**Deanship of Graduate Studies**
**Al-Quds University**

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

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


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## Thesis Approval

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Jerusalem-Palestine
1438/2018

## Dedication

I dedicate my work to those dearest to me, my family especially my father, my mother, sisters and brothers for their support and advice.

Thank you all.

**Declaration**

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or Institution.

Signed: Ahmad Abdelkader

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Date:10-1-2018

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## Abstract:

This study aimed to isolate 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 $C^{o}$.

Under the optimized conditions, this strain could degrade 88.6% of phenol (700 mg/L) within 96 h when the initial cell density was OD600 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, phenol 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.

# Table of Contents

## Contents

## List of Tables

## List of Figures

## List of Abbreviations

| Abbreviation | Full Word |
|---|---|
| **OMWW** | Olive mill wastewater |
| **OMW** | Olive mill waste |
| **SOMWW** | Solid olive mill waste water |
| **WHO** | World Health Organization |
| **EPA** | Environmental Protection Agency |
| **PAHs** | Polycyclic aromatic hydrocarbons |
| **LB** | Luria-Bertani Broth |
| **PCR** | Polymerase chain reaction |
| **DNA** | Deoxyribonucleic acid |
| **rRNA** | Ribosomal Ribonucleic acid |
| **µL** | Microliter |
| **M** | Molar |
| **PBS** | Phosphate buffer saline |
| **EDTA** | Ethylenediamintetraacetic acid |
| **TAE** | Tris acetate EDTA |
| **V** | Volt |
| **Bp** | Base pair |
| **°C** | Celsius or centigrade degree |
| **BLAST** | Basic local alignment search tool |
| **MW** | Molecular weight |
| **MSM** | Minimal salt medium |
| **Na2HPO4** | Sodium phosphate |
| **KH2PO4** | Potassium dihydrogen phosphate |
| **NH4Cl** | Ammonium chloride |
| **NaCl** | Sodium Chloride |
| **MgSO47.H2O** | Magnesium Sulfate Heptahydrate |
| **O.D** | Optical Density |
| **PPM** | Parts Per Million |

# Chapter One: Introduction and Literature Review

## 1.1 Olive Oil Industry

Olive oil extraction industries are mainly located in Mediterranean countries and seasonally accompanied by certain amounts of wastes( by-products). This includes olive mill wastewater (OMWW) and solid olive husk (OH) (Rouvalis and Iliopoulou–Georgudaki 2010, Asfi, Ouzounidou et al. 2012). Production of olive oil in the world is about 3 million metric tons per year. The biggest global producers of olive oil are Spain, Italy, and Greece, with an annual production of 1150, 560, and 370 thousand metric tons, respectively, followed by Tunisia and Turkey, with an annual production of 160 thousand metric tons each. Syria has been able to produce 150 thousand metric tons while Jordan produces about 26 thousand metric tons as show in figure 1.(Ntougias, Bourtzis et al. 2013) .

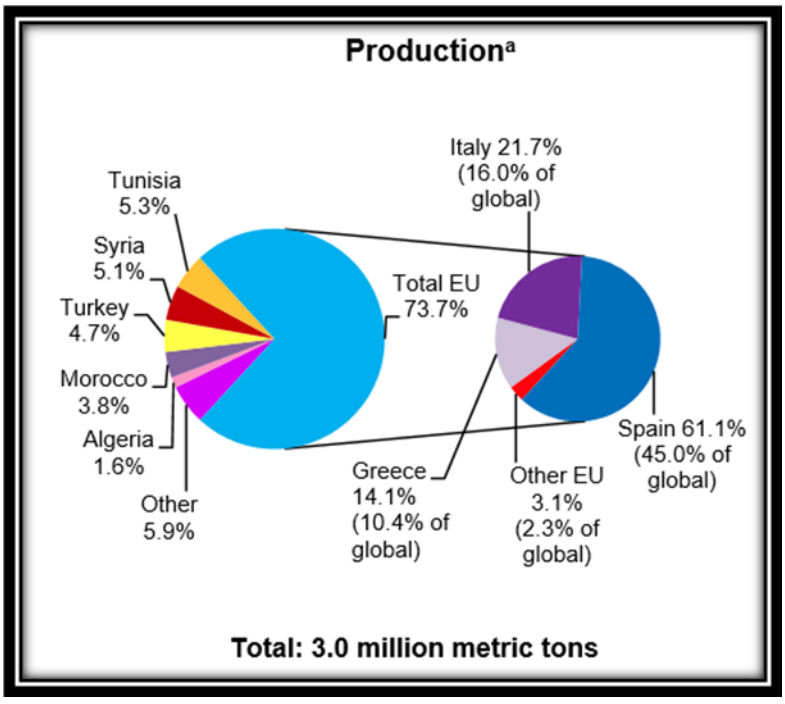


**Figure 1** Olive oil production by country.
(Source: International Olive Council (November 2012), accessed February 8, 2013.)

## 1.2 Olive Oil Industry in Palestine

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(UN 2008). Approximately 93% of the harvested olive is used for olive oil. The potential production of olive oil between 32,000 – 35,000 metric tons (UN 2008; Qutub et al., 2010). Olive oil production in Palestine is concentrated in the north and northwest regions. A total of 200 trees per hectare are planted in the northern half of Ramallah governorate and nearly in all of Jenin, Nablus, Tulkarem, Qalqilyia, and Salfit governorates (Figure 2).


**Figure 2** Most productive regions of the West Bank for olive cultivation

(Source: Qutub, et al. ( December 2010 ). Characterisation of the Main Palestinian Olive Cultivars and Olive Oil. EU / AFD)

## 1.3 Olive Mill Wastes

Three products are usually produced by traditional olive press extraction: the olive oil (20%) ,the olive husk (30%) which is wet solid waste and an aqueous waste called olive mill wastewater (OMWW) (50%) (Tsagaraki et al., 2007). The olive husk ( solid waste) remains after the first pressing of olives consist of olive pulps and olive stones. This waste is sometimes processed in seed oil factories, in order to extract any remnant oil. The exhausted olive husk can be used as solid fuels for heating and cooking purposes (Tsagaraki et al., 2007).

### 1.3.1 Olive Mill Wastewater (OMWW)

Annually, 7 to 30 million $m^3$ of OMWW is produced worldwide. In Palestine, more than 200 olive-mills are functioning by which 200 thousand $m^3$ of OMWW are generated per year (Shaheen et al., 2007). In fact, during the olive season, early October to late December, the waste water that produced by different olive-mills is disposed directly into the wadies such as, Wadi Zeimar in Nablus-Tulkarem region. This waste water can be mixed with the untreated flowing municipal wastewater or with rainwater(Shaheen et al., 2007).

OMWW has a considerable amount of reduced sugars, high phosphorus, organic and phenolic compounds that have a toxic action to some organisms which resist biological degradation (Aviani et al., 2009). Therefore, the disposal of the untreated OMWW into the open wadies and the water receiving bodies is considered as an urgent ecological problem that deteriorates the environment in Palestine. Hence, destroys the aquatic life and prevents its further development (Shaheen et al., 2007).

### 1.3.2 Toxicity of OMWW

The Major characteristics of OMWW are its undesirable color and odor, acidic pH (4.7), high salt concentration, total polyphenolics content, high chemical oxygen demand (COD) values, high organic matter and low water activity ($aw$). Several studies identified the major physiochemical characteristics of OMWW as shown Table (1) (Ntougias et al., 2013). The

The OMWW affects the microbial diversity in soil, inhibit seed germination and plant growth and change soil characteristics (Gad et al., 2008). Moreover, OMWW leads to severe problems for the whole ecosystem such as natural water bodies (ground water, surface aquatic reservoirs). Uncontrolled disposal methods of OMWW directly into sewer systems, valleys, rivers and lakes result in contamination of ground water and possess an environmental risk factor to aquatic organisms, including microorganisms, plants and fish. Thus, due to the toxicity and persistence of OMWW in the environment, it's 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 (Lakshmi et al., 2009; Namane et al., 2013). Bioremediation method was chosen because it is one of the alternative ways to deal with pollution, compared to other processes due to the potential of forming harmful by-product and costly such as activated carbon adsorption and ion exchange (Basha et al., 2010; Park et al., 2013).

**Table 1** Characteristics of olive mill wastewater OMWW. (Source: Morillo et al., 2009; Ntougia et al., 2013; Tziotzios et al., 2007)

| Characteristics | Range |
|---|---|
| **Solids (%)** | 6.33–7.19 |
| **Organic matter (g/L)** | 46.5–62. |
| **Lipids (g/L)** | 1.64–12.2 |
| **Phenols (g/L)** | 0.98–10. |
| **Carbohydrates (g/L)** | 1.4–16.1 |
| **COD (g/L)** | 67–178 |
| **BOD5 (g/L)** | 46–94 |
| **NaCl (g/L)** | 0.11–0.42 |
| **pH** | 4.2–5.17 |
| **EC (dS/m)** | 5.5–12 |
| **Mineral salts (%)** | 0.5 - 2% |

### 1.3.3 Phenolic Compounds

In Palestine, phenolic compounds contamination due to olive mill wastes which contaminate the soil in agricultural fields, surface and ground water is becoming a major contributor of pollution. Phenolic compounds can form hydrogen bonding so simplest phenol are liquid and soluble in water. Unless some group capable of producing color, phenols themselves are colorless (Basalat et al., 2012). Presence of phenolic compounds even at low concentration in the industrial waste water for examples olive mill waste water, adversely affects aquatic as well as human life directly or indirectly when disposed to public sewage, river or surface water. Sometimes these form complex compounds with metal ions, discharged from other industries, which are more carcinogenic in nature than the phenolic compounds. (Meikap et al., 1997).

Phenolic compounds founded naturally especially in plant kingdom. Plants oils contain some phenolic compounds and we can isolated it from these plants as oil of cloves, oil of nutmeg, oil of vanilla bean, oil of olives,….etc. Waste water of agro industrial activities contains phenolic compounds that are capable to reach to the resources of water. This 6 industry related to the manufacturing win, spirits, and olives oil that's very common in Mediterranean region, There are many Industrial resources of phenolic compounds. Nearly all phenol is made today, however, by process that start with cumene, isopropylbenzene. Cumene is converted by air oxidation into cumene hydroperoxide, which is converting by aqueous acid into phenol and acetone. Certain amount of phenol; as well as the cresols, is obtained from volatile and aqueous by-product material produce when convert coal into coke in the absence of air which called coal tar. (Basalat et al., 2012).

Olive mill wastes are increasing especially in the Mediterranean region due to increase olive trees and demand of olives oil worldwide. Processing of olive oil in the Mediterranean region are major contributors of pollution in that area (Basalat et al., 2012). Production of olive oil consider as a polluting industry generate two types of waste: first; a semi-solid effluent (Alzibar), second; solid waste (Gift). The improper disposal of these wastes cause negative impact to air, water and soil.

More than 30 phenolic compounds have been found in a semi-solid effluent (Alzibar) Tyrosol and hydroxytyrosol are the most abundant polyphenols present in a semi-solid effluent (Alzibar). Other groups of polyphenols are also found in a semi-solid effluent (Alzibar), like cinnamic, caffeic, p-coumaric, vanillic and ferulic acids. These compounds are contributing to the toxicity and antibacterial activity of a semi-solid effluent (Alzibar) which limit its microbial biodegradability (Basalat et al., 2012). The structures of the most abundant polyphenols in a semi-solid effluent (Alzibar) .

**Table 2** The main phenolic compounds in Olive mill wastewater. (Source: Kallel, Belaid et al. 2009)

| # | phenolic compounds | Percentages% |
|---|---|---|
| **1** | **4-Hydroxyphenylethanol (Tyrosol)** | **16.55** |
| **2** | **4-Hydroxybenzoic acid** | **1.35** |
| **3** | **Hydroxy-3-methoxyphenethylacohol (Homovanillyl alcohol)** | **4.25** |
| **4** | **4-hydroxy-3-methoxybenzoic acid (Vanillic acid)** | **1.02** |
| **5** | **3,4-Dihydroxyphenyl alcohol (Hydroxytyrosol)** | **70.93** |
| **6** | **3,4-Dihydroxybenzoic acid (Protocatechuic acid)** | **3.38** |
| **7** | **3,4-Dihydroxyphenylglycol** | **0.55** |
| **8** | **3,4-Dihydroxycinnamic acid (Caffeic acid)** | **1.93** |

## 1.4 Phenol

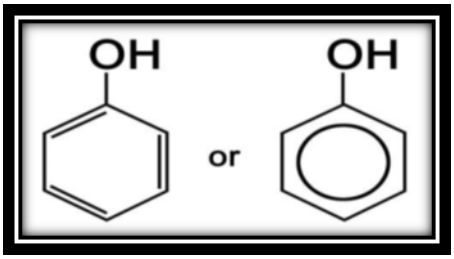


**Figure 3** Structure of Phenol (Source: Saluja et al.,2015)

Phenol is aromatic compound have one hydroxyl group attached on benzene ring, hygroscopic, crystalline solid with distinctive odor and acidic. Phenol very soluble in water, organic solvents and quite flammable. The specific terminology of phenol is its simplest member, monohydroxybenzene (C6H6OH), also benzenol and carbolic acids. The chemical formula for phenol, molecular weight and other physical properties are described in Table3.

**Table 3** Chemical and physical properties of phenol (Source: Saluja et al., 2015)

| **PROPERTY** | **PHENOL** |
|---|---|
| **Formula** | C6H5OH |
| **Molecular Weight(g/mole)** | 94.11 |
| **Water solubility(g/L at 25 °C)** | 87 |
| **Melting Point (°C)** | 43 |
| **Boiling Point (°C)** | 181.8 |

### 1.4.1 Toxicity of Phenol

Phenol is a major pollutant included in the list of Environmental Protection Agency EPA (1979) as reported by (Hooived et al., 1998). Phenol is toxic even at low concentration of about (0.1 mg/L), and the toxicity of phenols for microbial cells has been investigated (Rouvalis et al., 2010). Also, Phenol has serious health effects that can be both acute and chronic. Human Acute exposure to phenol causes damage and disorder of central nervous system(Saluja et al., 2015). It leads to collapse and progressively coma. A drop in body temperature occurs, which is known as hypothermia (Sundar et al., 2012). Mucus membrane also shows very sensitive action to phenol. It is followed by muscle weakness, tremors and noted by irritation of the skin and eyes(Saluja et al., 2015). Long-term exposure can lead to irregular breathing and respiratory arrest. At high doses conjunctival swelling, corneal whitening and finally blindness may occurs (Autrup, Calow et al. 2005) . Extreme contact with phenol can cause myocardial depression (Sundar et al., 2012).

The increased trend in environmental pollution may occur due to the population growth, urbanisation, the development of industrial sectors, the increasing of solid and hazardous waste discharged, and the effluent from wastewater treatment plant which leads to discharging of higher xenobiotic levels especially phenol and phenolic compounds (Tabrez et al.,2011; Krastanov et al., 2013). Based on the several studies , the major pollutants that have been identified in water sources are phenolic compounds, heavy metals, and pesticides(Akan et al., 2010; Wasi, et al., 2013).

It was estimated, on 2025, that two thirds of world's population will live in countries with moderate or severe water shortages (Azizullah et al., 2011). High levels of phenol in the environment can disrupt biological ecosystem and nutrient cycling (Gami et al., 2014; Cordova et al., 2009) because it was considered as a top list on the most hazardous chemical that is difficult to be removed (Al-Khalid et al., 2012) by human and other organism that makes them unable to tolerate it.

### 1.4.2 Chemical and biological methods for the removal of phenols

Phenolic wastes are treated by physicochemical methods such as, adsorption, ion exchange, evaporation, reverse osmosis, electrolytic oxidation, etc (Sundar et al., 2012). These methods are usually complex, expensive and produce hazardous end-products (Marrot et al., 2006). One of the known methods is Chemical oxidation which requires a reactor that operates at high temperature and high pressure. (Behera et al., 2009). A second method, the ion exchange adsorption method that commonly activated carbon use which is disposed by incineration. The process of incineration generates many new compounds such as dioxins and furans which have severe consequences on human health (Behera et al., 2009). Furthermore, solvent extraction that have been used for phenols recovery (e.g benzene, isopropyl ethyl and butyl acetate) may contaminate treated water (Saluja et al., 2015).. In addition to the presence of solvent in treated water, the high cost of solvent is another disadvantage for this method (Behera et al., 2009). Lastly, Membrane Processes are effective and economically appropriate to treat phenols (Villegas et al., 2016).

## 1.5 Phenol degradation by microorganisms

Biodegradation is a process by which microorganisms breakdown, metabolize or mineralize organic contaminants into less harmful non-hazardous substances. It has been universally accepted as an effective (with low cost) method that prevents environmental pollution. Bioremediation treatments involved microorganisms, as a single strain or co-cultures of two or more organisms, to remediate a waste product (Reshma et al., 2014). This process is used for removal and degradation of many environmental pollutants including the products of petroleum industry, hydrocarbon pollutants and olive mill wastes. Also, biodegradation by natural populations of microorganisms represents one of the major mechanisms by which petroleum can be removed from the environment (Das et al., 2010).

The focus on the microbial degradation of phenols in recent years has resulted in the isolation, culture, adaptation and enrichment of a number of microorganisms that can grow on the compound as a sole carbon and energy source. However, the process might not be fast

enough to prevent the ecological damage (Evans et al., 1947). Micro-organisms that degrade phenol were firstly isolated early in 1908(Evans et al., 1947). Recently, several bacterial species utilize phenol and other aromatic compounds as the sole carbon and energy source have been used in phenol biodegradation studies, including *Bacillus sp, Pseudomonas sp, Acinetobacter sp, Achromobacter sp* etc. *Fusarium sp, Phanerocheate chrysosporium, Corious versicolor, Ralstonia sp, Streptomyces sp* (Luckarift et al., 2011; Zhang et al., 2013; Karimi et al., 2016).. It is reported that phenol biodegradation efficiency could be further enhanced by the process of cell immobilization (Annadurai et al., 2000) by which immobilized cells could tolerate more phenol concentration, protected the bacteria against changes in temperature and pH and thus revealed better performance than free suspended cells (Anselmo, et al., 1985; Chitra et al., 1995). For examples immobilized *B.cereus* cells, for phenol degradation efficiency was higher degradation than that of the free cells at high phenol concentrations. (Ehrhardt et al., 1985;Banerjee et al., 2011) .

### 1.5.1 Physiological parameters affecting the degradation potential

The physiological parameters play a vital role in the growth and biodegradation behavior of any microorganism, but maximum growth is achieved only at the optimum conditions of these physiological parameters. Thus different physiological parameters that usually interfere in the biodegradation activity of a microbe are mainly: the incubation temperature pH and nutrient availability (Rajani et al., 2016)

### 1.5. 1.1 Effect of pH and temperature on phenol degradation potential

The internal environment of all living cell is believed to be approximately neutral. Almost all the living thing from microorganism to huge giants requires approximate neutral pH of 7 for their growth. Most organisms cannot tolerate pH values below 4.0 or above 9.0. No microorganism can survive as the acid and bases penetrates through the walls of cells interfering and disturbing the cell metabolism(Namane et al., 2013; Rajani et al., 2016).

