+	−	+	+	−	+	+	*K. oxytoca (100)*
S20	−	Bacilli	+	−	+	+	+	+	+	+	−	+	+	+	*Citrobacter freundii (88)*
S70	−	Coccobacilli	+	−	+	+	+/−	+	−	+	+	−	+	+	*Acinetobacter spp. (100)*
S30	−	Bacilli	+	−	+	−	+	+	−	+	+	−	+	+	*Providencia alcalifaciens(80)*
OM1		Bacilli	+	−	+	+	−	+	−	−	+	−	+	−	*Serratia odorifera* (90)
OM2	−	Bacilli	+	−	+	+	−	+	−	+	+	−	+	−	*Enterobacter intermedius* (42)

+/−: uncertain test result, MR: methyl red test, VP: Voges-Proskauer test, ID: identification (%): percentage of certainty of test results

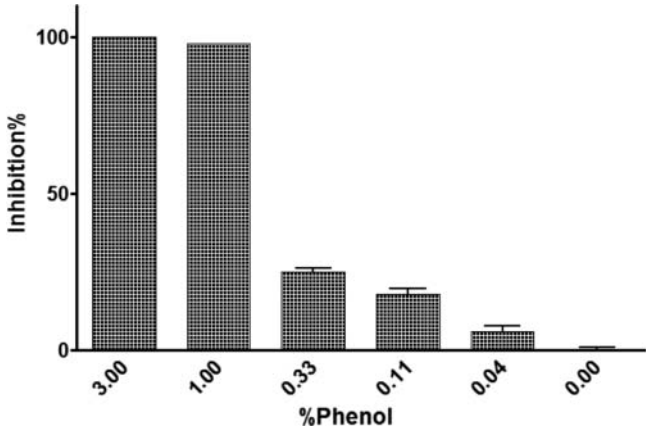

Figure 1. Phenol tolerance of the isolated bacteria J20 cultured in different concentrations of phenol. The percent inhibition of Alamar Blue reduction is plotted against phenol concentration.

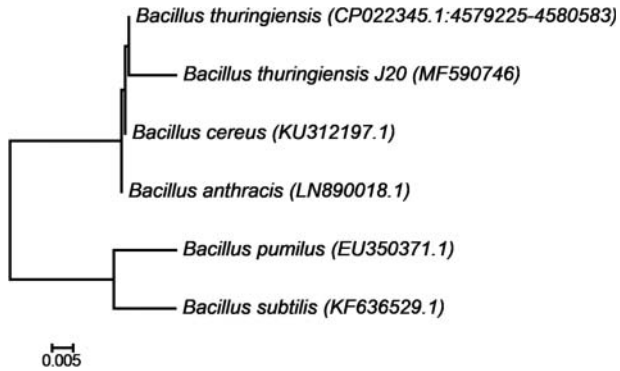

Figure 2. Phylogenetic analysis based on the 16SrRNA gene sequences of strain J20 and related bacterial strains imported from the GenBank database. Phylogenetic tree was constructed by the neighbor joining method with bootstrap of 1,000 replications using Mega 7 program.

It is reported that phenol degradation is affected by environment factors such as temperature and pH.[31] Therefore, the effect of pH and temperature on bacterial growth and phenol elimination was investigated after 96 h, keeping initial

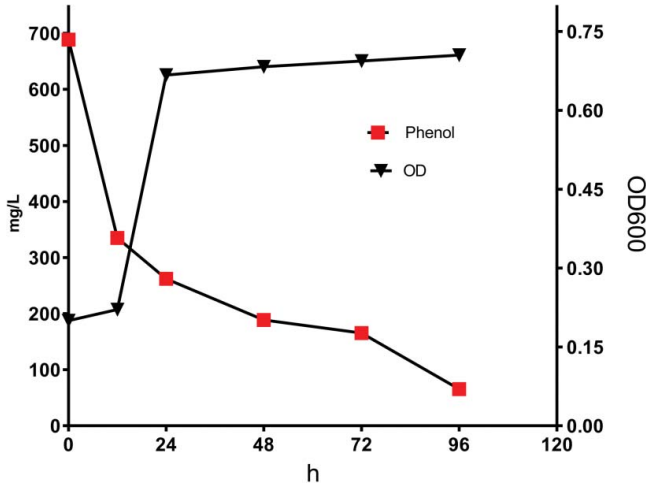

Figure 3. Growth curve of *Bacillus thuringiensis*-J20 and phenol degradation at 700 mg/L in MSM medium as function of time. OD_{600}; optical density measured at a wavelength of 600 nm.

