

**Bioremediation of Olive Mill Wastewater (Zibar) using
Phanerochaete chrysosporium and Potential use in Agriculture**

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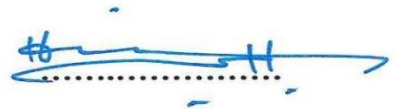
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Dedication

I dedicate this achievement to my beloved family, thank you to my parents and my brothers all by their names and value in my heart, Mohamad, Ahmad, Watheq, Amar, Emran and Ayham, and my beloved aunt Maram Abo-Amer, who have always given me love and their moral, spiritual, emotional and financial support.

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List of Abbreviations

1	Ph. <i>chrysosporium</i>	<i>Phanerochaete chrysosporium</i>
2	T Zibar	Zibar treated with OMWW2 for 2 weeks
3	COD	Chemical Oxygen Demand
4	WRF	White Rot Fungi
5	BOD	Biochemical Oxygen Demand
6	TSM	Total Suspended Matter
7	EC	Electrical Conductivity
8	TA	Tannic Acid
9	PDA	Potato Dextrose Agar
10	LME	Lignin Modifying Enzyme
11	KARC	Kadoorie Agriculture Research Center
12	PCR	Polymerase chain Reaction

Bioremediation of Olive Mill Wastewater (Zibar) using *Phanerochaete chrysosporium* and Possible Use in Agriculture

By: Sajida Iwissat

Supervised by: Dr. Mazen Salman

Summary

The manufacturing process of olive oil yields a black liquid waste called ‘olive mill wastewater’ (Zibar) creating a major environmental problem. Due to high levels of phytotoxic and antimicrobial compounds such as monomeric-polymeric phenols, volatile acids and polyalcohol, Zibar is toxic to plants and soil micro flora and can affect the soil quality. Proposed physio-chemical processes such as evaporation ponds or lagoons have not been efficient in decreasing the high toxicity of Zibar to reduce the ecological impact of Zibar, due to economic and technical reasons. Bioremediation using microorganisms is considered an environmentally compatible and least expensive alternative. In this work, four fungal isolates were tested for their efficacy in reducing the total polyphenols from Zibar. Erlenmeyer flasks (125 ml) each containing 25 ml of Zibar were inoculated each with 5 PDA discs (7 mm grown with the fungi). The flasks were placed on a rotary shaker at 150 rpm for two weeks, at optimum temperature for each isolate. Before extraction of total polyphenols, fungal biomass was

removed by filtration. After that, 2.5 ml of treated Zibar was diluted to 50% with distilled water, acidified to pH 2.0 with 5M HCl and extracted with ethyl acetate (1:1, v/v), which was evaporated under fuming hood for 24h. The residue was dissolved in 2.5 ml methanol and the volume was completed to 25 ml with distilled water. Total phenol concentration was determined spectrophotometrically at 725nm using Folin reagent. Results of this work showed that all fungal isolates were able to grow on Zibar. However, only one isolate showed significant reduction of total phenols. The concentration of phenols in the presence of isolate OMWW2 was 57.75 mg/ml compared to 159.27 mg/ml in the control untreated water. In addition to obtain percent of germination approximately 83% in 100% T Zibar, with no significant differences in germination percent compared with water-soaked seeds as control. While the germination percent in untreated Zibar was 0%. This isolate was identified by sequencing as *Paecilomyces* sp., F-BTUL-E1 isolate, in Eurotiomycetes class of fungi.

1 Introduction

1.1 General Introduction

The cultivation of olive trees and the production of olive oil have been known and affirmed practice in the Mediterranean region for more than 7000 years (Tsagaraki *et al.*, 2007). Olive cultivation and olive oil production are a part of the local heritage and rural economy throughout the Mediterranean regions (Paraskeva *et al.*, 2007). Each olive tree produces between 15 and 40 kg of olives per year. In 2012, approximately 2,903,676 tons of olive oil was produced worldwide (Hansen, 2014). Mediterranean countries alone produce about 98% of the total olive oil production. The global production of olive oil is currently estimated to be around 2.5 million metric tons annually.