On the other hand, temperature plays important role than nutrient availability in the degradation of organic pollutants. Most laboratory studies on phenol degradation have been carried out at an optimum temperature of 30°C.(Saluja et al., 2015).

## 1.6 Immobilization of bacterial cells

Biodegradation of phenol has been studied in detail using both pure and mixed cultures of suspended and immobilized bacteria (Ehrhardt et al., 1985). Immobilized cell technology has been widely applied in a variety of research and industrial applications. Immobilized cells offer the possibility of degrading higher concentrations of toxic pollutants than can be achieved with free cells. It is shown by several researches that immobilized microorganisms are better protected against phenolic compounds than free cells (Annadurai et al., 2000). The present bioremediation technology using different strains of bacteria under controlled environmental conditions has been proven to be useful to treat the hazardous waste containing phenol.

Furthermore, cell immobilization maintains continuous substrate degradation with concomitant cell growth and have been used for biodegradation of xenobiotic substances which is the current area of interest in the field of environmental studies (Sundar et al., 2012). Enhanced stability of the degradation potential, continuous operation and reuse of cells are some of the important advantages of the use of immobilization technique in the biodegradation studies.

The encapsulation technique is an attractive approach that have been used to encapsulate and deliver cells or bioactive moleculesand thus provide a protective shell for live cells. This method can be achieved by enveloping the biological components within various forms of spherical semi permeable membranes with a selective controlled permeability. (Jinchen Sun et al.,2013). Alginate beads,which are prepared under aqueous conditions via ionic crosslinking, are suitable for encapsulation of cells, growth factors and bioactive protein (Jinchen Sun et al.,2013).

## Objectives

The main objectives of our research study:

1-To isolate and identify phenol degrading bacteria from olive mill wastes in Bethlehem-southern Palestine.

2-To assess experimental factors affecting capability of bacterial isolates to degrade and utilize phenol as a sole source of carbon and energy (PH, temperature and cell density).

3- To investigate the efficacy of phenol biodegradation using immobilized bacterial cells in alginate beads.

## Significant of the study

Phenols pollutants from industrial wastewater in the environment cause several problems to ground water, soil, living organisms and many other ecological problems. Our study will focus on more cost effective applications of naturally occurring bacterial strains, isolated from olive mill in Palestine, for phenol degradation, where it pose an alarming problem due to its health effects on different organisms and human beings.

# Chapter Two: Materials and Methods

## 2.1 Sample collection

The OMWW, olive press (solid olive mill waste; SOMW) and soil samples were collected in October 2015 from olive mill in Bethlehem-southern Palestine as shown in Figure (4). Five samples (2 OMWW, 2 SOMW, 1 soil) were collected in sterile containers and delivered to the laboratory for enrichment in less than 24 hours shown in Appendix 1.

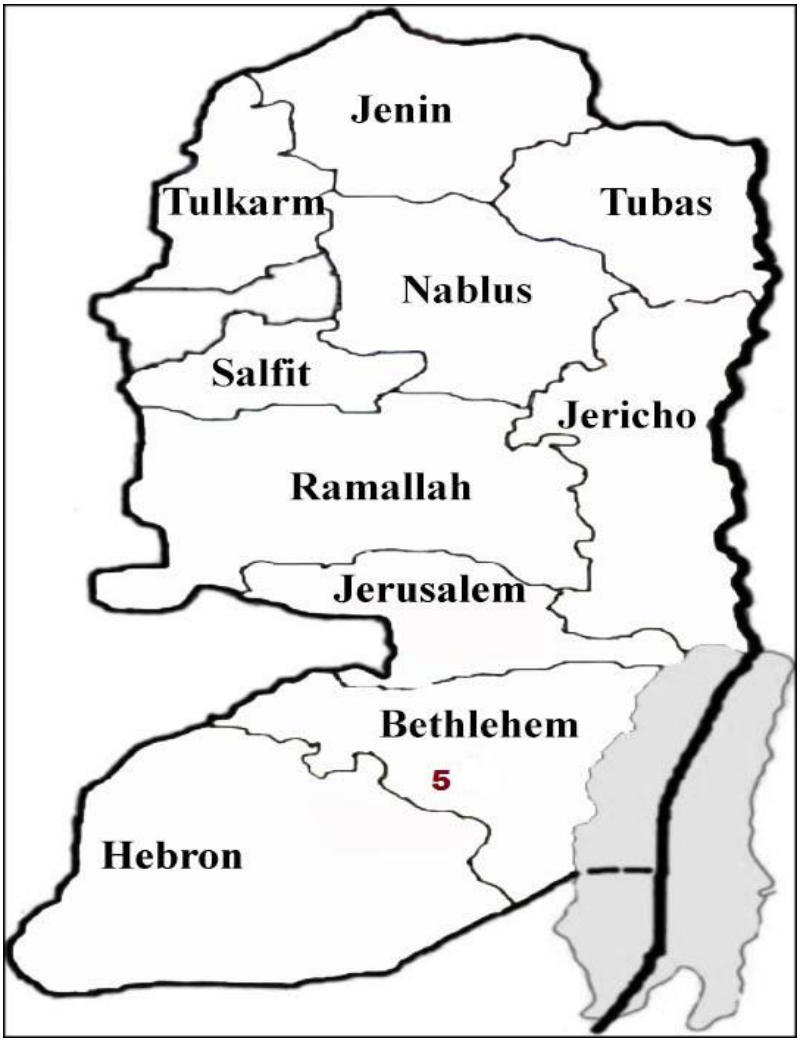


**Figure 4** Map of the West Bank-Palestine showing the site of sampling and number of collected samples.

## 2.2 Growth Media

Two types of growth media were used in the present study:

1- Luria-Bertani Broth (LB), contained 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1000 ml double distilled water.
2- Minimal salt medium ( MSM ) contained Na2HPO4 (6 g), KH2PO4 (3 g), NaCl (0.5 g), NH4Cl (1 g), CaCl2.2H2O (1 M) and MgSO47.H2O (1 M) in 1000 ml double distilled water.

## 2.3 Isolation and Identification of isolated bacteria

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 loop (0.1ml) from each sample observed with highest turbidity, inoculated into LB plates and incubated at 30C for 24 hours. The morphologically different colonies were sub cultured repeatedly in new LB plates to obtain the pure culture as described by (Soudi et al., 2011) (see Appendix 2).

## 2.4 Biochemical Identification

A battery of biochemical tests were used to identify bacterial isolates according to Bergey's manual of systematic bacteriology. Many tests have been known over years for the classification of microorganisms into families, genera, species and even sub specie. Several tests were done including Gram Staining test, Amylase test, Starch hydrolysis test, Urease test, Catalase test and Mannitol test.

### 2.4.1 Gram Staining

This test is done to differentiate between gram positive and gram negative organisms. Gram positive organisms contain a thick layer of protein-sugar complex called peptidoglycans in their cell wall whereas for gram negative, it only comprises of 10-20% of the cell wall.

The procedure is described below

- A drop of saline solution was placed on the sterile slide. a very small sample of a bacterial colony was gently mixed into the drop of saline to create a smear
- The bacterial smear was fixed on slide by heat.
- The slide was flooded with crystal violet stain as primary stain, rinsed with water.

and then the slide was flooded with iodine.

- It was again rinsed with water after 1 minute.
- It was then flooded with acetone alcohol as decolorizer and rinsed with water after 15 seconds.
- It was rinsed with safranin as secondary stain and again rinsed with water after 1 minute.
- Then the slide was blot dry and observed under microscope.
  - Gram positive bacteria will be stained in purple while Gram negative bacteria will be stained in red

### 2.4.2 Amylase Test

This test is done to determine if the organism is capable of breaking down starch into maltose through the activity of the extra-cellular α-amylase enzyme.

The procedure followed was;

- A sterile loop was used to pick up a few colonies of the pure culture plate. then it was streaked across a starch plate in the form of a line across the width of the plate followed by incubation for 48 hours at 37°C.
- Three drops of iodine were added on the colonies, the resultswas recorded after 15 minutes.
  - Clear zones around colonies indicate Amylase positive isolates while Purple/blue around colonies indicate Amylase negative

### 2.4.3 Catalase Test

This is used to test for the presence of the enzyme catalase. This enzyme mediates the breakdown of hydrogen peroxide ($H_2O_2$) into oxygen and water

A loopful of colony of pure culture was picked up and kept on a clean glass slide, 2 drops of 3% $H_2O_2$ was added and waited for 15 seconds.

- Immediate bubbling (O2 formed) indicating catalase positive bacteria while lack of catalase was evident by a lack of bubble production indicating catalase negative bacteria

### 2.4.4 Mannitol Test

This test is used to determine the ability of an organism to grow in 7.5% NaCl and ferment mannitol as a carbon source. If mannitol is fermented an acidic end products can drop the pH of the medium which can be detected by phenol red that turned yellow at pH <6.8 or orange/pink at pH >8.4.

A Mannitol salt agar plate was streaked with a light line of inoculum from the pure culture of the test organism using a sterile loop. It was then incubated at 30°C for at least 48 hours. The change of culture color to yellow indicating a positive test whereas to red /orange indicating a negative test.

### 2.4.5 Urease Test

The urease test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. The surface of a urea agar slant was streaked with a portion of well isolated colony. The cap was left on loosely and incubated the test tube at 35°C for 48 hours.

- Positive: Color changes from light orange to pink. (Organism produces urease)
- Negative: No color change.

## 2.5 DNA Extraction

The bacterial broth was taken and centrifuged at 8000 ×g for 10 min. The obtained pellet was washed with PBS; the DNA of each pellet was extracted using (QIAGEN GmbH, Hilden, Germany). DNA concentration were measured by nanodrop (Thermo Scientific NanoDrop 1000) and kept frozen at -20 until further use.

### 2.5.1 Amplification of DNA

A fragment 16S rRNA gene (1500 bp) was amplified with primer of ribosomal RNA primers set (pA: AGAGTTTGATCCTGGCTCAG and pH': AAGGAGGTGATCCAGCCGCA). as previously described (Hall et al., 1999). PCR reactions were performed in 25-µl PCR ready mix (One TaqR HS Quick -Load, Biolabs, Jerusalem), containing 1.µM of each set of primers and 2 µl of the extracted DNA. Nuclease-free water was used as Negative control in PCR run. The PCR amplification program performed by thermocycler (Professional, Thermocycler gradients, Biometra) included an initial denaturation step of 15 min at 95 ℃, followed by 35 cycles of denaturation at 95 ℃ for 30 s, annealing at 53 ℃ for 45 s, and elongation at 72 ℃ for 45 s and final extension 72 ℃ for 7 m.

### 2.5.2 Gel Electrophoresis

The PCR products were loaded into 2 % agarose gel (Agarose LE, Analytical gradient, Promega, Spain). The gel was prepared by dissolving 2 g of agarose in 100 ml solution of 1X Tris-acetate EDTA buffer (TAE) (40 mM of Tris acetate and 1mM EDTA). The agarose was dissolved in Erlenmeyer flask using microwave for about 1 minute till completely dissolved, and then 3.5µl of 10 mg/ml (0.35µg/ml) of Ethidium Bromide was added for DNA staining. The gel was poured in the gel tray in the casting chamber (Bio-Rad Laboratories Inc., USA). 5µl of PCR products were loaded into the gel. DNA marker ladder of 1000bp (Thermo scientific Lithuania)

was used in each run. The gel was run at 100V for 45 minutes. The gel images were captured using MiniLumi 1.4 gel documentation system from (DNR Bio-Imaging SystemsLtd, Israel).

### 2.5.3 DNA Sequencing

The PCR products were purified and sent for sequencing from both direction at HyLab sequencing service (Rehovot, Israel). The obtained sequence was arranged and aligned using Bioedit sequence alignment editor software. BLAST software (http://blast.ncbi. nlm. nih.gov/Blast.cgi) were used for comparing and the obtained nucleotide sequences with the reference strains in the GenBank. Phylogenetic tree construction was carried out using a MEGA 7 software.

## 2.6 Phenol Tolerance Experiment

To examine the toxicity of phenol on bacterial growth, the cells were grown on LB broth at 30C for 24hrs, cells were treated with different concentrations of Phenol (starting by 3% phenol) and incubated at 30C for 24 hrs. The same bacterial cells cultured in the LB medium with no treatment and broth medium devoid of bacterial inoculums served as positive and negative controls, respectively. 10% of Alamar blue (AB) was directly added to culture medium of treated and control cells. The redox reaction, in which AB is reduced by the cells, was measured by absorbance readings at 540 and 630 nm using flouroskan at various time intervals (3hr and 5 hrs).

## 2.7 Growth assessment and Phenol degradation

The ability of isolated bacteria to grow on phenol -as the sole source of carbon- was studied by culturing in LB to med-log phase (OD600 ≈0.50). Then cells were harvested by centrifugation at 4000 rpm for 15 min, washed and suspended with MSM medium then adjusted to 0.2 OD600. Phenol was used as a sole source of carbon and added directly to MSM at a concentration of 700ppm. The Growth behavior of the isolates was monitored spectro photometrically at 600 nm for 24, 48, 72 and 96 h. Similar procedure was carried out for control solution without having the cells in the solution. 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. The phenol OD was measured at 510 nm based on condensation of phenol with 4-aminoantipyrine followed by oxidation with alkaline potassium ferricyanide giving a red color(Der Yang et al., 1975). The amount of phenol removal by bacteria is obtained in accordance with standard curve of phenol. Different phenol concentrations were prepared to establish phenol standard curve. The curve was used to determine the phenol concentration in ppm of the tested samples using this equation($y = 0.0003x + 0.0084$ $R^2 = 0.9894$) (see Appendix 5).

## 2.8 Evaluation of phenol elimination at different experimental conditions

The effect 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 ppm of phenol.

## 2.9 Microtoxcicity Test

25 ml of waste water were syringe filtered ( 0.2 um Whatman, Life sciences) inoculated by 0.5 ml of phenol degrading bacteria (J20) (OD600 0.2) and incubated at 30C for 5 days. Treated and untreated olive waste water samples were diluted in a series of gradient dilutions at 0, 20, 40, 60, 80, and 100% using sterile LB medium. Diluted samples were inoculated with 200ul of an overnight culture of *E. coli* (ATCC25922; OD600nm=1.2).

All samples were incubated for 24 hours at 37 °C. Standard plate count was conducted by plating a volume of 100µl of each dilution of the treated and untreated olive waste water samples on MacConkey agar, selective for Gram- negative bacilli. MacConkey agar plates, 4 mm thick and 9 mm in diameter, were incubated aerobically for 24 hours at 37 °C. Number of pink colonies indicating *E. coli* was counted on each agar plate. Number of colonies over 300 was considered too numerous to count (TNTC). Numbers of colony-forming units (CFU) per ml were count based on the formula: No. CFU/ml= No. colonies/(dilution x volume of suspension), and plotted as dilution versus CFU/ml x $10^6$ as shown in Appendix 8.

## 2.10 Immobilization of bacterial cells in alginate matrix

The phenol-degrading bacteria were harvested after 24 h of growth in LB culture medium. The cell pellet was obtained by centrifugation at 4000 rpm for 10 min and subsequently re-suspended in 10 ml phosphate buffered saline (PBS). A stock of 4% (w/v) sodium alginate was prepared in the MSM medium. Ten milliliters of bacterial cell suspension (0.2OD600) was added to 50 ml of sterilized alginate solution and mixed by stirring on a magnetic stirrer. This alginate cell mixture was ejected drop by drop into a cold sterile 0.1 M calcium chloride solution (CaCl2) as shown in Appendix 7. The beads were then rinsed with MSM followed by distilled water to remove residual CaCl2. The biodegradation study was done at the same condition described for the free cells as described by (Usha et al., 2010).

## 2.11 Stability study

To examine the stability of the immobilized cell, the beads containing the immobilized 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 in section ( 2.10 ) .

## 2.12 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 $\leq 0.05$.

# Chapter Three: Results

## 3.1 Microorganisms Identification

### 3.1.1 Biochemical and Molecular Identification of the Isolated Bacteria

We successfully isolated eleven strains from an olive mill in south Palestine (Six isolates were obtained from solid olive mill waste, two isolates from OMWW and three isolates soil. All isolates were identified based on its morphological and biochemical characteristics as shown in Table 4. Among them, two strains were Gram positives and nine were Gram negatives

Gram staining of representative samples were shown in Fig (5 and 6). The identity of each isolate was confirmed by amplifying 400bp fragment of 16srRNA followed by sequencing.

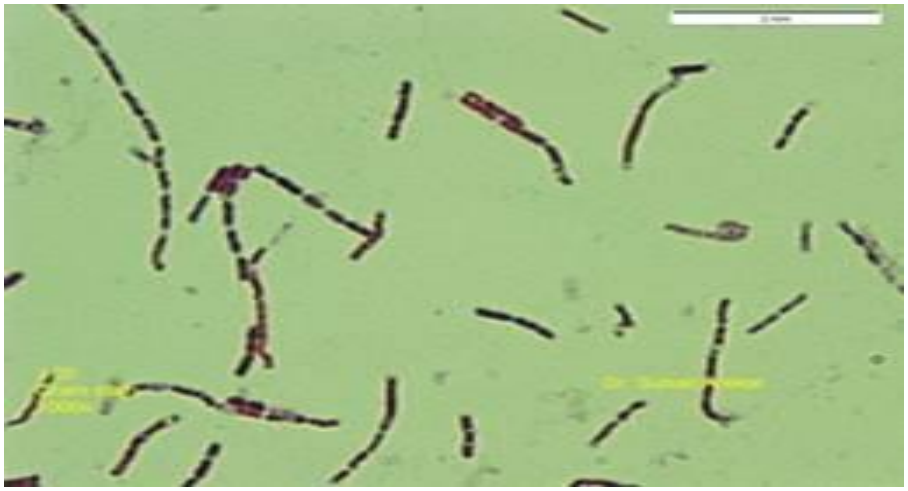

**Figure 5.** Gram stain showing Gram positive bacilli.

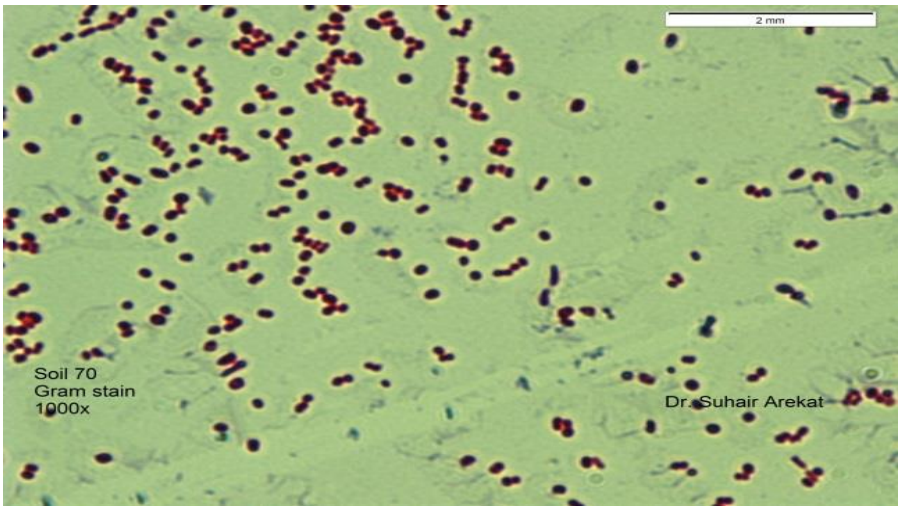


**Figure 6.** Gram stain showing Gram negative cocci.

The identity of the isolated bacteria based on sequencing and biochemical analysis were shown in table 4 and Appendix 3. Three strains (*Enterobacter, K. pneumonia and enterobacteriaceae*) were reported to be pathogenic and excluded from the study while eight strains were environmental bacteria and further investigated.