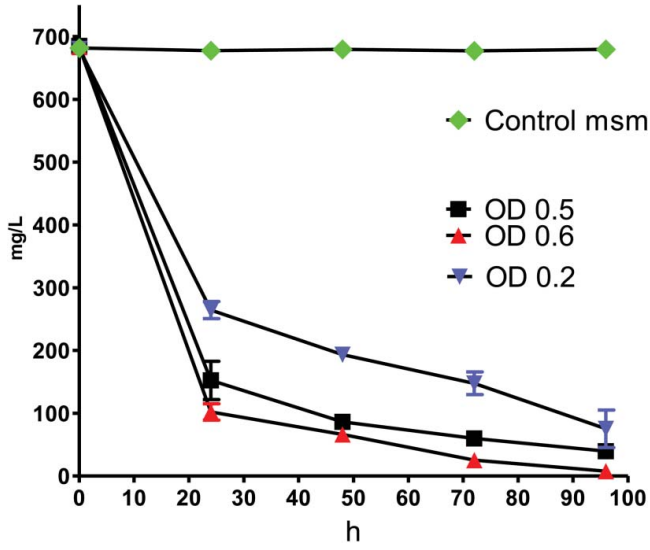

Figure 4. Effect of three inoculation sizes (0.2, 0.5 and 0.6) on the biodegradation of 700 mg/L phenol by *Bacillus thuringiensis*-J20.

concentration of phenol at 700 mg/L and starting inocula with 0.2 OD_{600}. The bacterial strain could grow within a range of pH 5.5–9.5 and temperature of 25–42 °C. The optimal pH and temperature for the highest phenol elimination was 6.57 at 30 °C. Increasing the incubation temperature from 25 °C to 30 °C resulted in an increase in the percentage of phenol degradation from 74.5% to 88.6%, but above 30 °C, there was a decrease from 88.6% to 62.1% (Fig. 5A). These results indicate that the phenol degradation is temperature-dependent in agreement with previous studies, which revealed that most favorable temperature range for the phenol-degrading bacteria was 25 °C to 30 °C.[16,32] Furthermore, the concentration of phenol decreased more rapidly at pH 6.57 as compared to higher and lower pH values (Fig. 5B).

Phenol degradation by immobilized cells in alginate

Biodegradation of phenol at initial concentration 700 mg/L was carried out by alginate-immobilized cells of J20 and compared to free cells. The phenol-degradation efficiency by free cells was less compared to immobilized cells, 88.6% of phenol was eliminated within 96 h by free cells, whereas the immobilized cells were able to utilize 97.2% of the same amount of phenol within 96 h. A biodegradation of 100% was obtained at 120 h by immobilized cells compared to 88.6% by free cells (Fig. 6A). This was probably because the immobilized cells were protected by the alginate beads in line with other studies that demonstrated that immobilized cells of *Acenitobacter, Aeromonas* and *Pseudomonas spp.* were better at phenol degradation than those of free cells.[21,33,34] Alginate beads without bacterial cells (negative control) showed no detectable loss in phenol concentration. Furthermore, when a higher concentration of phenol was used (1,400 mg/L), 74% of phenol was removed in the first 48 h and then very slow degradation was monitored followed by plateau phase (Fig. 6B). These findings may be due to rupture of gel beads that led to a substantial loss of the protection against phenol and thus loss of the cell viability at these concentrations. The effect of storage time on phenol degradation by alginate-