The manufacturing process of olive oil yields a black liquid waste called ‘olive mill wastewater’ (called Zibar in Palestine) that consists of vegetation water and water used in the various stages of the oil extraction process (Petrotos *et al.*, 2014). Around 10 to 30 million m³ of Olive Mill Waste Water are annually produced worldwide (McNamara *et al.*, 2008; Hansen, 2014). The production of the huge amounts of the Zibar has a significant environmental impact (McNamara *et al.*, 2008; Rengaraj *et al.*, 2002). While

Zibar contains plant nutrients such as macronutrients including N, P, K, Ca and Mg and organic matter, it can result in the accumulation of phytotoxic compounds and salts in soil and can potentially contaminate aquifers (Santi *et al.*, 2008). Zibar can also have a deleterious effect on soil porosity and pH (Anastasiou *et al.*, 2011; McNamara *et al.*, 2008). Untreated Zibar can alter the microbial composition of the soil through their antibacterial activity, therefore prevents its use in agriculture (Laconi *et al.*, 2007; El-Hadrami *et al.* 2004; Barakat *et al.*, 2010).

The safe disposal of this waste is of serious environmental concern, because this wastewater can't be sent to ordinary wastewater treatment systems. Moreover, due to its complex compounds, Zibar is recalcitrant and needs to be detoxified before it can be used in agricultural and other industrial processes (Hansen, 2014).

Zibar is often disposed in sewage or dispersed into the soil, causing water and soil pollution (Laconi *et al.*, 2007). Phytotoxic and antimicrobial properties of Zibar have been associated with monomeric phenols.

Moreover, the dark color of Zibar attributes to the polymerization of tannins and low molecular weight of phenolic compounds (Amaral *et al.*, 2012).

Due to high levels of phytotoxic and antimicrobial compounds such as monomeric-polymeric phenols, volatile acids and polyalcohol, Zibar is inappropriate for using in irrigation and fertilizing purposes in agriculture (Barakat *et al.*, 2010).

Different physical and chemical methods have been developed for phenol degradation in Zibar. However, most of these methods are costly and inefficient in decreasing the high toxicity of Zibar and reduce its ecological impact. In addition to that, these methods might produce other toxic byproducts and do not alleviate the toxicity or high chemical oxygen demand (COD) of Zibar (McNamara *et al.*, 2008; Rengaraj *et al.*, 2002).

Bioremediation is considered the most environmentally compatible and least expensive methods (Mantzavinos and Kalogerakis, 2005). The process depends on using microorganisms to clean up contaminated soil and groundwater by degrading or transforming hazardous chemical contaminants to less toxic compounds (Arun *et al.*, 2008). Significant reduction of phenolic compounds by effective bioremediation process allows safe and

economical disposal and uses of Zibar onto land or into surface waters (Laconi *et al.*, 2007). As another benefit, bioremediation may produce valuable products including an excellent fertilizer (Aytar *et al.*, 2011). Several studies indicated that the white rot fungi are amongst the most popular microbes known to degrade phenolic compounds, lignin and lignin-like compounds to carbon dioxide and water (Salman *et al.*, 2014; Ahmadi *et al.*, 2006).

In Palestine, olive production is the backbone of the Palestinian agriculture. It contributes to the economic and social well-being of Palestinian households. There were 295 olive presses in Palestine in 2016, and the total quantity of olives pressed in 2016 was 841476 tons with an extraction rate of olive presses in Palestine was 23.9% producing about 274 thousand cubic meter Zibar. The majority of Zibar (44.3%) is disposed in Tight Cesspit. Zibar also spread to land and discharges into surface waters, which affect the chemical and physical properties of the soil. (Palestinian Central Bureau of Statistics, 2017). The aim of this work was to search for economical and environmentally safe methods to detoxify Zibar.

1.2 Objectives

- Isolation and identification of local Palestinian *Ph. chrysosporium* and other fungal isolates for Zibar detoxification.
- Determination of optimal condition (e.g. pH, temperature, fungal dose. *etc.*) for Zibar treatment.
- Determination of the chemical content of treated Zibar (COD, pH, EC and polyphenols).
- Studying the potential use of treated Zibar as liquid fertilizer and its impact on plant growth (tomato was chosen as a model plant).
- Evaluation of the effect of treated Zibar on soil microflora (colony-forming unit (CFU) in the soil).

2 Literature Review

2.1 Zibar Characteristics

The quantitative and qualitative composition of Zibar is variable due to several reasons including climatic conditions, olive variety, ripeness of olives, use of pesticides and fertilizers, and extraction processes (Santi *et al.*, 2008). Zibar contains some of organic constituents such as phenolic compounds, sugars, and some organic acids. The most common sugars in Zibar are fructose, mannose, glucose, saccharose, sucrose, and some pentoses (Niaounakis and Halvadakis, 2006). Zibar also contains inorganic compounds such as potassium (~ 4 g=L) magnesium, nitrogen, calcium, phosphorous and iron. Depending on the extraction process, the main anions are Cl, F, PO₄, and SO₄. The composition of Zibar, as reported by a number of authors, is summarized in Table (1).