**Table 4** Morphological and biochemical characteristics of eleven isolates obtained from olive mill/Palestine.

| Bacterial Spp. | Gram stain | Shape | Catalase | Oxidase | Glucose ferm. | Lactose-sucrose ferm. | Indole | Motility | Hydrogen sulfide | Gas | Simmon's Citrate | Urease | MR | VP | ID (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SL2Y | + | V | - | + | + | + | - | - | - | - | - | - | ND | ND | *Exigobacterium* |
| SL3C1 | - | Cocci | + | - | - | - | - | - | - | - | - | - | ND | ND | *Acinetobacter spp/.* |
| J20 | + | Bacilli | + | + | + | - | + | + | - | + | - | - | + | + | *B. thuringiensis.* |
| J70 | - | Bacilli | + | - | + | + | + | + | - | + | + | - | + | + | *K oxytoca (100)/ Klebsiela sp* |
| Soil 20 | - | Bacilli | + | - | + | + | + | + | + | + | - | + | + | + | *Klebsiela oxytoca* |
| Soil 70 | - | Coccobacilli | + | - | + | + | +/- | + | - | + | + | - | + | + | *acinetobacter schindleri* |
| Soil 30 | - | Bacilli | + | - | + | - | + | + | - | + | + | - | + | + | *enterobacteriaceae sp* |
| JO1 | - | Bacilli | + | - | + | - | - | - | - | - | - | - | + | - | *K. pneumonia ssp ozaenae sp.* |
| MO2L | - | Bacilli | + | - | + | + | + | + | - | + | + | + | + | - | *Aeromonas jandaei* |
| OMW1 | | Bacilli | + | - | + | + | - | + | - | - | + | - | + | - | *Enterobacter sp.* |
| OMW2 | - | Bacilli | + | - | + | + | - | + | - | + | + | - | + | - | *Enterobacter sp.* |

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

### 3.2 Phenol tolerance of the isolated bacteria

Eight isolates were investigated to check the effect of phenol toxicity. At high concentration of Phenol, 100% growth inhibition was observed at a concentration of 1% and 3% . At 0.33% phenol concentration; the growth inhibition was only 20% for J20 and 30% for S70. These 2 isolates had a tolerance of phenol concentration as high as 1100 ppm as shown in Figure7. J20 was isolated from olive (SOMW), it is a Gram positive bacillus, motile, non-lactose ferments while S70 was Gram negative Coccobacillus, motile, lactose ferments isolated from soil olive mill . The biochemical characteristics of both strains were shown in Table (3). Both strains showed growth under aerobic conditions with the optimum temperature of 30C.

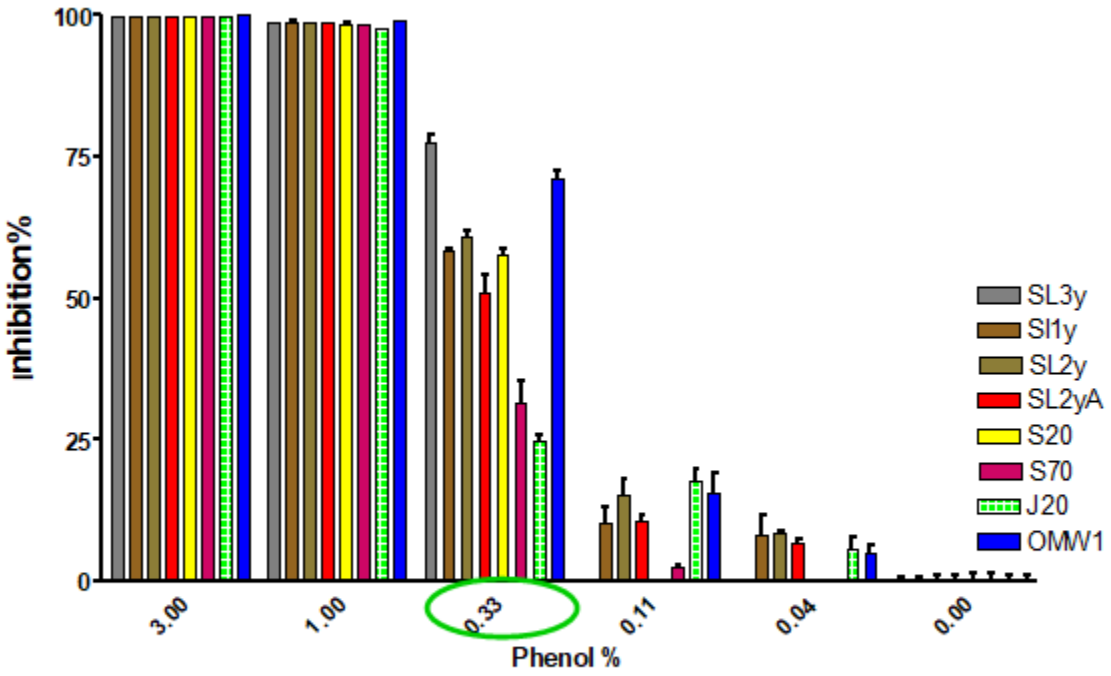


**Figure 7.** Phenol tolerance of the eight isolates bacteria grown under varied concentrations. The X axis represented phenol concentration; Y axis represented growth inhibition**.**

### 3.3 Molecular identification of J 20 isolate

Further identification was done by 16S rRNA PCR targeting a larger fragment of 1500 bp Figure (8). BLAST analysis revealed 99% nucleotide sequence homology with the nucleotide sequence of the reference strain of *Bacillus thuringiensis* (CP022345.1) with 100% coverage of 1393 bp The obtained sequence was deposited in the GeneBank (accession number MF590746), Figure (9). Based on the phylogenetic analysis, the strain was classified in the bacillus genera which belong 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* in Figure (10).

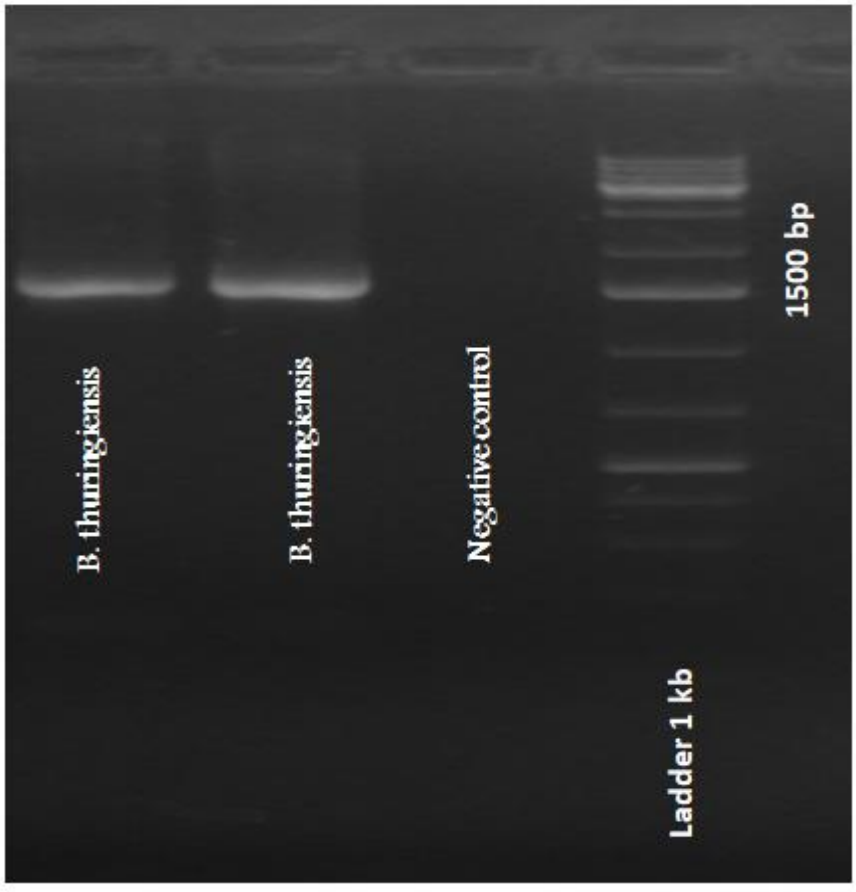


**Figure 8:** Amplification of bacterial DNA (J20) targeting1500 bp of 16S rRNA gene.

AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGAT
TAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACGCTGCCCCTGCGG
CTGGAATACCTCCTTGAAACCGGGGCTAATACCGGATAACATTTTGAACTGCATGGTTCGAAATTGAA
AGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCA
CCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCG
TGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGC
TGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGA
AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAAAGGAAAGTGG
AATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTG
GTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAAATACCCTGGTAGTCCAC
GCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAACTTAACCCATTAAGC
ACTCCCCCTGGGGAGTACGGCCGCAAGGGTGAAACTCAAAGGAATTGAGGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCC
TAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCAC
TCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATG
ACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATC
TCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAA
TCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCT

**Figure 9** 16srRNA sequence (1500bp) of J20 isolate in FASTA format obtained by direct sequencing.

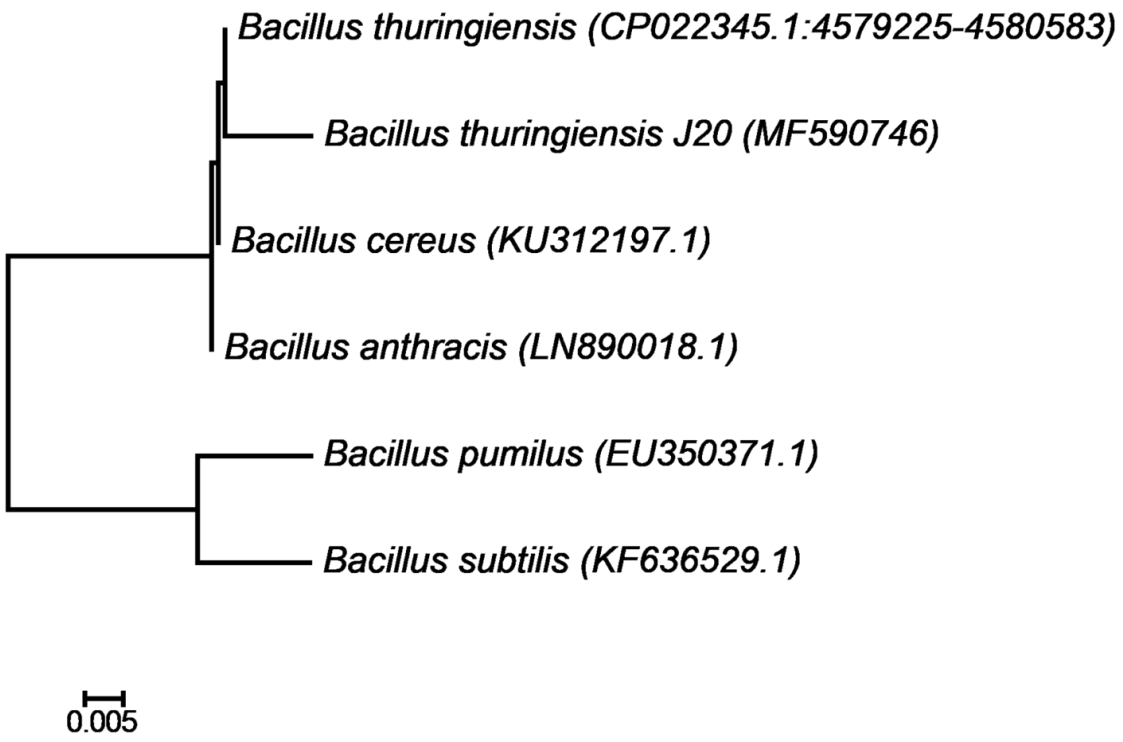


**Figure 10.** Phylogenetic analysis based on the 16S rRNA gene sequences of strain J20 and related bacterial strains imported from the GenBank database

### 3.4 Growth assessment of J20 and S70 using MSM medium

The growth of J20 and S70 was monitored for 96h using MSM media supplemented with 700 ppm phenol and compared to bacterial growth without phenol . Our results showed that the turbidity of the bacterial broth of J20( as measured by OD) was increased in the first 48h compared to those without phenol and then decreased by time (Figure11).The ability of S70 to grow in the presence of phenol was lower than J20 cells as shown in Figure11 and thus excluded from the study. The exact measurement of OD for both strain with and without phenol were shown in table 5.

**Table 5.** Assessment of bacterial growth of J20 and S70 isolates with and without phenol at OD 600 nm

| Time of incubation | Strains | | | |
|---|---|---|---|---|
| **Hours** | **J20P*** | **J20w*** | **S70P** | **S70w** |
| 0 | 0.2 | 0.2 | 0.2 | 0.2 |
| 24 | 0.221 | 0.148 | 0.187 | 0.181 |
| 48 | 0.241 | 0.147 | 0.182 | 0.166 |
| 72 | 0.215 | 0.134 | 0.174 | 0.126 |
| 96 | 0.165 | 0.104 | 0.100 | 0.076 |

P: indicate the presence of phenol in the cultured medium, w: without phenol

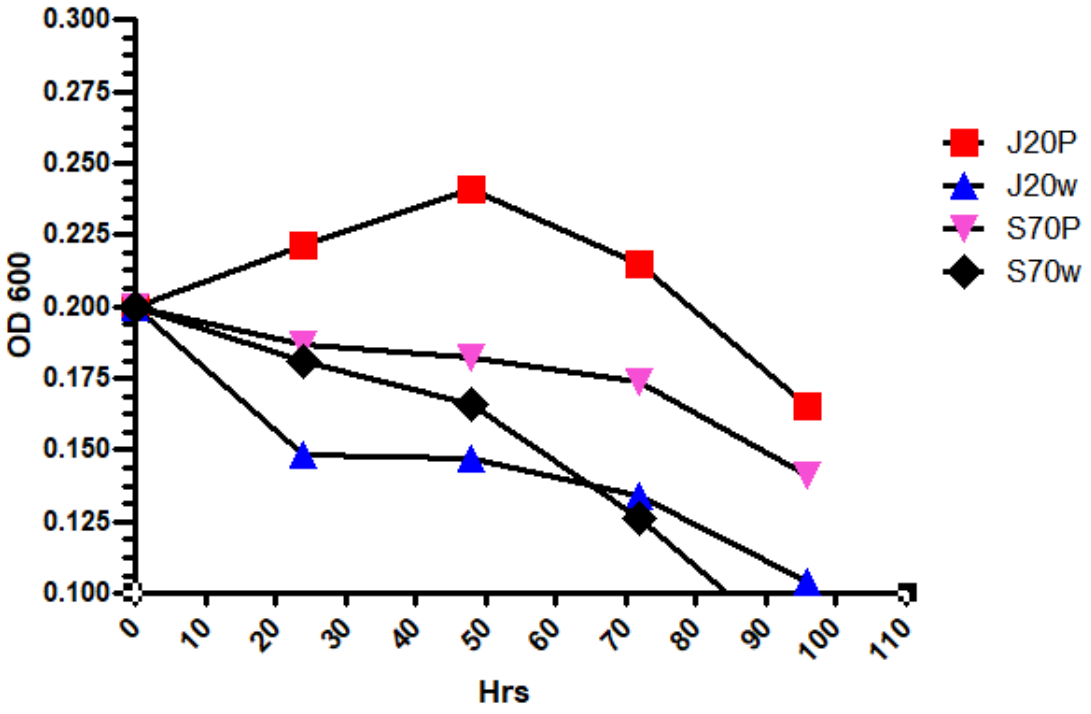


**Figure 11:** Growth assessment of J20 and S70 in MSM medium

### 3.5 Growth assessment using MSM media and Phenol degradation by free cells of J20

Significant negative correlation between phenol concentration and cell numbers was observed (P=0.03, r=-0.8), the decrease of phenol concentration was accompanied by increase in cell growth as noted by increasing broth turbidity and optical density cells of J20 isolate can degrade 88.6% of the phenol within 96 hrs as shown in Figure (12).

To evaluate the accuracy and precision of the method, the samples were analyzed in duplicates with each batch as shown in Table 6

**Table 6** measurements and phenol degradation in ppm of J20 (*Bacillus thuringiensis*) at OD 600nm

| **Incubation time in hours** | **OD 600(1)** | **OD600(2)** | **Mean** | **SD** |
|---|---|---|---|---|
| 0 | 0.200 | 0.200 | 0.200 | 0.000 |
| 12 | 0.221 | 0.285 | 0.253 | 0.045 |
| 24 | 0.667 | 0.556 | 0.6115 | 0.078 |
| 48 | 0.683 | 0.631 | 0.6565 | 0.037 |
| 72 | 0.694 | 0.662 | 0.678 | 0.022 |
| 96 | 0.705 | 0.677 | 0.691 | 0.019 |
| **Phenol concentration in ppm** | | | | |
| **Incubation time in hours** | **J20 (1)** | **J20 (2)** | **Mean** | **SD** |
| 0 | 688.7 | 688 | 688.3 | 0.49 |
| 12 | 335.0 | 355 | 345 | 14.1 |
| 24 | 262.0 | 315 | 288.5 | 37.4 |
| 48 | 188.7 | 269 | 228.8 | 56.7 |
| 72 | 165.3 | 212 | 188.6 | 33 |
| 96 | 65.3 | 89 | 77.1 | 16.7 |

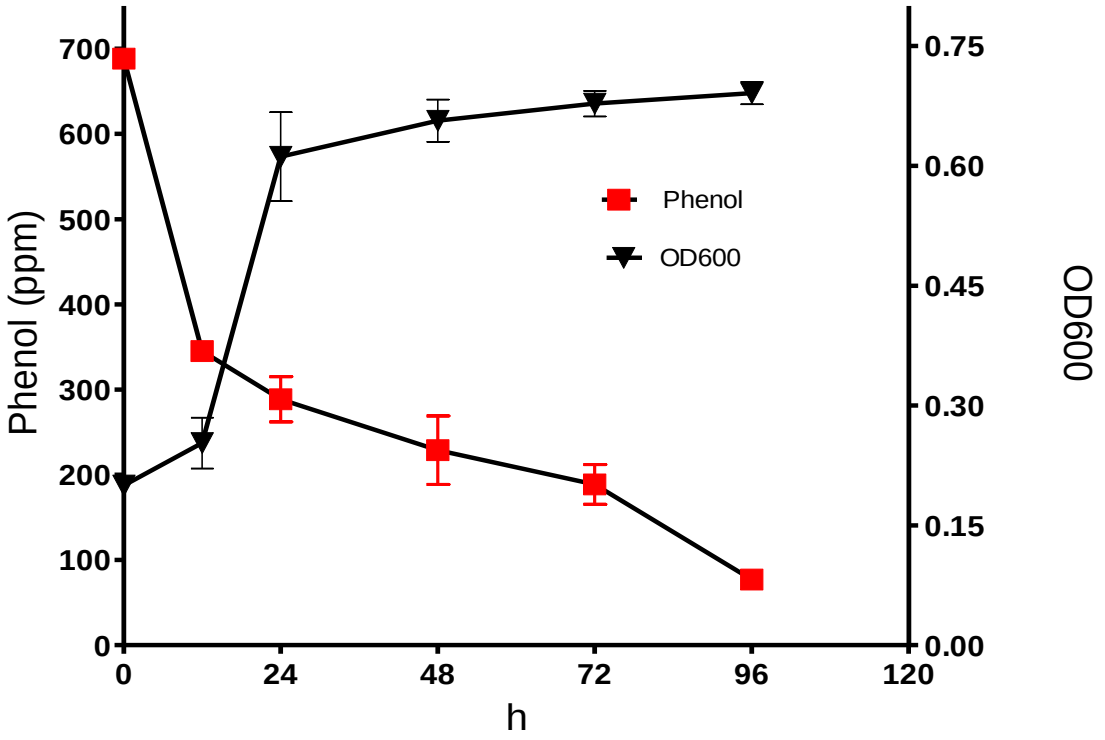


**Figure 12:** Growth curve of *Bacillus thuringiensis*- J20 and phenol degradation at 700ppm in MSM medium as function of time. OD600; opitical density measured at wavelength 600 nm. ppm; part per million. h; hours.