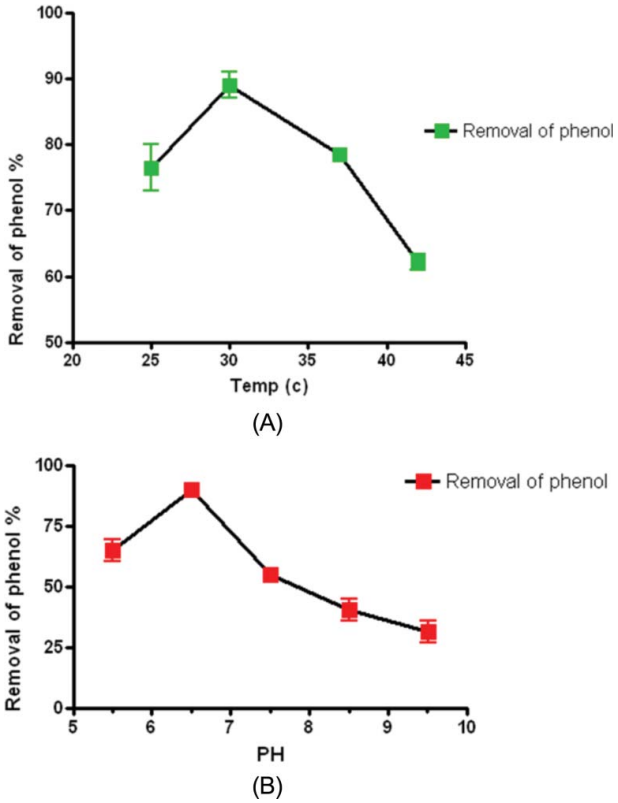

Figure 5. Effect of pH and temperature on phenol biodegradation at initial phenol concentration of 700 mg/L. (A) Effect of temperature on the removal of phenol; (B) Effect of pH on the removal of phenol.

immobilized cells was also investigated. Storage of immobilized cells significantly reduced its capability to degrade phenol at 700 mg/L compared to control group ($P = 0.0005$, $r = 0.8$). Phenol degradation efficiency was reduced to 72% when the immobilized beads were stored for 35 days at 4 °C (Fig. 6C). Thus, *B. thuringiensis*-J20 performed less well than *B. cereus* cells that can be stored for one month without compromising their phenol-degrading capacity.[24] However, more investigation is needed to examine the reusability of J20 entrapped cells over time and the effect of immobilization parameters like alginate concentration and inoculum size.

Detoxification test

The evaluation of a detoxification efficiency of wastes should not rely only on observation of phenol elimination, but also monitoring how the microbial community is affected by the detoxified wastes. Several studies have already been conducted to evaluate OMWW toxicity on microorganisms; most of bioremediation techniques used diluted OMWW.[35,36] In the current study, the J20 isolate was used to treat crude OMWW and this treatment was assessed on the growth of *E. coli* as compared to untreated OMWW. Figure 7 showed that increasing the percentages of untreated OMWW from 20% to 40% (V/V) resulted in sharp decrease in *E. coli* cell density. Moreover, no cells were able to grow in 60% or more of untreated OMWW sample, while 150 colonies grew in 100% J20-treated OMWW sample. Interestingly, a decrease of phenol concentration from