Table 1. Influence of the production process on Zibar composition.

Parameter	Press process	3-phase	2-phase	Reference
L Zibar/ olives		900-1,500	50-70	Aktas <i>et al.</i> (2001)
		500-1,500		Rozzi and Malpei (1996)
		500-1,400		Sierra <i>et al.</i> (2001)
	400	1,000		Aragon and Karagouni (2000)
	400-600	1,000-1,200	85-110	Caputo <i>et al.</i> (2003)
(%of olives weight)	50	80-110		Mulinacci <i>et al.</i> (2001)
pH	4.5-5	4.7-5.2		Azbar <i>et al.</i> (2004)
	4.5±0.3	4.8±0.3		Aktas <i>et al.</i> (2001)
	4.5-5	4.5-5		Caputo <i>et al.</i> (2003)
COD (g/L)	120-130	40		Azbar <i>et al.</i> (2004)
	65.7±27.1	103.4±19.5	5-25	Aktas <i>et al.</i> (2001)
	125	50		Caputo <i>et al.</i> (2003)
BOD (g/L)	90-100	33		Azbar <i>et al.</i> (2004)
	90	40		Caputo <i>et al.</i> (2003)
TSM % (g/L)	0.1	0.9		Azbar <i>et al.</i> (2004)
	0.1	0.9		Caputo <i>et al.</i> (2003)
	2.7±1.1	27.6±5.1		Aktas <i>et al.</i> (2001)
VSS (%) (g/L)	10.5	2.6		Azbar <i>et al.</i> (2004)
	2.5±1.1	24.5±5		Aktas <i>et al.</i> (2001)
TS (%) (g/L)	12	3		Azbar <i>et al.</i> (2004)
	44.4±13.8	78.2±13.6		Aktas <i>et al.</i> (2001)
Sugars (%) (g/L)	2-8	1		Azbar <i>et al.</i> (2004)
	2.2±1.7	4.7±1.8		Aktas <i>et al.</i> (2001)
Total N (%) (mg/L)	5-2	0.28		Azbar <i>et al.</i> (2004)
	1.8	0.3		Caputo <i>et al.</i> (2003)
	43.7±33.9	78.8±39.6		Aktas <i>et al.</i> (2001)
Polyphenols (%)	1-2.4	0.5		Azbar <i>et al.</i> (2004)
	1.7	0.63		Caputo <i>et al.</i> (2003)

There are more than 30 different phenolic compounds have been detected in large quantities Zibar. These compounds divided into Phenolic monomers, flavonoids, not autoxidated tannins, and other compounds with $MW \leq$

10kDa and Medium and high MW (MW>10kDa) dark colored polymers resulting from the polymerization and autoxidation of phenolic compounds of the first group. The color of Zibar depends on the ratio between these two groups (Lesage-Meesen et al., 2001).

During the olive oil extraction process, the separation of oil from water is different and the major proportion of these compounds goes to Zibar aqueous phase from the olive pulp, due to the chemical characteristics of polyphenols that are water-soluble. Zibar phenolic content varies depending on several factors, including type of olive, stage of maturity, and most important type of production process. (Lesage-Meesen *et al.*, 2001).

Some Zibar constituents such as Hydroxytyrosol, 2-hydroxytyrosol, tyrosol, oleanolic acid, and maslinic acid, flavonoids, anthocyanins, and tannins, are considered as natural antioxidants with considerable commercial and economic interest. Hydroxytyrosol that is found in Zibar acts against both gram negative and gram positive bacteria. It could be used as a food preservative, in agriculture for the protection of olive trees, and in cosmetics industry in antiaging preparations (Allouche *et al.*, 2004; Visioli *et al.*, 1999).

2.2 Zibar and the environment

In terms of pollution effect, 1m³ of Zibar is equivalent to 100-200 m³ of domestic sewage water. In addition to its characteristics mentioned in Table (1), Zibar has a strong offensive smell and color, which prohibit its direct discharge into fresh and coastal waters or onto land. Uncontrolled disposal of Zibar into the environment (Figure1) and water bodies leads to severe problems for the whole ecosystem and especially for the natural water bodies (Fiorentino *et al.*, 2004). Zibar also has an abundant content of phosphorus, reduced sugars, high phenolic load that has a toxic effect on many organisms. Some aquatic organisms become severely poisoned even at exposures corresponding to one liter of untreated Zibar into 100,000 liters of circulating water. Some microorganisms that metabolize sugars develop more rapidly at the expense of other living organisms. It also accelerates algal growth due to the high phosphorus content resulting in eutrophication (Fiorentino *et al.*, 2004).