### 3.6 Effect of Cell Density on Phenol Degradation

To study the effect of cell density on phenol degradation; different concentration of J 20 cells of 0.2, 0.5 and 0.6 OD were used. Our results showed a decrease in the absorbance at 510nm by time indicating a decrease in phenol concentration (Figure13). Our results showed a significant variation between initial cell density and biodegradation of 700ppm phenol ($P=0.0003$, $r^2 =0.8$), when an initial cell concentration of 0.6 $OD_{600}$ was used 0.2 $OD_{600}$. As shown in figure (13) a cell density of 0.6 OD had highest biodegradation efficiency. The phenol of 700ppm concentration was completely degraded within 96hr at 0.6 $OD_{600}$. To ensure accuracy and precision of the measurements, three samples from each dilution and control samples were analyzed with each batch as shown in Table 7.

**Table 7** Phenol biodegradation (ppm) by J20 (*Bacillus thuringiensis*) using three different cell density.

| Time of incubation | | | | | |
|---|---|---|---|---|---|
| **Hrs** | **0.5 OD $_{600}$ J20 (1) (ppm)** | **0.5 OD $_{600}$ J20 (2) (ppm)** | **0.5 OD $_{600}$ J20 (3) (ppm)** | **Mean** | **SD** |
| 0 | 689 | 685 | 682 | 685.3 | 3.51 |
| 24 | 119 | 159 | 179 | 152.3 | 30.55 |
| 48 | 75 | 95 | 89 | 86.33 | 10.26 |
| 72 | 59 | 52 | 69 | 60.00 | 8.544 |
| 96 | 32 | 42 | 45 | 39.66 | 6.80 |
| **Hrs** | **0.6 OD $_{600}$ J20 (1) (ppm)** | **0.6 OD $_{600}$ J20 (2) (ppm)** | **0.6 OD $_{600}$ J20 (3) (ppm)** | **Mean** | **SD** |
| 0 | 689 | 682 | 679 | 683.33 | 5.131 |
| 24 | 89 | 102 | 115 | 102.0 | 13.00 |
| 48 | 55 | 69 | 75 | 66.33 | 10.26 |
| 72 | 19 | 25 | 32 | 25.33 | 6.50 |
| 96 | 2 | 12 | 9 | 7.66 | 5.131 |
| **Hrs** | **0.2 OD $_{600}$ J20 (1) (ppm)** | **0.2 OD $_{600}$ J20 (2) (ppm)** | **0.2 OD $_{600}$ J20 (3) (ppm)** | **Mean** | **SD** |
| 0 | 672.000 | 682 | 688 | 680.66 | 8.082 |
| 24 | 262 | 279 | 252 | 264.33 | 13.65 |
| 48 | 189 | 199 | 192 | 193.33 | 5.13 |
| 72 | 165 | 129 | 149 | 147.66 | 18.03 |
| 96 | 65 | 52 | 109 | 75.33 | 29.87 |
| **Hrs** | **Control sample *** | **Control sample *** | **Control sample *** | **Mean** | **SD** |
| 0 | 675 | 689 | 695 | 686.33 | 10.26 |
| 24 | 672. | 685 | 682 | 679.66 | 6.80 |
| 48 | 675 | 677 | 679 | 677.00 | 2.00 |
| 72 | 682 | 675 | 672 | 676.33 | 5.13 |
| 96 | 685 | 672 | 665 | 674.00 | 10.14 |

***Control samples:** Minimal salt medium ( MSM ) contain phenol only.

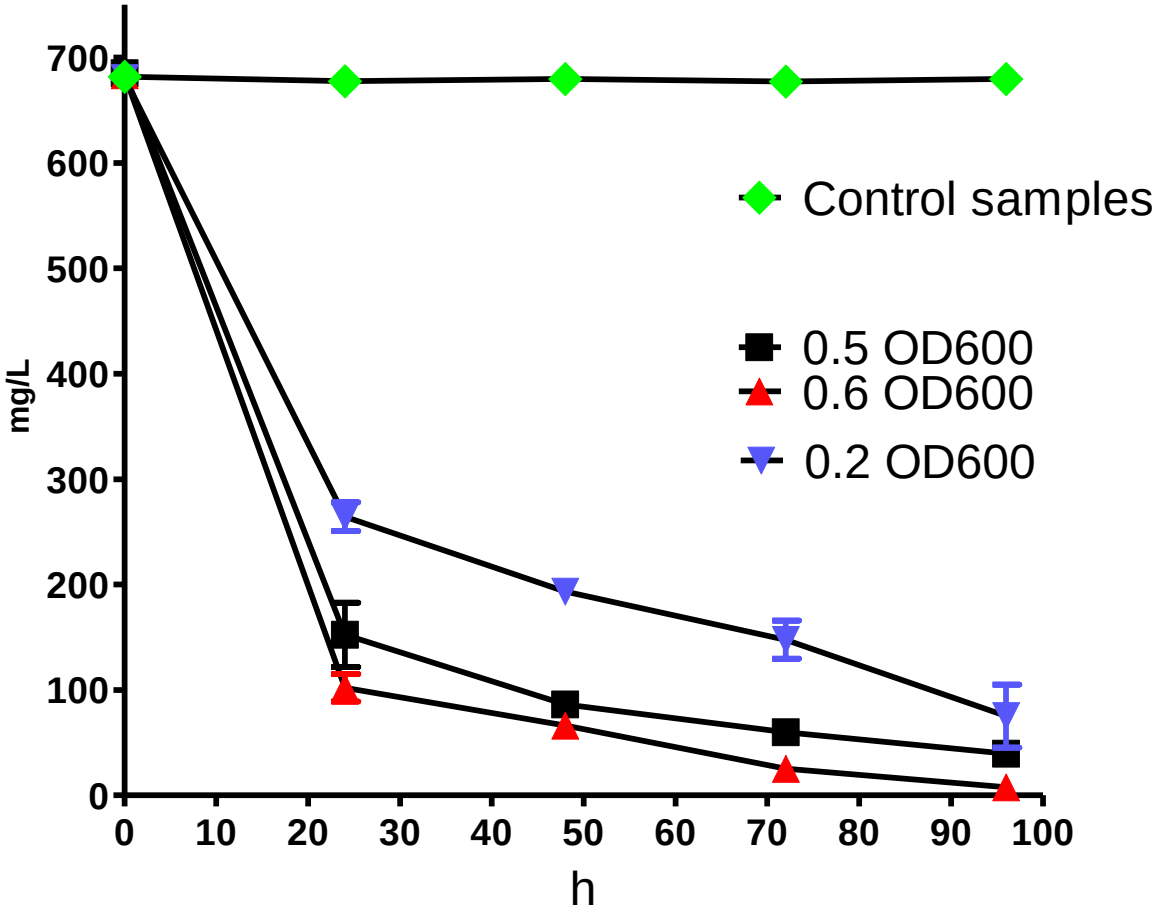


**Figure 13** Effect of three inoculation sizes (0.2, 0.5 and 0.6) on the biodegradation of 700ppm phenol by *Bacillus thuringiensis*- J20 .

### 3.7 Effect of Incubation Temperature and pH on Phenol elimination

To investigate the effect of incubation temperature on phenol degradation, different incubation temperature (25°C, 30°C, 37°C and 42 °C ) were used at pH 6.57. Cells were able to utilize phenol over a wide range of incubation temperatures. The optimal temperature for the highest phenol elimination was at 30°C. Increasing the incubation temperature from 25°C to 30°C resulted in an increase in the percentage of phenol degradation from 70.5% to 90.6 % as shown in Figure (14). High temperature (42°C) negatively affected the activity of the bacterial growth and hence slowed its biodegradation capabilities to 62.1% as shown in Figure (14). The experiment was done at initial concentration of 700ppm phenol.

The effect of pH on the growth of the phenol degradation was also studied compared to other pH values (pH5.5, pH6.5, pH7.5, pH8.5 and pH9.5) at 30°C., the optimal pH for the highest phenol elimination was 6.57 resulted in an increase in the percentage of phenol degradation about 90 % as shown in (Figure 15). Furthermore, Phenol concentration decreased faster at pH 6.57 compared to other pH values indicating high degradation rate..

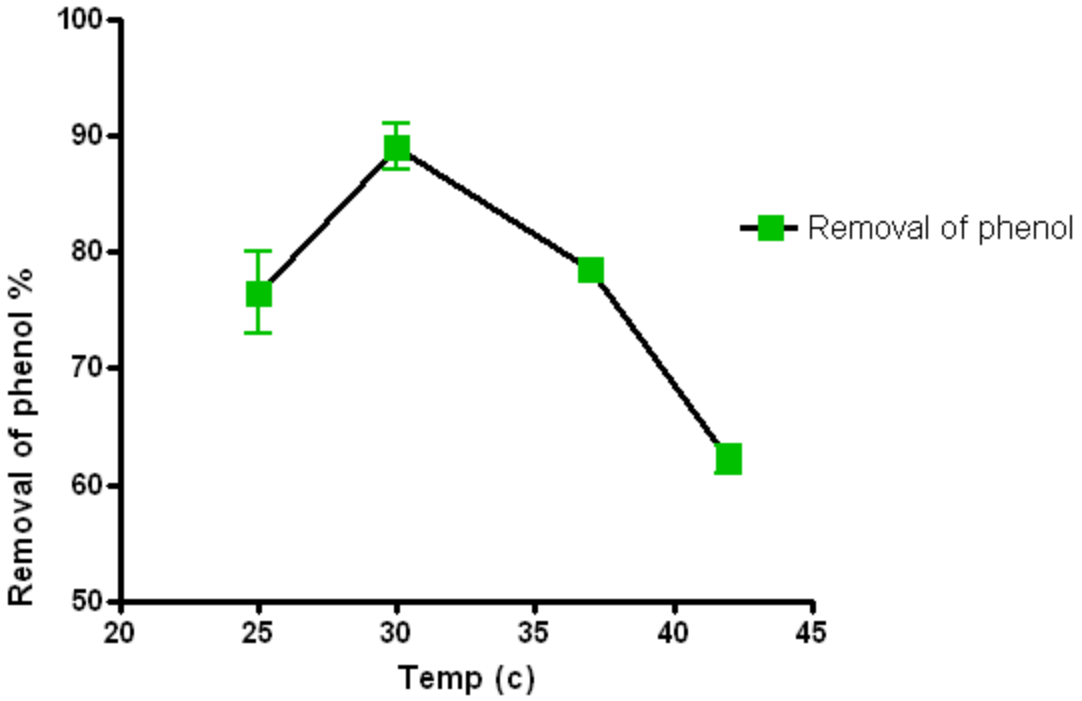


**Figure 14** Effect of temperature on the removal of phenol at initial phenol concentration of 700ppm.

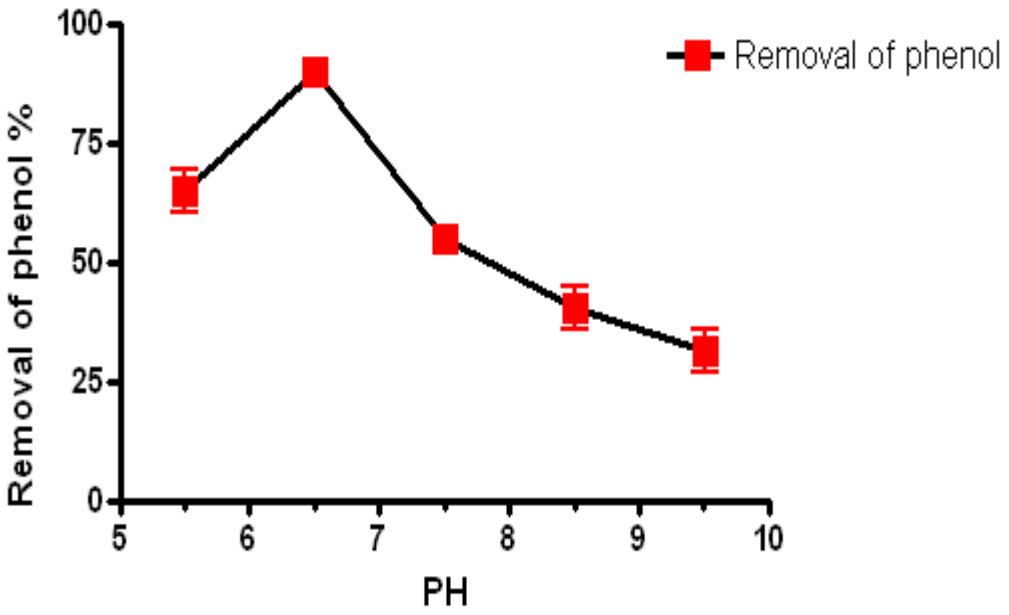


**Figure 15** Effect of pH on removal of phenol at initial phenol concentration of 700ppm

### 3.8 Phenol degradation by immobilized cells in alginate

The biodegradation of phenol at initial concentration 700ppm was carried out by alginate-immobilized cells of J20 and compared to free cells. The concentration of phenol in ppm was recorded by time as describe in Table (8). The phenol-degradation efficiency by free cells was less compared to immobilized cells, 90.6% of phenol was eliminated at 96 hrs by free cells whereas the immobilized cells were able to utilize 97.2% of the same amount of phenol within 96hrs. The phenol of 700ppm concentration was completely degraded by immobilized cells within 120hr as shown in Figure (16).

**Table 8** Biodegradation of phenol (ppm) at initial concentration 700ppm using alginate-immobilized cells of J20 versus free cells.

| **Time of incubation** | | | | |
|---|---|---|---|---|
| **Hrs** | **Imm J20 (1)** | **Imm J20 (2)** | **Mean** | **SD** |
| 0 | 688 | 688 | 688 | 0 |
| 24 | 335 | 365 | 350 | 21.21 |
| 48 | 59 | 69 | 64 | 7.071 |
| 72 | 35 | 49 | 42 | 9.89 |
| 96 | 19 | 29 | 24 | 7.07 |
| 120 | 2 | 5 | 3.5 | 2.12 |
| **Hrs** | **Free J20 (1)** | **Free J20 (2)** | Mean | SD |
| 0 | 689 | 688 | 688.5 | 0.707 |
| 24 | 262 | 349 | 305.5 | 61.51 |
| 48 | 189 | 252 | 220.5 | 44.54 |
| 72 | 165 | 192 | 178.5 | 19.09 |
| 96 | 65 | 149 | 107 | 59.39 |
| 120 | 65 | 109 | 87 | 31.11 |
| **Hrs** | **Control sample *** | **Control sample *** | Mean | SD |
| 0 | 675 | 689 | 682 | 9.89 |
| 24 | 672. | 688 | 680 | 11.31 |
| 48 | 687 | 677 | 682 | 7.07 |
| 72 | 682 | 675 | 678.5 | 4.94 |
| 96 | 685 | 673 | 679 | 8.48 |
| 120 | 679 | 676 | 677 | 2.12 |

Imm J20: immobilized J20 cells .

Free J20: free cells

Control samples: Alginate beads without bacterial cells with phenol only

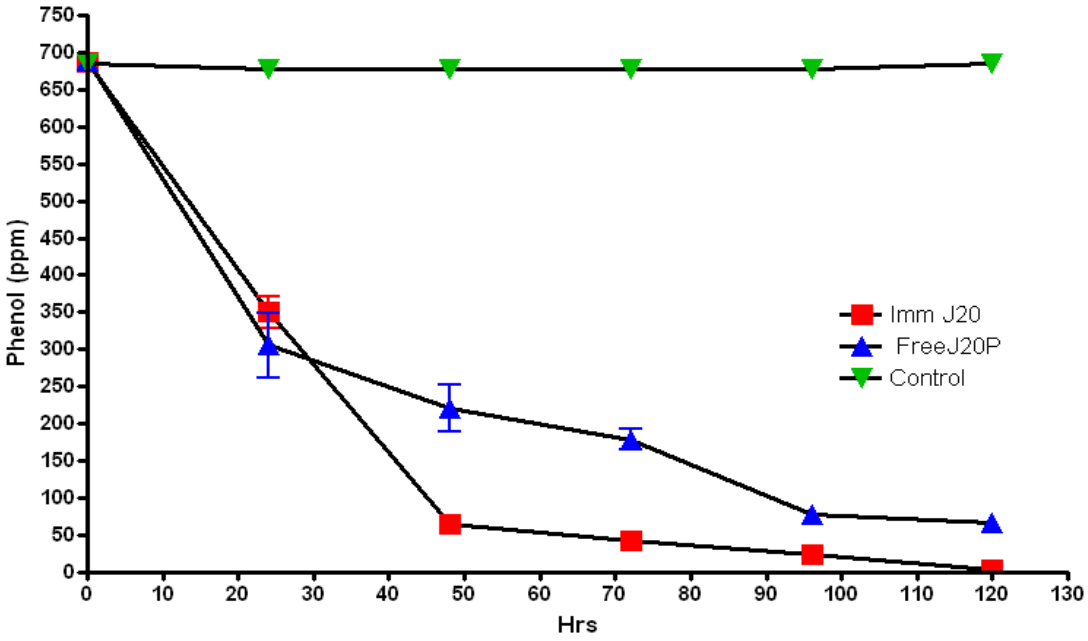


**Figure 16** Phenol degradation profiles of free and alginate-immobilized J20 at phenol concentration of 700ppm

The effect of storage time on phenol degradation by alginate-immobilized cells was investigated, storage of immobilized cells significantly reduced its capability to degrade phenol at 700ppm 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 as shown in (Figure 17).