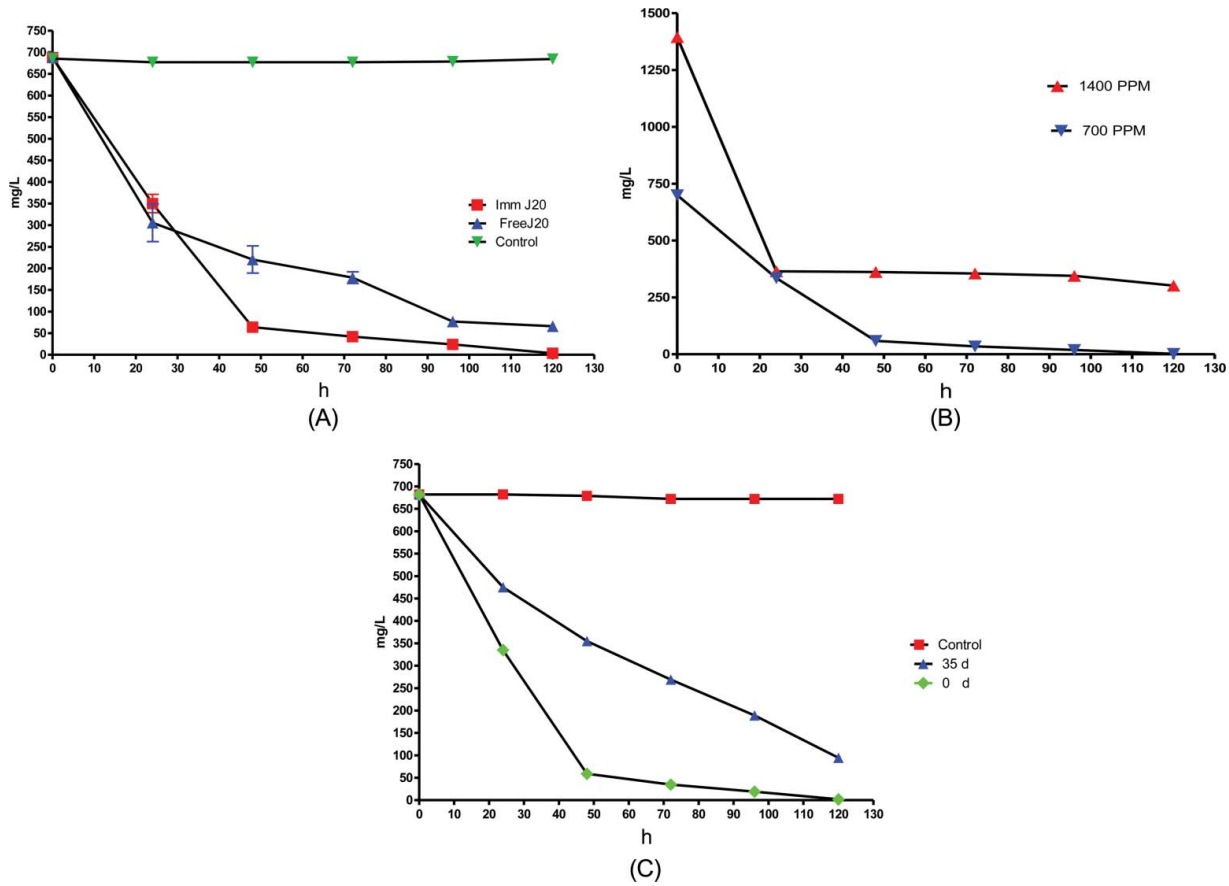

Figure 6. (A) Phenol degradation profiles of free and alginate-immobilized J20 at phenol concentration of 700 mg/L. (B) Phenol degradation profiles of alginate-immobilized J20 at two different initial phenol concentrations. (C) Phenol degradation by alginate-immobilized J20 at 700 mg/L, pre-storage (day 0) and after 35 days of storage at 4 °C. The control represents alginate beads without bacterial cells.

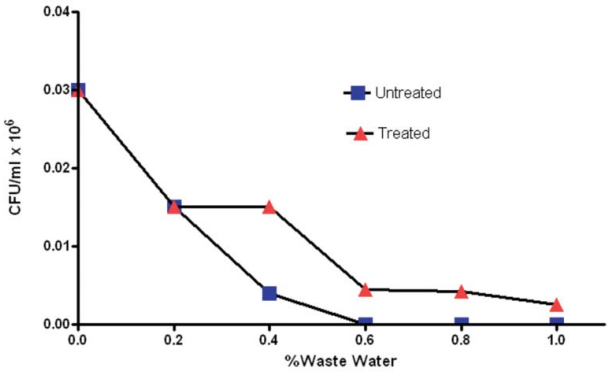

Figure 7. Growth curves of *E. Coli* (ATCC 25922) in the presence of different percentages of J20-treated and untreated olive mill waste water (OMWW) serially-diluted in LB medium.

109 mg/L (before treatment) to 42 mg/L (after treatment with J20) was observed within 5 days indicating that the phenol was partially consumed (61%) by J20 and thus reduce the polluting charge of crude none diluted OMWW. We believe that the bacterial viability and biodegradation efficiency were affected by the pH of OMWW sample (4.47) as it was not adjusted prior to biological treatment. Furthermore, several phenolic compounds with variable concentrations and other toxic inhibiting compounds have been detected in OMWW samples,[37,38] which may have different degradation patterns and hence affect the degradation efficiency of J20.

Conclusion

We report here on the characterization of phenol-degrading bacteria, isolated from an olive mill in Palestine and identified as *B. thuringiensis*. Our study provided useful guidelines in evaluating potential phenol biodegraders isolated from environment. The phenol-degradation efficiency is affected by the condition of the medium, such as pH and temperature. The *B. thuringiensis*, J20 isolate, can be used for the bioremediation, which may be a cheap and efficient method to eliminate phenol-contaminants. The efficiency of phenol degradation was better with alginate-immobilized than free cells at 30 °C and pH 6.57.

Effective biotreatment of OMWW is needed to reduce phenolic compounds allowing for safe disposal of OMWW into soil and surface waters. However, further experiments are needed to study the effective application of immobilization techniques on phenol degradation efficiency and to assess the reuse of these immobilized cells for repeated batch degradation of phenol.

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