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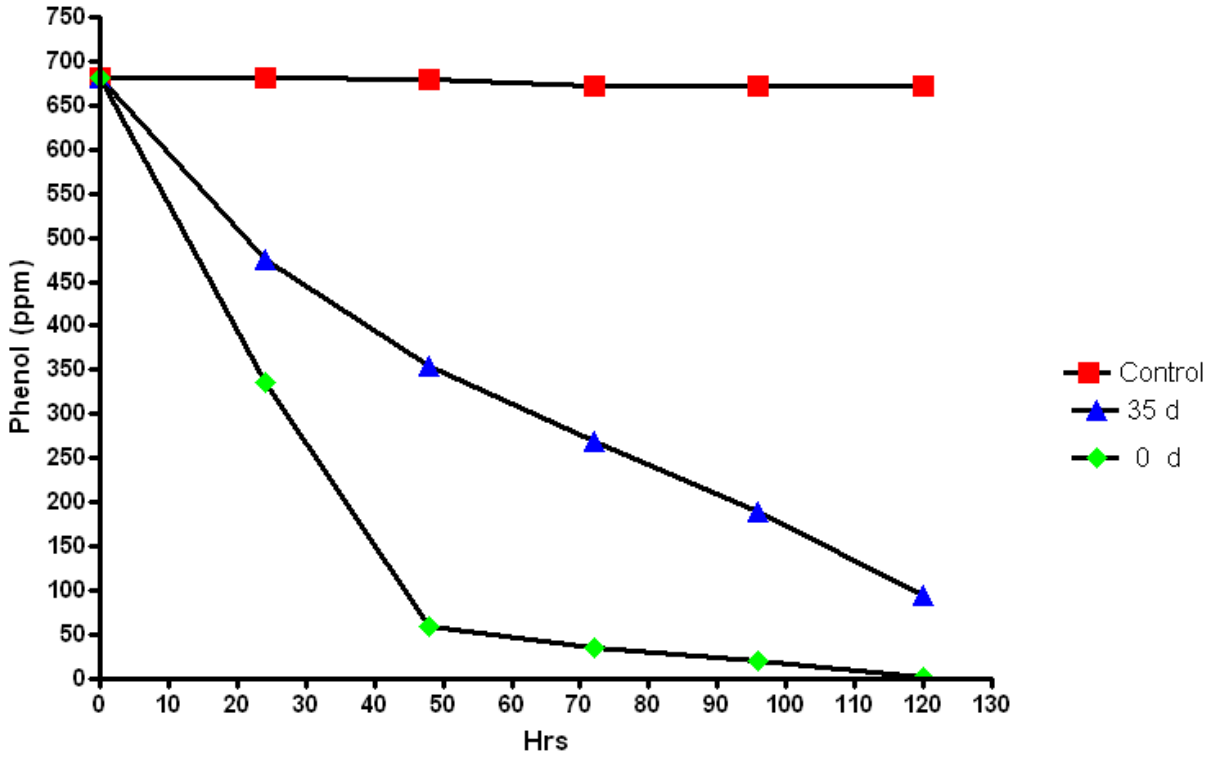



**Figure 17** Phenol degradation by alginate-immobilized J20 at 700ppm, pre-storage (day 0) and after 35 days of storage at 4°C. The control represents alginate beads without bacterial cells.

Alginate beads without bacterial cells (negative control) showed no detectable loss in phenol concentration due to evaporation or adsorption on the beads. When higher concentration of phenol was used (1400ppm), 74% of phenol was removed in the first 48hrs and then very slow degradation was monitored as shown in (Figure 18).

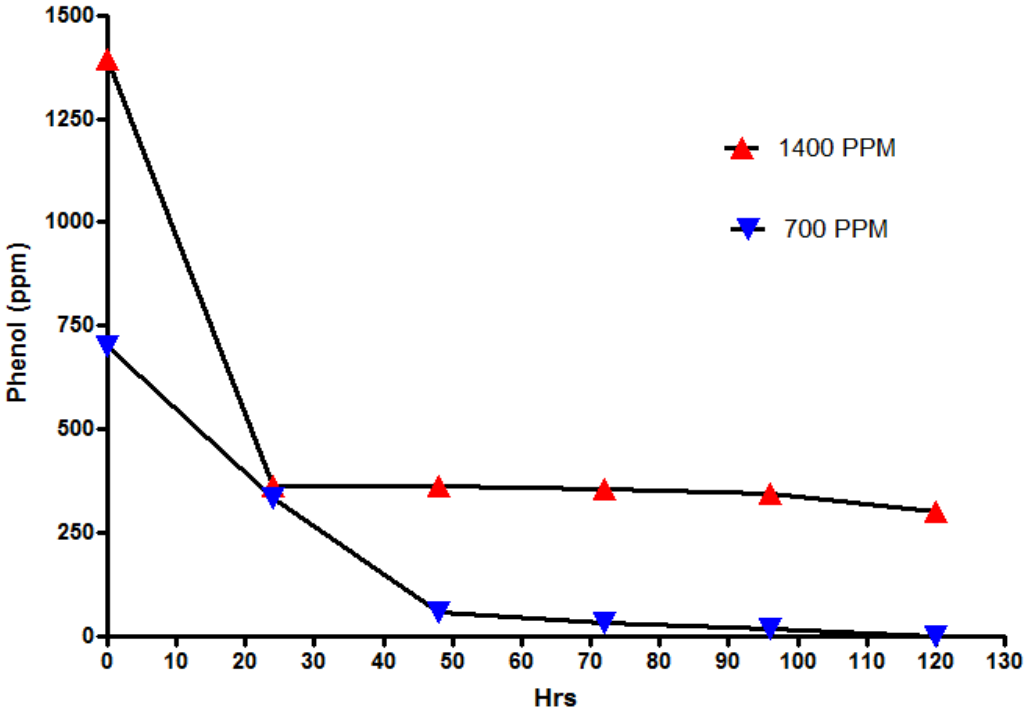



**Figure 18** Phenol degradation profiles of alginate-immobilized J 20 at two different initial phenol concentrations.

### 3.9 Detoxification test

In the current study, the J20 isolate was used to treat OMWW and this treatment was assessed on the growth of *E. coli* as compared to untreated OMWW. The phenol concentrations in the wastewater were determined before and after treatment with J20 cells using 4-aminoantipyrine in the colorimetric assay as described in the materials and methods. A decrease in phenol concentration from 189 ppm (before treatment) to 9 ppm ( after treatment with J20) was observed within 120h. The data of treated and untreated OMWW samples at different dilutions were correlated by the same curve as shown in Figure (19). It is clear that increasing the percentages of untreated OMWW from 20 to 40% (V/V) resulted in sharp decrease in CFU.

Moreover, no cells were able to grow in 60% or more of untreated OMWM sample while 100 colonies were grown in 100% of treated OMW sample.

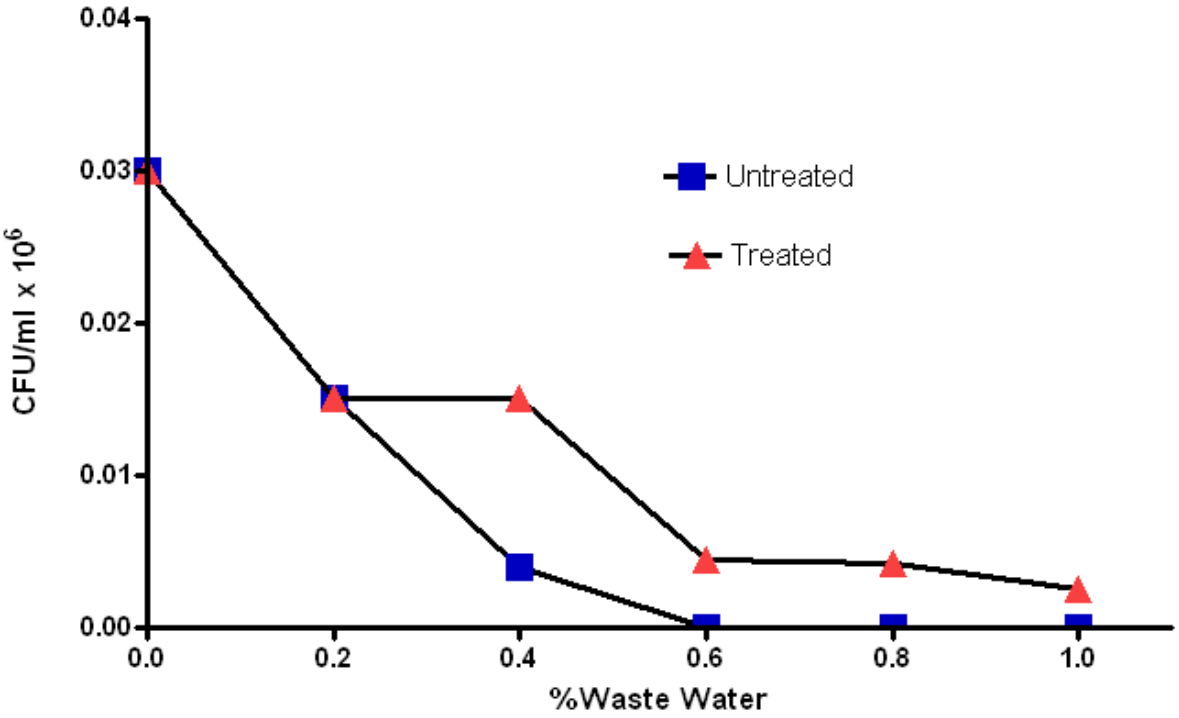



**Figure 19** Growth curves of E. Coli in the presence of different percentages of untreated and treated OMWW.

## Chapter Four: Discussion

Worldwide a huge amount of wastewater worldwide contaminated with toxic organic substances was generated from the industrial sector. The increase of phenol pollutants compounds in industrial wastewater in the environment causes several problems to ground water, soil, living organisms and many other ecological problems. The conventional methods that have been for treatment of phenol usually complex, expensive and produce hazardous end-products (Hooived et al., 1998).In Palestine, around 200 thousand m3 per year of OMWW are produced and disposed in wadies and water receiving bodies resulting in ecological and envrinmenta problems. Therefor, our research have focused on the isolation of phenol degrading bacteria from olive mill waste and attempted to be used in phenol biodegradation studies in Palestine.

Biodegradation is being preferred over the other physio-chemical treatment methods of phenol due to its potential to degrade phenol completely and overcome the disadvantages presented by other processes, as it is cheaper, produces no harmful products and most importantly maintains phenol concentration below the toxic limit. Different microbes have shown the ability to break down phenol completely and utilize it for energy production (Saluja et al., 2015). Furthermore, denitrifying microorganisms capable of using phenol as a sole source of carbon and energy were readily isolated from sediments from different geographic locations, including a pristine environment and environments exposed to xenobiotic compounds (Palula et al.,1998).

In this study, we successfully isolated a bacterium strain from SOMW which was capable to utilize phenol as the sole source of carbon and energy. Based on biochemical tests, 16srRNA-PCR and phylogentic analysis, this strain was identified as *Bacillus thuringiensis* ( designated as J20). Increasing levels of phenol was used to determine phenol tolerance of the J20 isolate. We noted thatthe bacterial growth was significantly reduced with increasing phenol concentrations ($P = 0.03$). However, the isolate expressed a tolerance to phenol concentration as high as 1,100 mg/L indicating that it was a good candidate in phenol biodegradation study.

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 (Kamal et al., 2008; Das et al., 2014; Das et al.,2010). 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 ( Kafilzadeh et al., 2013).

Furthermore, several bacteria were discovered to have excellent capability of phenol degradation, including *Bacillus sp*, *Pseudomonas* sp, *Acinetobacte*r sp, *Achromobacter sp* etc. *Fusarium sp*, *Phanerocheate chrysosporium*, *Corious versicolor*, *Ralstonia sp* and *Streptomyces sp* (Luckarift et al., 2011; Zhang et al., 2013; Karimi et al., 2016). However, isolation of native microbial species from local polluted environments has been reported to be more adaptive and efficient than non-indigenous microorganisms as biodegrades. Therefore, isolation of new phenol-degrading bacteria is recommended for the bioremediation of the phenol-contaminated sites in various regions (Silva et al., 2009;Liu et al., 2016).

In this study, we experimentally monitored the course of biodegradation of phenol using free J20 cell. . Our results showed that free cells of the J20 isolate can degrade 88.6% of the phenol within 96 h. Repeated measures of ANOVA showed significant variation between the initial cell density inoculum and biodegradation rate ofphenol at concentration of 700 mg/L ($P = 0.0003$, $r^2 = 0.8$) suggesting that high cell concentrations enhance the biodegradation of phenol, which is in agreement with other studies reported that high bacterial density resulted in a high degradation rate of toxic compounds (Godjevargova et al., 2003)

It is reported that Physiological conditions (such as incubation temperature pH, oxygen content and substrate concentration) can also play an important role in the growth and biodegradation efficiency of bacteria. All biological reactions that involved in the degradation pathway have an optimum temperature and pH and thus will not have the same metabolic turnover at different conditions (Shawabkeh et al.,2007). Each bacterium has a specific temperature range for growth, for example *Corynebacterum glutamicum* has been reported to degrade phenol at temperature range 30-37 C (Bhargavi, et al. 2016). *Acetobacter sp*, has been used to effectively degrade phenol at 33 C optimal tempreture (Zeng, Cao et al. 2014).

In this study,. we observed that the phenol degradation- by J20- was temperature dependent, the optimal was at 30C, which supports and agrees previous studies revealed that most favorable temperature range for the phenol-degrading bacteria was from 25 to 30℃ (Monteiro et al.,2000; Zhang et al., 2013). Furthermore, the concentration of phenol decreased more rapidly at pH 6.57 as compared to higher and lower pH values indicating that 6.57 was optimal for J20 In contrast, *Halomonas camisalis* has been reported to degrade phenol at alkaline pH between 8 and 11 (Alva et al., 2003) where *Weingella ameriacna* degrade phenol at merely natural pH 7.5 (Khleifat et al., 2006). Further, Kiebsilla oxytoca degrade phenol at acidic pH 6.8.(Shawabkeh et al., 2007). However, It is reported that most organisms cannot tolerate pH values below 4.0 or above 9.0 which is believed to be species specific. Therefore, follow-up of the medium pH can be used as indicator for phenol degradation effceincy as they respond differently to different pH (Alva et al., 2003).

It is reported that immobilization of bacterial cells can be a good alternative to exhibit higher degradation efficiency at high phenol concentrations. Therefore, we investigated the biodegradation potential of both immobilized and free cells of J20. Indeed, a biodegradation of 100% was obtained at 120 hrs by immobilized cells compared to 88.6% by free cells. This probably because the immobilized cells were protected by the alginate beads which is in consistence with other studies showed that immobilized cells of *Acenitobacter, Aeromonas and Pseudomonas spp.* were better phenol-biodegraders than those of free cells (El-Naas et al., 2009; Mollaei, Abdollahpour et al., 2010; Namane et al., 2013). Furthermore, when a higher concentration of phenol was used (1,400 mg/L), very slow degradation was monitored after 48h followed by plateau phase, 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 (Lee et al., 2010). On the other hand, Storage of immobilized cells significantly reduced its capability to degrade phenol at 700 mg/L compared to control group. . This is in contrast to a previous report which demonstrated that entrapped *B. cereus* cells can be stored for one month without compromising their phenol-degrading capacity(Banerjee et al., 2011) . 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.

Several studies have already been conducted to evaluate OMWW toxicity on microorganisms.(Paixao et al., 1999, Letnik et al., 2015). In the current study, the J20 isolate was used to treat OMWW and this treatment was assessed on the growth of *E. coli* as compared to untreated OMWW. We have noticed that no cells were able to grow in 60% or more of untreated OMWW sample, while 150 colonies grew in 100% J20-treated OMWW sample indicating that J20 was able to reduce the polluting charge of crude none diluted OMWW. However, the phenols in the crude OMWW sample were partially consumed (61%) after treatment by J20. 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 sample (Leouifoudi et al., 2014, Dragičević et al., 2010) which may have different degradation patterns and hence affect the degradation efficiency of J20. The phenol-degradation capability of J20 strain makes it an organism of choice for the bioremediation of phenol-contaminated environments in Palestine.

## Conclusions

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 bio-treatment 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.

## Study Limitations

No universal method that can be applied to the different olive industries, the solution is dependent on pH of OMWW, extraction process, and other specific local conditions. Our recent work with *B. thuringiensis* J20, highlights the potential of this strain to be used as a good alternative strain for OMWW bioremediation compared to the *B. thuringiensis* strains already described in other studies of OMW treatments. In the crude OMWW treatment by J20, it was achieved a reduction of 60 %. However, the OMWW is still considered slightly toxic to microorganisms to be discharged directly in the environment. Therefore, these results are promising for further research in this area.

**Recommendations:**

A common way of dealing with the OMWW in Palestine as in many Mediterranean countries was to discharge directly into Wadies or sewage network (if available) which is unacceptable without a pretreatment. The treatment of OMWW produced from olive oil production is still a major challenge facing the olive-oil producing countries. At the moment, the management options of OMWW in Palestine are limited due to the traditional oil extraction processes, the economic and social conditions, and the seasonal limitations. Therefore, a new strategy for OMWW management must be adapted by the Palestinian water Authority. Transport systems to transfer the OMWW from the olive-mill sites to the centralized treatment plants are needed.

## References


Alva, V. A. and B. M. Peyton (2003). "Phenol and catechol biodegradation by the haloalkaliphile Halomonas campisalis: influence of pH and salinity." Environmental science & technology **37**(19): 4397-4402.

Akan, J., F. Abdulrahman, O. Sodipo, A. Ochanya and Y. Askira (2010). "Heavy metals in sediments from River Ngada, Maiduguri Metropolis, Borno State, Nigeria." Journal of Environmental Chemistry and Ecotoxicology 2(9): 131-140.

Annadurai, G., S. M. Balan and T. Murugesan (2000). "Design of experiments in the biodegradation of phenol using immobilized Pseudomonas pictorum (NICM-2077) on activated carbon." Bioprocess Engineering **22**(2): 101-107.

Anselmo, A., M. Mateus, J. Cabral and J. Novais (1985). "Degradation of phenol by immobilized cells ofFusarium flocciferum." Biotechnology letters 7(12): 889-894.

Al-Khalid, T. and M. H. El-Naas (2012). "Aerobic biodegradation of phenols: a comprehensive review." Critical Reviews in Environmental Science and Technology 42(16): 1631-1690.

Asfi, M., G. Ouzounidou and M. Moustakas (2012). "Evaluation of olive oil mill wastewater toxicity on spinach." Environmental Science and Pollution Research **19**(6): 2363-2371.

Autrup, H., P. Calow, W. Dekant, H. Greim, H. Wojciech, C. Janssen, B. Jansson, H. Komulainen, O. Ladefoged and J. Linders (2005). "Scientific Committee on Health and Environmental Risks opinion on: Environmental Risk Assessment of non Biodegradable Detergent Surfactants under Anaerobic Condition." Scientific Committee on Health and Environmental Risks.

Aviani, I., M. Raviv, Y. Hadar, I. Saadi and Y. Laor (2009). "Original and residual phytotoxicity of olive mill wastewater revealed by fractionations before and after incubation with Pleurotus ostreatus." Journal of agricultural and food chemistry **57**(23): 11254-11260.

Azizullah, A., M. N. K. Khattak, P. Richter and D.-P. Häder (2011). "Water pollution in Pakistan and its impact on public health—a review." Environment International 37(2): 479-497.

Aladham, R. A. F. (2012). "Removal of polyphenols from olive mill wastewater using activated olive stones." Unpublished Master Thesis). An-Najah National University, Palestine.

Banerjee, A. and A. K. Ghoshal (2011). "Phenol degradation performance by isolated Bacillus cereus immobilized in alginate." International Biodeterioration & Biodegradation **65**(7): 1052-1060.

Banerjee, A. (2011). Biodegradation of Phenolic and Petroleum Wastewater by Isolated Bacillus cereus.

Balasundram, N., K. Sundram and S. Samman (2006). "Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses." Food chemistry 99(1): 191-203.

Basha, K. M., A. Rajendran and V. Thangavelu (2010). "Recent advances in the biodegradation of phenol: a review." Asian J Exp Biol Sci 1(2): 219-234.

Basalat, N. H. J. (2012). Treatment of Organic Phenolic Contaminant In wastewater Using Activated Carbon From Cypress Products, Faculty of Graduate Studies Treatment of Organic Phenolic Contaminant In wastewater Using Activated Carbon From Cypress Products By Nadeem Hamdallah Jameel Bassalat Supervisor Dr. Shehdeh Jodeh Dr. Ahmad Abu Obed This Thesis is Submitted in Partial Fulfillment of the Requirements for the Degree of Master in Environmental Science, Faculty of Graduate Studies, An-Najah National University.

Behera, N. (2009). Treatment of industrial effluents in a bioreactor.

Bhargavi, B. J., M. M. Rafi and D. M. Rao (2016). "Effect of physicochemical conditions on phenol degradation by Corynebacterium glutamicum: A focus on phenol catabolic enzymes."

Caetano, M., C. Valderrama, A. Farran and J. L. Cortina (2009). "Phenol removal from aqueous solution by adsorption and ion exchange mechanisms onto polymeric resins." Journal of Colloid and Interface Science **338**(2): 402-409.

Chitra, S., G. Sekaran, S. Padmavathi and C. Gowri (1995). Removal of phenol from wastewater using a biocatalyst. Proceedings of the National Symposium Frontiers in Applied Environmental Microbiology.

Cordova-Rosa, S., R. Dams, E. Cordova-Rosa, M. Radetski, A. Corrêa and C. Radetski (2009). "Remediation of phenol-contaminated soil by a bacterial consortium and Acinetobacter calcoaceticus isolated from an industrial wastewater treatment plant." Journal of hazardous materials 164(1): 61-66.

Das, N. and P. Chandran (2010). "Microbial degradation of petroleum hydrocarbon contaminants: an overview." Biotechnology research international **2011**.

Das, P., Sinha, S. and Mukherjee, S.K. (2014) Nickel bioremediation potential of Bacillus thuringiensis KUNi1 and some environmental factors in nickel removal. Bioremediation journal 18(2), 169-177.

Der Yang, R. and A. E. Humphrey (1975). "Dynamic and steady state studies of phenol biodegradation in pure and mixed cultures." Biotechnology and Bioengineering **17**(8): 1211-1235.

Dragičević, T., M. Hren, M. Gmajnić, S. Pelko, D. Kungulovski, I. Kungulovski, D. Čvek, J. Frece, K. Markov and F. Delaš, 2010: Biodegradation of olive mill wastewater by Trichosporon cutaneum and Geotrichum candidum. *Archives of Industrial Hygiene and Toxicology,* **61,** 399-405.

Działo, M., J. Mierziak, U. Korzun, M. Preisner, J. Szopa and A. Kulma (2016). "The potential of plant phenolics in prevention and therapy of skin disorders." International journal of molecular sciences 17(2): 160.

Evans, W. (1947). "Oxidation of phenol and benzoic acid by some soil bacteria." Biochemical Journal 41(3): 373.

Ehrhardt, H. and H. Rehm (1985). "Phenol degradation by microorganisms adsorbed on activated carbon." Applied Microbiology and Biotechnology **21**(1-2): 32-36.

El-Naas, M. H., S. A. Al-Muhtaseb and S. Makhlouf (2009). "Biodegradation of phenol by Pseudomonas putida immobilized in polyvinyl alcohol (PVA) gel." Journal of Hazardous Materials **164**(2): 720-725.

Evans, W. (1947). "Oxidation of phenol and benzoic acid by some soil bacteria." Biochemical Journal **41**(3): 373.

Gad, N. S. and A. S. Saad (2008). "Effect of environmental pollution by phenol on some physiological parameters of Oreochromis niloticus." Global Veterinaria **2**(6): 312-319.

Gami, A. A. (2014). "Phenol and its toxicity." Journal of Environmental Microbiology and Toxicology 2(1).
Krastanov, A., Z. Alexieva and H. Yemendzhiev (2013). "Microbial degradation of phenol and phenolic derivatives." Engineering in Life Sciences 13(1): 76-87.

Godjevargova, T., D. Ivanova, Z. Alexieva and N. Dimova (2003). "Biodegradation of toxic organic components from industrial phenol production waste waters by free and immobilized Trichosporon cutaneum R57." Process biochemistry **38**(6): 915-920.

Hall, V., G. O'Neill, J. Magee and B. Duerden (1999). "Development of amplified 16S ribosomal DNA restriction analysis for identification of Actinomyces species and comparison with pyrolysis-mass spectrometry and conventional biochemical tests." Journal of clinical microbiology **37**(7): 2255-2261.

Hooived, M., D. J. Heederik, M. Kogevinas, P. Boffetta, L. L. Needham, D. G. Patterson and H. B. Bueno-de-Mesquita (1998). "Second follow-up of a Dutch cohort occupationally exposed to phenoxy herbicides, chlorophenols, and contaminants." American journal of epidemiology **147**(9): 891-899.

İzli, G. "Phenolic compounds change in Table Olives."

KAFIlzADeh, F. and S. MoKhtArI (2013). "Isolation and Identification of Phenol Degrading Bacteria from Mangrove Sediments in the Persian Gulf (Asaluyeh) and their Growth Kinetics Assay." Biomedical & Pharmacology Journal **6**(2): 189-196.

Kallel, M., C. Belaid, T. Mechichi, M. Ksibi and B. Elleuch (2009). "Removal of organic load and phenolic compounds from olive mill wastewater by Fenton oxidation with zero-valent iron." Chemical Engineering Journal 150(2-3): 391-395.

Krastanov, A., Z. Alexieva and H. Yemendzhiev (2013). "Microbial degradation of phenol and phenolic derivatives." Engineering in Life Sciences 13(1): 76-87.

Karimi, M. and M. Hassanshahian (2016). "Isolation and characterization of phenol degrading yeasts from wastewater in the coking plant of Zarand, Kerman." brazilian journal of microbiology **47**(1): 18-24.

Keith, L. and W. Telliard (1979). "ES&T special report: priority pollutants: Ia perspective view." Environmental science & technology **13**(4): 416-423.

Kumaran, P. and Y. Paruchuri (1997). "Kinetics of phenol biotransformation." Water Research **31**(1): 11-22.

Khleifat, K. M. (2006). "Biodegradation of phenol by Ewingella americana: Effect of carbon starvation and some growth conditions." Process Biochemistry 41(9): 2010-2016.

Lakshmi, M. C., V. Sridevi, E. Neharika, C. Beena, M. N. Rao and A. Swamy (2009). "Effect of temperature and carbon source on phenol degradation by Pseudomonas aeruginosa

(NCIM 2074) and Pseudomonas desmolyticum (NCIM 2028) and their comparison." International Journal of Chemical Sciences **7**(4): 2591-2601.

Leouifoudi, I., Zyad, A., Amechrouq, A., Oukerrou, M.A., Mouse, H.A. and Mbarki, M. (2014) Identification and characterisation of phenolic compounds extracted from Moroccan olive mill wastewater. Food Science and Technology (Campinas) 34(2), 249-257.

Letnik, I., Avrahami, R., Rokem, J.S., Greiner, A., Zussman, E. and Greenblatt, C. (2015) Living composites of electrospun yeast cells for bioremediation and ethanol production. Biomacromolecules 16(10), 3322-3328.

Lee, S. Y., B.-N. Kim, J.-H. Han, S.-T. Chang, Y.-W. Choi, Y.-H. Kim and J. Min (2010). "Treatment of phenol-contaminated soil by Corynebacterium glutamicum and toxicity removal evaluation." Journal of hazardous materials **182**(1): 937-940.

Liu, Z., W. Xie, D. Li, Y. Peng, Z. Li and S. Liu (2016). "Biodegradation of Phenol by Bacteria Strain Acinetobacter Calcoaceticus PA Isolated from Phenolic Wastewater." International journal of environmental research and public health **13**(3): 300.

Luckarift, H. R., S. R. Sizemore, K. E. Farrington, P. A. Fulmer, J. C. Biffinger, L. J. Nadeau and G. R. Johnson (2011). "Biodegradation of medium chain hydrocarbons by Acinetobacter venetianus 2AW immobilized to hair-based adsorbent mats." Biotechnology progress **27**(6): 1580-1587.

Marrot, B., A. Barrios-Martinez, P. Moulin and N. Roche (2006). "Biodegradation of high phenol concentration by activated sludge in an immersed membrane bioreactor." Biochemical Engineering Journal **30**(2): 174-183.

Meikap, B. and G. Roy (1997). "Removal of Phenolic Compounds From Industrial Waste Water by SemifluidizedBed Bio-Reactor."

Mollaei, M., S. Abdollahpour, S. Atashgahi, H. Abbasi, F. Masoomi, I. Rad, A. S. Lotfi, H. S. Zahiri, H. Vali and K. A. Noghabi (2010). "Enhanced phenol degradation by Pseudomonas sp. SA01: gaining insight into the novel single and hybrid immobilizations." Journal of hazardous materials **175**(1): 284-292.

Monteiro, Á. A., R. A. Boaventura and A. r. E. Rodrigues (2000). "Phenol biodegradation by Pseudomonas putida DSM 548 in a batch reactor." Biochemical Engineering Journal **6**(1): 45-49.

Namane, A., F. Amrouche, H. Hamdi, O. Ali, D. Hank and A. Hellal (2013). "BIODEGRADATION OF PHENOLIC WASTEWATERS." Environmental Engineering and Management Journal **13**(1): 1-6.

Ntougias, S., K. Bourtzis and G. Tsiamis (2013). "The microbiology of olive mill wastes." BioMed research international **2013**.

Paixao, S., E. Mendonça, A. Picado and A. Anselmo (1999). "Acute toxicity evaluation of olive oil mill wastewaters: a comparative study of three aquatic organisms." Environmental toxicology **14**(2): 263-269.

Park, M.-R., D.-J. Kim, J.-W. Choi and D.-S. Lim (2013). "Influence of immobilization of bacterial cells and TiO2 on phenol degradation." Water, Air, & Soil Pollution 224(3): 1473.

Peikert, B., G. Schaumann, Y. Keren, N. Bukhanovsky, M. Borisover, M. Garfha, J. Shoqeric and A. Dag (2015). "Characterization of topsoils subjected to poorly controlled olive oil mill wastewater pollution in West Bank and Israel." Agriculture, Ecosystems & Environment **199**: 176-189.

Qutub, M., S. Ali, M. Mutawea, M. Abed, T. Arabasi, F. Pierini and E. Lodolini (2010). "Characterisation of the main Palestinian olive cultivars and olive oil." National and international, Governmental or Civil Society Institutions and Organization **1**.

Rajani, V. and J. Reshma (2016). "FACTORS AFFECTING PHENOL DEGRADATION POTENTIAL OF MICROBES-A REVIEW."

Reshma, J., V. S. G. Thanga and A. Mathew (2014). "PHENOL DEGRADATION BY BACTERIA ISOLATED FROM COIR RETTING BEDS OF SOUTH KERALA COAST." Journal of Global Biosciences **3**(6): 935-940.

Rouvalis, A. and J. Iliopoulou–Georgudaki (2010). "Comparative assessment of olive oil mill effluents from three-phase and two-phase systems, treated for hydrogen production." Bulletin of environmental contamination and toxicology **85**(4): 432-436.

Ruiz-Ordaz, N., J. C. Ruiz-Lagunez, J. H. Castanon-Gonzalez, E. Hernandez-Manzano, E. Cristiani-Urbina and J. Galindez-Mayer (2001). "Phenol biodegradation using a repeated batch culture of Candida tropicalis in a multistage bubble column." REVISTA LATINOAMERICANA DE MICROBIOLOGIA-MEXICO- **43**(1): 19-26.

Saluja, M. (2015). Isolation and characterization of phenol degrading organisms from soil sample containing traces of crude oil, National Institute of Technology Rourkela.

Sundar, M. S. (2012). "Microbial degradation of phenol: A comparative study." Master of Technology (Research). Department of Biotechnology & Medical Engineering National Institute of Technology, Rourkela Orissa-769008, India.

Shaheen, H. and R. Abdel Karim (2007). "Management of olive-mills wastewater in Palestine. An-Najah Univ." J. Res.(N. Sc.) **21**: 64-83.

Shawabkeh, R., K. M. Khleifat, I. Al-Majali and K. Tarawneh (2007). "Rate of biodegradation of phenol by Klebsiella oxytoca in minimal medium and nutrient broth conditions." Bioremediation Journal **11**(1): 13-19.

Silva, Í. S., E. d. C. dos Santos, C. R. de Menezes, A. F. de Faria, E. Franciscon, M. Grossman and L. R. Durrant (2009). "Bioremediation of a polyaromatic hydrocarbon contaminated soil by native soil microbiota and bioaugmentation with isolated microbial consortia." Bioresource Technology **100**(20): 4669-4675.

Sun, J. and H. Tan (2013). "Alginate-based biomaterials for regenerative medicine applications." Materials **6**(4): 1285-1309.

Soudi, M. R. and N. Kolahchi (2011). "Bioremediation potential of a phenol degrading bacterium, Rhodococcus erythropolis SKO-1." Progress in Biological Sciences 1(1): 31-70.

Tsagaraki, E., H. N. Lazarides and K. B. Petrotos (2007). Olive mill wastewater treatment. Utilization of By-products and Treatment of Waste in the Food Industry, Springer**:** 133-157.

Tabrez, S. and M. Ahmad (2011). "Oxidative stress-mediated genotoxicity of wastewaters collected from two different stations in northern India." Mutation Research/Genetic Toxicology and Environmental Mutagenesis 726(1): 15-20.

UN (2008). "The Olive Harvest in the West Bank and Gaza." United Nations.

Usha, M., M. Sanjay, S. Gaddad and C. Shivannavar (2010). "Degradation of h-acid by free and immobilized cells of Alcaligenes latus." Brazilian Journal of Microbiology **41**(4): 931-945.

Villegas, L. G. C., N. Mashhadi, M. Chen, D. Mukherjee, K. E. Taylor and N. Biswas (2016). "A short review of techniques for phenol removal from wastewater." Current Pollution Reports **2**(3): 157-167.

Wasi, S., S. Tabrez and M. Ahmad (2013). "Toxicological effects of major environmental pollutants: an overview." Environmental monitoring and assessment 185(3): 2585-2593.

Zeng, H.-Y., X.-L. Cao, L.-B. Xiong, X.-L. Cai, L.-Q. Huang, C.-Y. Zhang and Y.-Q. Li (2014). "Microbiological degradation of phenol using two co-aggregating bacterial strains." Environmental earth sciences **71**(3): 1339-1348.

Zhang, Y., D. Lu, T. Ju, L. Wang, S. Lin, Y. Zhao, C. Wang, H. He and Y. Du (2013). "Biodegradation of phenol using Bacillus cereus WJ1 and evaluation of degradation efficiency based on a graphene-modified electrode." Int J Electochem Sci **8**: 504-519.

Zorpas, A. A., V. J. Inglezakis, M. Stylianou and V. Irene (2010). "Sustainable treatment method of a high concentrated NH3 wastewater by using natural zeolite in closed-loop fixed bed systems." Open Environmental Sciences **4**(1).

Zeinat Kamal, M., Nashwa, A., Mohamed, A.I. and Sherif, E.-N. (2008) Biodegradation and detoxification of malathion by of Bacillus thuringiensis MOS-5. Aus J Basic Appl Sci 2(3), 724-732.

# Appendixes

**Appendix 1.1 Samples were collected from Bait Sahour olive mill. Bait Sahour -Bethlehem-southern -West Bank - Palestine.**


**Appendix 1.2 Olive Mill Wastewater samples collected.**

**Appendix 1.3 Solid Olive Mill Waste (olive press) samples collected.**

**Appendix 1.4 Soil samples from olive mill were collected.**

**Appendix 2 Isolation Bacteria from olive mill samples.**

**Appendix 3: Full sequences for each isolate was confirmed by amplifying 400 bp fragment of 16srRNA:**

> Full SL2Y *Exigobacterium*

GGCAGCAGTA GGGAATCTTC CACAATGGAC GAAAGTCTGA TGGAGCAACG CCGCGTGAAC GATGAAGGCC
TTCGGGTCGT AAAGTTCTGT TGTAAGGGAA GAACAAGTGC CGCAGGCAAT*GGCGGCACCT TGACGGTACC*
*TTGCGAGAAA GCCACGGCTA ACTACGTGCC AGCAGCCGCG GTAATACGTA GGTGGCAAGC GTTGTCCGGA*
*ATTATTGGGC GTAAAGCGCG CGCAGGCGGC CTCTTAAGTC TGATGTGAAA GCCCCCGGCT CAACCGGGGA*
*GGGCCATTGG AAACTGGGAG GCTTGAGTAT AGGAGAGAAG AGTGGAATTC CACGTGTAGC GGTGAAATGC*
*GTAGAGATGT GGAGGAACAC CAGTGGCGAA GGCGACTCTT TGGCCTATAA CTGACGCTGA GGCGCGAAAG*
*CGTGGGGAGC AAACAGGATT AGATACCCTG GTAGTCCACG CCGTAAACGA TGAGTGCTAG GTGTTGGAGG*
*GTTTCCG*CCC TTCAGTGCTG AAGCTAACGC ATTAAGCA

>Full SL3C1 *Acinetobacter*

GGCAGCAGT GGGGAATATT GGACAATGGG GGGAACCCTG ATCCAGCCA*T GCCGCGTGTG TGAAGAAGGC*
*CTTTTGGTTG TAAAGCACTT TAAGCGAGGA GGAGGCTACC GAGATTAATACTCTTGGATA GTGGACGTTA*
*CTCGCAGAAT AAGCACCGGC TAACTCTGTG CCAGCAGCCG CGGTAATACA GAGGGTGCAA GCGTTAATCG*
*GATTTACTGG GCGTAAAGCG CGCGTAGGTG GCCAATTAAG TCAAATGTGA AATCCCCGAG CTTAACTTGG*
*GAATTGCATT CGATACTGGT TGGCTAGAGT ATGGGAGAGG ATGGTAGAAT TCCAGGTGTA GCGGTGAAAT*
*GCGTAGAGAT CTGGAGGAAT ACCGATGGCG AAGGCAGCCA TCTGGCCTAA TACTGACACT GAGGTGCGAA*
*AGCATGGGGA GCAAACAGGA TTAGATACCC TGGTAGTCCA TGCCGTAAAC GATGTCTACT AGCCGTTGGG*
*GCCTTTGAGG CTTTAGTGGC GCAGCTAACG CGATAAGTAG ACCGCCTGGG GAGTACGGTC GCAAGACTAA*
*AACTCAAATG AATTGACGGG GGCCCGCACA AGC*GGTGGAG CATGTGGTTT AATTCGATGC AACGCGAANA
NCCCCTACA

> FULL J20 Isolate *(bacillus cereus)*

TCCTACGGGA GGCAGCAGTA GGGAATCTTC CGCAATGGAC GAAAGTCTGA CGGAGCAACG CCGCGTGAGT
GATGAAGGCT TTCGGGTCGT AAAACTCTGT TGTTAGGGAA GAACAAGTGC TAGTTGAATAAGCTGGCACC
TTGACGGTAC CTAACCAGAA AGCCACGGCT AACTACGTGC CAGCAGCCGC GGTAATACGT AGGTGGCAAG
CGTTATCCGG AATTATTGGG CGTAAAGCGC GCGCAGGTGG TTTCTTAAGT CTGATGTGAA AGCCCACGGC
TCAACCGTGG AGGGTCATTG GAAACTGGGA GACTTGAGTG CAGAAGAGGA AAGTGGAATT CCATGTGTAG
CGGTGAAATG CGTAGAGATA TGGAGGAACA CCAGTGGCGAAGGCGACTTT CTGGTCTGTA ACTGACACTG
AGGCGCGAAA GCGTGGGGAG CAAACAGGAT TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGAGTGCTA
AGTGTTAGAG GGTTTCCGCC CTTTAGTGCGAAGTTAACG CATTAAGCAC TCCGCCTGGG GAGTACGGCC
GCAAGGCTGA AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT AATTCGAAGC
AACGCGAAGA

>Full J70 *Klebsiela oxytoca*

*GGGGNNNTGCANGGGCGCAAGCCTGAGCAGCCTGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAG*
*CGGGGAGGAAGGCGGTGAGGTTAATAACCTCATCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCC*
*AGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAA*
*GTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTA*
*GAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGAC*
*TGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATT*
*TGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGT*
*TAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCA*

>Full Soil 20 *Klebsiela oxytoca*

GGNNATGCATGGGCGCAAGCCTGAGCAGCCTGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCA*GCG*
*GGGAGGAAGGCGATGAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAG*
*CAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGT*
*CGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTGGAGTCTTGTAGAGGGGGGTAGA*
*ATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTG*
*ACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGACTTG*
*GAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTA*
*AAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGACCCCCT*
*ACA*

>Full Soil 70 *Acinetobacter schindleri*

TGGGCGGANCCTGATCCNCCTGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGC
TCCTTTAGTTAATACCTAAAGTGAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGG
TAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTTTTAAGTCGGATGTGA
AATCCCTGAGCTTAACTTAGGAATTGCATTCGATACTGGAAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGT
GTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGG
TACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGGTGGGG
CCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTACACCCCCTGGGGAGTACGGTCGGAGACTAAAACTCGAAT
GAGTTGACGGGGGCCCGCACAGCCGGTGGAGCATGTGATTTAATTCCATGCGCCGCGCACGACCGTGC

>Full Soil 30 *Enterobacteriaceae sp*

CATGGGCGCAGCCTGAGCAGCCTGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAA
GGCGATGCGGTTAATAACCGCGTCAATTGACGTTACCCGCAAAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCG
GTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTG
AAATCCCCGGGCTCAACCTGGGAACTGCATCCGAAACTGGCAGGCTTGAGTCTTGTAGAGGGGGGTAGAATTCCAGG
TGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAG
GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGT
GCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAA
ATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAA

>Full JO1 *Klebsiela. Pneumonia*

GCAGCCTGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGGTGAGGTTAATA
ACCTCATCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGC
AAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAA
CCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGC
GTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGC
TTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCA

> FULL OMW1 *Enterobacter*

TCCTACGGGA GGCAGCAGTG GGGAATATTG CACAATGGGC GCAAGCCTGA TGCAGCCATG CCGCGTGTAT
GAAGAAGGCC TTCGGGTTGT AAAGTACTTT CAGCGGGGAG GAAGGCGTTG AGGTTAATAA *CCTCAGCGAT*
*TGACGTTACC CGCAGAAGAA GCACCGGCTA ACTCCGTGCC AGCAGCCGCG GTAATACGGA GGGTGCAAGC*
*GTTAATCGGA ATTACTGGGC GTAAAGCGCA CGCAGGCGGT CTGTCAAGTCGGATGTGAAA TCCCCGGGCT*
*CAACCTGGGA ACTGCATTCG AAACTGGCAG GCTAGAGTCT TGTAGAGGGG GGTAGAATTC CAGGTGTAGC*
*GGTGAAATGC GTAGAGATCT GGAGGAATAC CGGTGGCGAAGGCGGCCCCC TGGACAAAGA CTGACGCTCA*
*GGTGCGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG GTAGTCCACG CCGTAAACGA TGTCGACTTG*
*GAGGTTGTGC CCTTGAGGCG TGGCTTCCGG AGCTAACGCG TTAAGTCGAC CGCCTGGGGA GTACGGCCGC*
*AAGGTTAAAA CTCAAATGAA TTGACGGGGG CCCGCACAAG CGGTGGAGCA TGTGGTT*TAA TTCGATGCAA
CGCGAAGAAC CCTACA

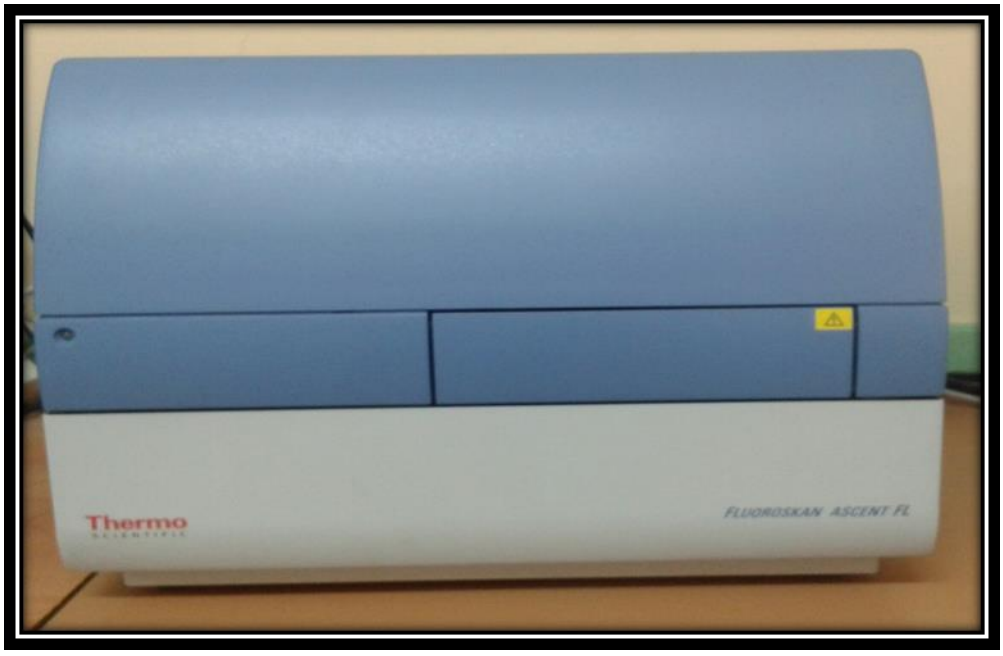
>FULL OMW2 *Enterobacter*

TCCTACGGGA GGCAGCAGTG GGGAATATTG C*ACAATGGGC GCAAGCCTGA TGCAGCCATG CCGCGTGTAT*
*GAAGAAGGCC TTCGGGTTGT AAAGTACTTT CAGCGGGGAG GAAGGCGTTG AGGTTAATAA CCTCAGCGAT*
*TGACGTTACC CGCAGAAGAA GCACCGGCTA ACTCCGTGCC AGCAGCCGCG GTAATACGGA GGGTGCAAGC*
*GTTAATCGGA ATTACTGGGC GTAAAGCGCA CGCAGGCGGT CTGTCAAGTC GGATGTGAAA TCCCCGGGCT*
*CAACCTGGGA ACTGCATTCG AAACTGGCAG GCTAGAGTCT TGTAGAGGGG GGTAGAATTC CAGGTGTAGC*
*GGTGAAATGC GTAGAGATCT GGAGGAATAC CGGTGGCGAA GGCGGCCCCC TGGACAAAGA CTGACGCTCA*
*GGTGCGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG GTAGTCCACG CCGTAAACGA TGTCGACTTG*
*GAGGTTGTGC CCTTGAGGCG TGGCTTCCGG AGCTAACGCG TTAAGTCGAC CGCCTGGGGA GTACGGCCGC*
*AAGGTTAAAA CTCAAATGAA TTGACGGGGG CCCGCACAAG CGGTGGAGCA TG*TGGTTTAA TTCGATGCAA
CGCGAAGAAC CCTAC

>FULL MO2L *Aeromonas jandaei*

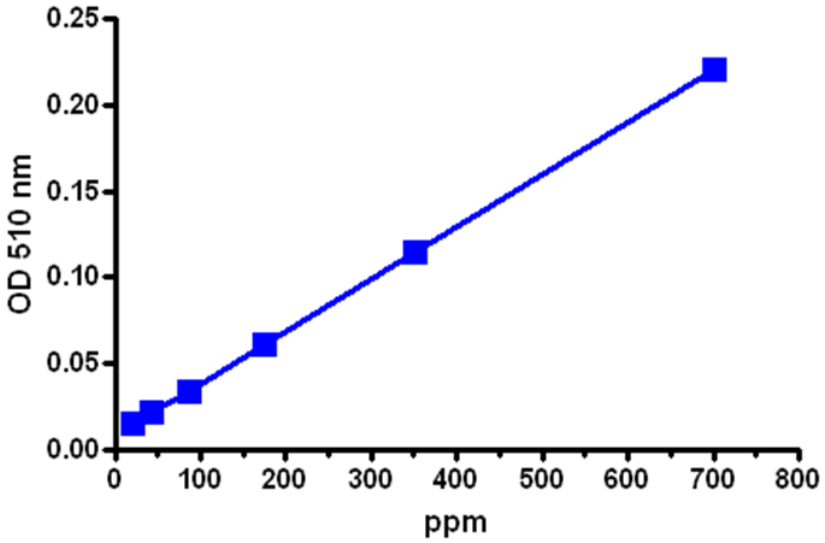
CCTACGGGA GGCAGCAGTG GGGAATATTG CACAATG*GGG GAAAC*CCTGA T*GCAGCCATG CCGCGTGTGT*
*GAAGAAGGCC TTCGGGTTGT AAAGCACTTT CAGCGAGGAG GAAAGGTCAG TAGCTAATAT CTGCCGGCTG*
*TGACGTTACT CGCAGAAGAA GCACCGGCTA ACTCCGTGCC AGCAGCCGCG GTAATACGGA GGGTGCAAGC*
*GTTAATCGGA ATTACTGGGC GTAAAGCGCA CGCAGGCGGT TGGATAAGTT AGATGTGAAA GCCCCGGGCT*
*CAACCTGGGA ATTGCATTTA AAACTGTCCA GCTAGAGTCT TGTAGAGGGG GGTAGAATTC CAGGTGTAGC*
*GGTGAAATGC GTAGAGATCT GGAGGAATAC CGGTGGCGAA GGCGGCCCCC TGGACAAAGA CTGACGCTCA*
*GGTGCGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG GTAGTCCACG CCGTAAACGA TGTCGATTTG*
*GAGGCTGTGT CCTTGAGACG TGGCTTCCGG  AGCTAACGCG TTAAATCGAC CGCCTGGGGA GTACGGCCGC*
*AAGGTTAAAA CTCAAATGAA TTGACGGGGG CCCGCACAAG CGGTGGAGCA TGTGGTTT*AA TTCGATGCAA
CGCGAAGACC CCTACA

**Appendix4 Flouroskan use for  measured of absorbance readings at 540 and 630 nm.**



**Appendix5: Stander Curve**

To establish stander curve used different phenol concentration, after Optical Densities (ODs) have been collected, resulting using this equation{y = 0.0003x + 0.0084 $R^2$ = 0.9894} is used to convert OD 510nm to part per million (ppm) as shown blow.




**Appendix 6 Spectrophotometr use for measured phenol OD(600nm &510nm).**

**Appendix 7 Cell immobilized sodium alginate beads**

**Appendix 8 Microtoxcicity Test.**

**عزل و وصف البكتيريا المحللة للفينول من نفايات معاصر الزيتون .**

**إعداد : احمد عبد الباسط جميل عبد القادر .**

**المشرف الأول : د. زياد عابدين .**

**المشرف الثاني : د. سهير عريقات .**

الملخص

**مركبات الفينول منتشره على نطاق واسع كملوثات للبيئة وذلك ببسب وجودها في النفايات السائلة في العديد من العمليات الصناعية ،وذلك يتضمن صناعة زيت الزيتون .**

**تم عزل سلالة J20 التي تستخدم الفينول كمصدر وحيد للكربون ومصدر لطاقة بنجاح من معاصر زيت الزيتون في فلسطين . استنادا إلى خصائصها المورفولوجية والبيوكيميائية، تم وصف سلالة J20 بأنها غرام-موجبة ، الهوائية، عصوية الشكل .**

**و كشف تحليل تسلسل للجين *16srRNA* أن هذه السلالة تنتمي إلى *Bacillus thuringiensis*.**

**وكانت درجة الحموضة ودرجة الحرارة المثلى لتحلل الفينول بوساطة هذا النوع من البكتيريا من سلالة J20 ( *Bacillus thuringiensis* )، 6.57 و30 درجة مئوية ، على التوالي.**

**هذه البكتيريا يمكن أن تتحلل 90.6٪ من الفينول في غضون 96 ساعة عندما كان التركيز الأولي للفينول 700 جزء في المليون. ويزداد معدل تحلل الفينول بزيادة كثافة الخلايا للبكتريا وايضا بزيادة وقت التحلل .**

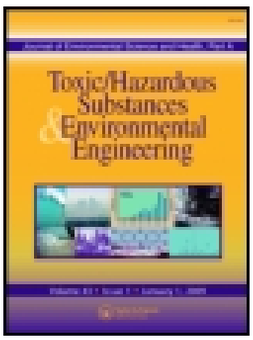
**أظهرت الدراسة أنه عندما يتم تجيمد خلايا بكتيريا سلالة J20 داخل alginate beads يكون أداها افضل في تحليل الفينول مقارنة مع الخلايا العادية من سلالة J20 عند تحليل كمية 700 جزء في المليون من الفينول.**

**حيث كانت كفاءة تحلل الفينول بواسطة الخلايا العادية من سلالة J20 أقل مقارنة مع خلايا بكتيريا من سلالة J20 التي تم تجميدها داخل alginate beads ،حيث تم تحلل 88.6% من الفينول في غضون 96 ساعة بواسطة الخلايا العادية من سلالة بينما خلايا بكتيريا سلالة J20 التي تم تجميدها داخل**

**alginate beads كانت قادرة على تحليل 97.2% من الفينول ،ومن نفس الكمية من الفينول في غضون 96 ساعة ،وهذا ربما بسبب حماية البكتيريا داخل alginate beads.**

**علاوه على ذلك كان تأثير وقت التخزين على تحلل الفينول بواسطة خلايا بكتيريا من سلالة J20 التي تم تجميدها داخل alginate beads تم تخزينها على 4 درجات مئوية ،ولمده 35 يوم ، مقارنة مع خلايا تم تجميدها داخل alginate beads ولكن بدون تخزين .حيث تم تحلل 97.2 % من الفينول بواسطة البكتيريا الجديدة المجمدة داخل alginate beads،بينما تم تحلل 72% من الفينول بواسطة الخلايا التي تم تخزينها لمدة 35 يوم على 4 درجات مئوية .**

**بالتالي البكتيريا التي تم عزلها من سلالة J20 ( *Bacillus thuringiensis* )، قادرة على تحليل الفينول بشكل فعال . وبالتالي يمكن توفير طريقة واعدة واقتصادية للحد من مركبات الفينول الملوثة للبيئة .**



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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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**KEYWORDS**
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 $\mu$m 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 $\mu$L 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 $\mu$L 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 $\leq 0.05$.

## 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

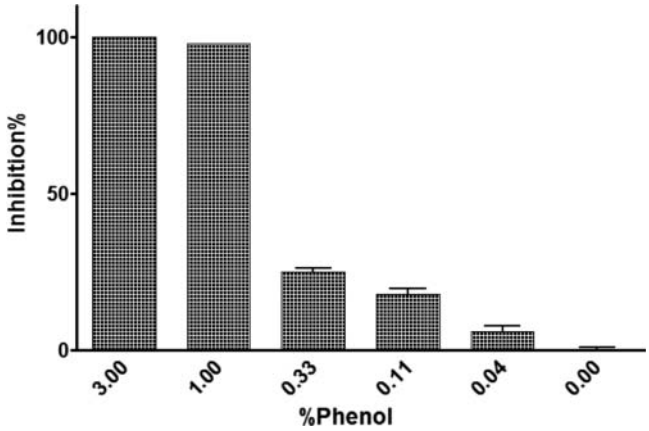


**Figure 1.** Phenol tolerance of the isolated bacteria J20 cultured in different concentrations of phenol. The percent inhibition of Alamar Blue reduction is plotted against phenol concentration.

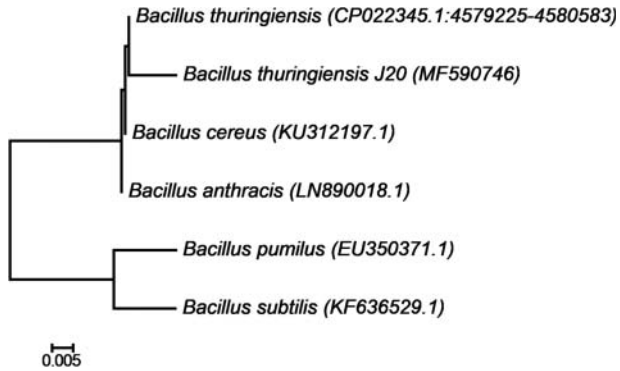


**Figure 2.** Phylogenetic analysis based on the 16SrRNA gene sequences of strain J20 and related bacterial strains imported from the GenBank database. Phylogenetic tree was constructed by the neighbor joining method with bootstrap of 1,000 replications using Mega 7 program.

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

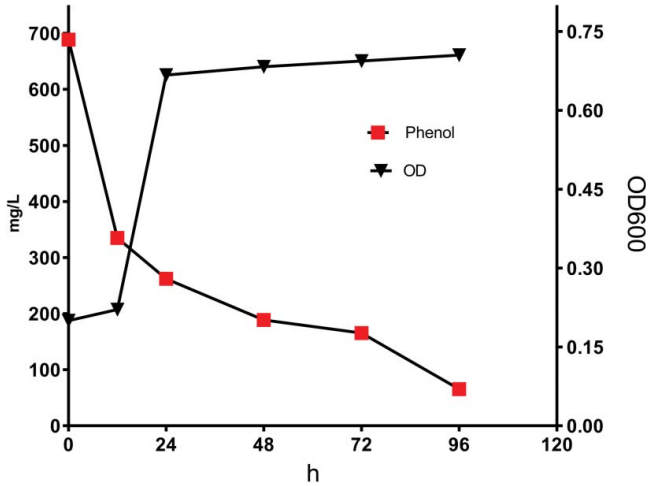


**Figure 3.** Growth curve of *Bacillus thuringiensis*-J20 and phenol degradation at 700 mg/L in MSM medium as function of time. $OD_{600}$; optical density measured at a wavelength of 600 nm.

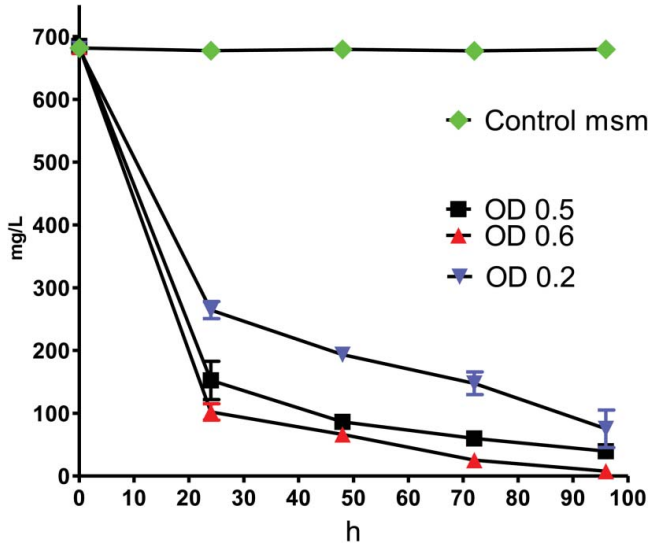


**Figure 4.** Effect of three inoculation sizes (0.2, 0.5 and 0.6) on the biodegradation of 700 mg/L phenol by *Bacillus thuringiensis*-J20.

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-

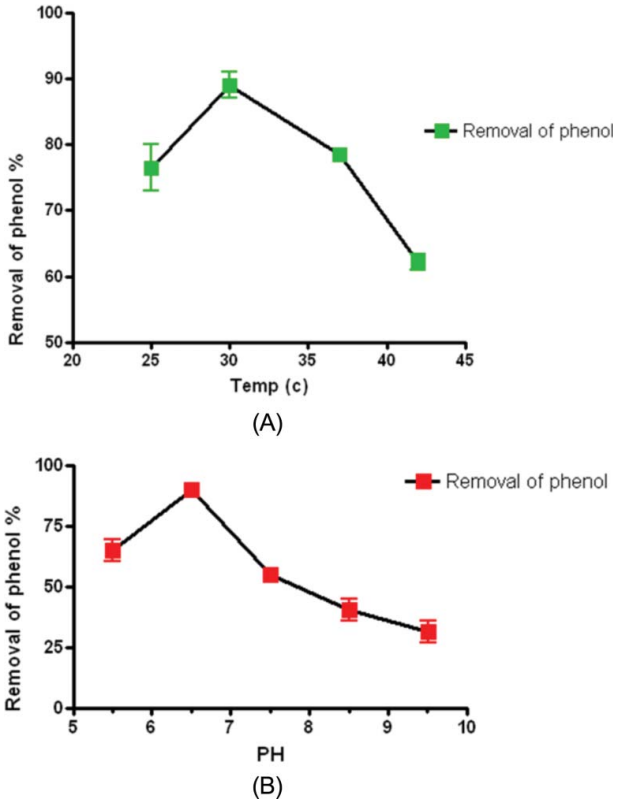


**Figure 5.** Effect of pH and temperature on phenol biodegradation at initial phenol concentration of 700 mg/L. (A) Effect of temperature on the removal of phenol; (B) Effect of pH on the removal of phenol.

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

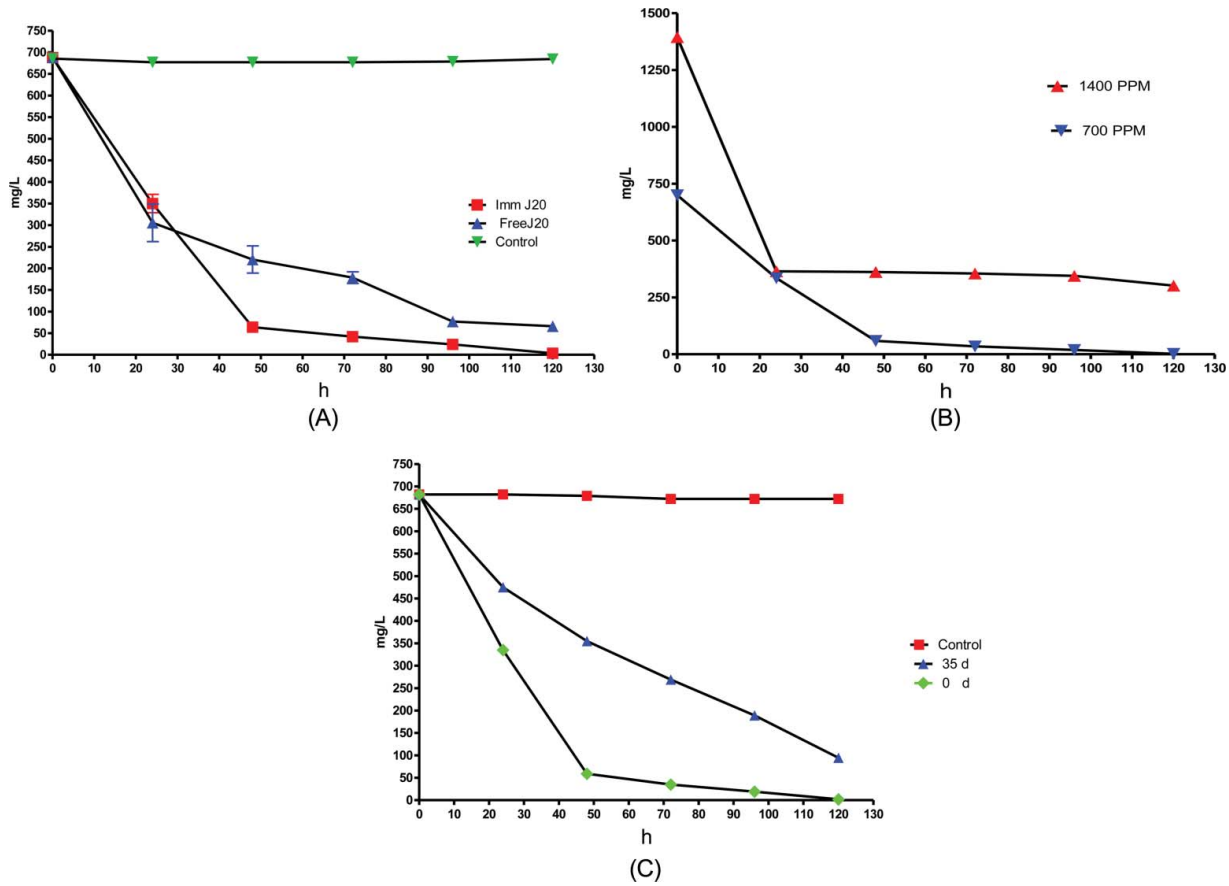


**Figure 6.** (A) Phenol degradation profiles of free and alginate-immobilized J20 at phenol concentration of 700 mg/L. (B) Phenol degradation profiles of alginate-immobilized J20 at two different initial phenol concentrations. (C) Phenol degradation by alginate-immobilized J20 at 700 mg/L, pre-storage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

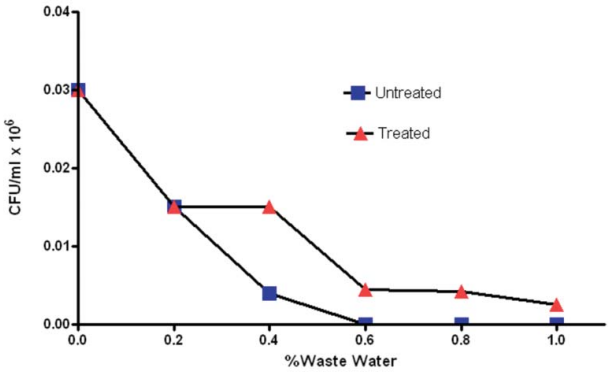


Figure 7. Growth curves of *E. Coli* (ATCC 25922) in the presence of different percentages of J20-treated and untreated olive mill waste water (OMWW) serially-diluted in LB medium.

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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## References

[1] Rouvalis, A.; Iliopoulou–Georgudaki, J. Comparative Assessment of Olive Oil Mill Effluents From Three-Phase And Two-Phase Systems, Treated for Hydrogen Production. *Bull. Environ. Contam. Toxicol.* **2010**, *85*(4), 432–436.
[2] Asfi, M.; Ouzounidou, G.; Moustakas, M. Evaluation of Olive Oil Mill Wastewater Toxicity on Spinach. *Environ. Sci. Pollut. Res.* **2012**, *19*(6), 2363–2371.
[3] UN. *The Olive Harvest in the West Bank and Gaza*. United Nations Development Programme, World Food Programme, UN Office for the Coordination of Humanitarian Affairs, The World Bank, **2008**.
[4] Coskun, T.; Debik, E.; Demir, N. M. Treatment of Olive Mill Wastewaters by Nanofiltration and Reverse Osmosis Membranes. *Desalination* **2010**, *259*(1), 65–70.
[5] Borja, R.; Alba, J.; Banks, C. J. Impact of the Main Phenolic Compounds of Olive Mill Wastewater (OMW) on the Kinetics of Acetoclastic Methanogenesis. *Process Biochem.* **1997**, *32*(2), 121–133.
[6] Keith, L.; Telliard, W., ES&T Special Report: Priority Pollutants: I-a Perspective View. *Environ. Sci. Technol.* **1979**, *13*(4), 416–423.
[7] Hooived, M.; Heederik, D. J.; Kogevinas, M.; Boffetta, P.; Needham, L. L.; Patterson, D. G.; Bueno-de-Mesquita, H. B. Second Follow-Up of a Dutch Cohort Occupationally Exposed to Phenoxy Herbicides, Chlorophenols, and Contaminants. *Am. J. Epidemiol.* **1998**, *147*(9), 891–899.
[8] Nassar, N. N.; Arar, L. A.; Marei, N. N.; Ghanim, M. M. A.; Dwekat, M. S.; Sawalha, S. H. Treatment of olive Mill Based Wastewater by Means Of Magnetic Nanoparticles: Decolourization, Dephenolization and COD Removal. *Environ. Nanotechnol., Monit. Manage.* **2014**, *1*, 14–23.
[9] Lakshmi, M. C.; Sridevi, V.; Neharika, E.; Beena, C.; Rao, M. N.; Swamy, A. Effect of Temperature and Carbon Source on Phenol Degradation by *Pseudomonas aeruginosa* (NCIM 2074) and *Pseudomonas desmolyticum* (NCIM 2028) and Their Comparison. *Int. J. Chem. Sci.* **2009**, *7*(4), 2591–2601.
[10] Marrot, B.; Barrios-Martinez, A.; Moulin, P.; Roche, N. Biodegradation of High Phenol Concentration by Activated Sludge in an Immersed Membrane Bioreactor. *Biochem. Eng. J.* **2006**, *30*(2), 174–183.
[11] Narayan, A.; Pushpa, A. Enzyme Based Processes for Removal of Phenol From Waste Water: Current Status and Future Challenges. *J. Environ. Res. Dev.* **2012**, *7*, 724–728.
[12] Nazari, K.; Esmaeili, N.; Mahmoudi, A.; Rahimi, H.; Moosavi-Movahedi, A. Peroxidative Phenol Removal From Aqueous Solutions Using Activated Peroxidase Biocatalyst. *Enzyme Microb. Technol.* **2007**, *41*(3), 226–233.
[13] Chakraborty, S.; Bhattacharya, T.; Patel, T.; Tiwari, K. Biodegradation of Phenol by Native Microorganisms Isolated From Coke Processing Wastewater. *J. Environ. Biol.* **2010**, *31*(3), 293–6.
[14] Reshma, J.; Thanga, V. S. G.; Mathew, A. Phenol Degradation by Bacteria Isolated From Coir Retting Beds of South Kerala Coast. *J. Global Biosci.* **2014**, *3*(6), 935–940.
[15] Karimi, M.; Hassanshahian, M. Isolation and Characterization of Phenol Degrading Yeasts From Wastewater in the Coking Plant of Zarand, Kerman. *Braz. J. Microbiol.* **2016**, *47*(1), 18–24.
[16] Zhang, Y.; Lu, D.; Ju, T.; Wang, L.; Lin, S.; Zhao, Y.; Wang, C.; He, H.; Du, Y. Biodegradation of Phenol Using *Bacillus cereus* WJ1 and Evaluation of Degradation Efficiency Based on a Graphene-Modified Electrode. *Int. J. Electochem. Sci.* **2013**, *8*, 504–519.
[17] Luckarift, H. R.; Sizemore, S. R.; Farrington, K. E.; Fulmer, P. A.; Biffinger, J. C.; Nadeau, L. J.; Johnson, G. R. Biodegradation of Medium Chain Hydrocarbons by *Acinetobacter venetianus* 2AW Immobilized to Hair-Based Adsorbent Mats. *Biotechnol. Progress* **2011**, *27*(6), 1580–1587.
[18] Liu, Z.; Xie, W.; Li, D.; Peng, Y.; Li, Z.; Liu., S. Biodegradation of Phenol by Bacterium Strain *Acinetobacter calcoaceticus* PA Isolated from Phenolic Wastewater. *Int. J. Environ. Res. Public Health* **2016**, *13*(3), 300.
[19] Annadurai, G.; Balan, S. M.; Murugesan, T. Design of Experiments in the Biodegradation of Phenol Using Immobilized *Pseudomonas pictorum* (NICM-2077) on Activated Carbon. *Bioprocess Eng.* **2000**, *22*(2), 101–107.
[20] Ehrhardt, H.; Rehm, H. Phenol Degradation by Microorganisms Adsorbed on Activated Carbon. *Appl. Microbiol. Biotechnol.* **1985**, *21*(1–2), 32–36.

[21] Namane, A.; Amrouche, F.; Hamdi, H.; Ali, O.; Hank, D.; Hellal, A. Biodegradation of Phenolic Wastewaters. *Environ. Eng. Manage. J.* **2013**, *13*(1), 1–6.
[22] Hall, V.; O'Neill, G.; Magee, J.; Duerden, B. Development of Amplified 16S Ribosomal DNA Restriction Analysis for Identification of *Actinomyces* Species and Comparison With Pyrolysis-Mass Spectrometry and Conventional Biochemical Tests. *J. Clin. Microbiol.* **1999**, *37*(7), 2255–2261.
[23] Der Yang, R.; Humphrey, A. E. Dynamic and Steady State Studies of Phenol Biodegradation in Pure and Mixed Cultures. *Biotechnol. Bioeng.* **1975**, *17*(8), 1211–1235.
[24] Banerjee, A.; Ghoshal, A. K. Phenol Degradation Performance by Isolated *Bacillus Cereus* Immobilized in Alginate. *Int. Biodeterior. Biodegradation* **2011**, *65*(7), 1052–1060.
[25] Zeinat Kamal, M.; Nashwa, A.; Mohamed, A.I.; Sherif, E. N. Biodegradation and Detoxification of Malathion by of *Bacillus thuringiensis* MOS-5. *Aus. J. Basic Appl. Sci*. **2008**, *2*, 724–732.
[26] Das, P.; Sinha, S.; Mukherjee, S. K. Nickel Bioremediation Potential of *Bacillus thuringiensis* KUNi1 and Some Environmental Factors in Nickel Removal. *Biorem. J.* **2014**, *18*, 169–177.
[27] Das, N.; Chandran, P. Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview. *Biotechnol. Res. Int*. **2010**, *2011*, 1–13.
[28] Kaflizadeh, F.; Mokhtari, S. Isolation and Identification of Phenol Degrading Bacteria From Mangrove Sediments in the Persian gulf (Asaluyeh) and Their Growth Kinetics Assay. *Biomed. Pharmacol. J.* **2013**, *6*(2), 189–196.
[29] Silva, Í. S.; dos Santos, E. d. C.; de Menezes, C. R.; de Faria, A. F.; Franciscon, E.; Grossman, M.; Durrant, L. R. Bioremediation of a Polyaromatic Hydrocarbon Contaminated Soil by Native Soil Microbiota and Bioaugmentation with Isolated Microbial Consortia. *Bioresour. Technol.* **2009**, *100*(20), 4669–4675.
[30] Godjevargova, T.; Ivanova, D.; Alexieva, Z.; Dimova, N. Biodegradation of Toxic Organic Components From Industrial Phenol Production Waste Waters by Free and Immobilized *Trichosporon cutaneum* R57. *Process Biochem*. **2003**, *38*(6), 915–920.
[31] Shawabkeh, R.; Khleifat, K. M.; Al-Majali, I.; Tarawneh, K. Rate of Biodegradation of Phenol by *Klebsiella oxytoca* in Minimal Medium and Nutrient Broth Conditions. *Biorem. J*. **2007**, *11*(1), 13–19.
[32] Monteiro, Á. A.; Boaventura, R. A.; Rodrigues, A. R. E. Phenol Biodegradation by *Pseudomonas putida* DSM 548 in a Batch Reactor. *Biochem. Eng. J*. **2000**, *6*(1), 45–49.
[33] Mollaei, M.; Abdollahpour, S.; Atashgahi, S.; Abbasi, H.; Masoomi, F.; Rad, I.; Lotfi, A. S.; Zahiri, H. S.; Vali, H.; Noghabi, K. A. Enhanced Phenol Degradation by *Pseudomonas* sp. SA01: Gaining Insight into the Novel Single and Hybrid Immobilizations. *J. Hazard. Mater*. **2010**, *175*(1), 284–292.
[34] El-Naas, M. H.; Al-Muhtaseb, S. A.; Makhlouf, S. Biodegradation of Phenol by *Pseudomonas putida* Immobilized in Polyvinyl Alcohol (PVA) Gel. *J. Hazard. Mater*. **2009**, *164*(2), 720–725.
[35] Paixao, S.; Mendonça, E.; Picado, A.; Anselmo, A. Acute Toxicity Evaluation of Olive Oil Mill Wastewaters: A Comparative Study of Three Aquatic Organisms. *Environ. Toxicol*. **1999**, *14*(2), 263–269.
[36] Letnik, I.; Avrahami, R.; Rokem, J.S.; Greiner, A.; Zussman, E.; Greenblatt, C. Living Composites of Electrospun Yeast Cells for Bioremediation and Ethanol Production. *Biomacromolecules* **2015**, *16*, 3322–3328.
[37] Leouifoudi, I.; Zyad, A.; Amechrouq, A.; Oukerrou, M. A.; Mouse, H. A.; Mbarki, M. Identification and Characterisation of Phenolic Compounds Extracted From Moroccan Olive Mill Wastewater. *Food Sci. Technol (Campinas)* **2014**, *34*(2), 249–257.
[38] Dragičević, T.; Hren, M.; Gmajnić, M.; Pelko, S.; Kungulovski, D.; Kungulovski, I.; Čvek, D.; Frece, J.; Markov, K.; Delaš, F. Biodegradation of Olive Mill Wastewater by *Trichosporon cutaneum* and *Geotrichum candidum*. *Arch. Ind. Hyg. Toxicol*. **2010**, *61*, 399–405.