Figure 1. Discharging of Zibar in the environment (Source: <http://www.prosodol.gr>).

Soil physical and chemical properties such as porosity and pH are affected as a result of discharging of Zibar directly into soil (Niaounakis and Halvadakis, 2006). Zibar contains high concentration of potassium which affects the cation exchange capacity of the soil, leading to change of environmental conditions for soil microorganisms and consequently to changes in the fertility of the soil. Other possible negative effects of Zibar include the immobilization of available nitrogen and decreased available magnesium, perhaps because of the antagonistic effect on potassium (Niaounakis and Halvadakis, 2006).

Phenolic content and some organic acids in Zibar are the causes of plant toxicity and antimicrobial activity. Direct application of Zibar on plants inhibits the germination of different seeds and early plant growth of different vegetable species and may cause fruit and leaf abscission. However,

different reactions are shown by different types of crops in response to Zibar and some crops may tolerate certain amount of Zibar during early growing stages (Rinaldi *et al.*, 2003).

2.3 Treatment Options of Zibar

Several methods have been proposed to solve the Zibar problems, such as physicochemical treatments (e.g. precipitation/flocculation, ultrafiltration and reverse osmosis, adsorption, chemical oxidation processes and ion exchange), thermal processes (combustion and pyrolysis), agronomic applications (e.g. land spreading), extraction of valuable compounds (e.g. antioxidants, residual oil, sugars), animal-breeding methods (e.g. direct utilization as animal feed or following protein enrichment) and biological treatments which considered as the most environmentally compatible and the least expensive methods (Mantzavinos and Kalogerakis, 2005; Niaounakis and Halvadakis, 2006).

These processes aim at “cleaning” the waste to allow its safe, subsequent disposal in water or soil reservoirs. Two different approaches have been developed for Zibar biological treatment: aerobic and anaerobic processes (Assas *et al.*, 2002). In contrast to, some of negative results are observed in the anaerobic conditions such as namely the difficulty of removing phenols

with high molecular weight, the need for a long period for the adaptation of microorganisms and the high costs for the storage (Assas *et al.*, 2002; Marques, 2001).

Early studies focused on the use of specific bacterial species, including *Bacillus pumilus*, *Azotobacter chroococcum*, *Azotobacter vinelandii*, *Arthrobacter sp.*, *Pseudomonas putida*, *Ralstonia sp.* and different bacterial consortia. In general, aerobic bacteria appeared to be very effective against some low-molecular-mass phenolic compounds but are relatively ineffective against the more complex polyphenolics (McNamara *et al.*, 2008). Several strains of filamentous fungi have shown interesting capacities for the removal of toxic Zibar compounds. A variety of white-rot fungi have been used including *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleurotus spp.*, *Funalia trogii* and *Lentinus edodes*. According to a recent review, fungi - including white rot fungi - are more effective than bacteria for the degradation of phenols in Zibar (Morillo *et al.*, 2009). The high effectiveness of fungi relies upon the structure of the aromatic compounds present in Zibar, which are similar to that of many lignin monomers, and only a few microorganisms, mainly white rot fungi, are able to degrade lignin efficiently by producing ligninolytic enzymes such as lignin peroxidases, manganese peroxidases and laccases (Morillo *et al.*, 2009).

2.4 Bioremediation of Zibar using filamentous fungi

Most studies related to Zibar bioremediation focus on the use of filamentous fungi with the capability to degrade the toxic phenolic fraction of Zibar. These are mostly fungi that produce Lignin modifying enzyme (LMEs), which mediate the oxidation of phenolic compounds (Morillo *et al.*, 2009).

Bioremediation techniques for Zibar include aerobic and anaerobic digestion. Anaerobic digestion is carried out by a series of anaerobic microorganisms, mainly bacteria, in the absence of molecular oxygen. These microorganisms have lower growth rates than aerobic microorganisms (Hamdi, 1996). Anaerobic digestion of Zibar proceeds in three phases. During the hydrolytic phase, complex organic materials are hydrolyzed and subsequently used as substrate by acidogenic microorganisms. In the acidogenic phase, volatile fatty acids, H₂ and CO₂ are produced. These are subsequently substrates for methanogenic bacteria. During the methanogenic phase, methane is produced either by decarboxylation of acetic acid or from the reduction of H₂ and CO₂ (Fadil *et al.*, 2003). The method has low energy requirements, produces less sludge and allows for energy recovery, as, during the final stage, methane gas is produced which can be used for energy production (Hamdi, 1996). However, phenolics removal is often unsatisfactory (Di Gioia *et al.*, 2002) and the antimicrobial properties related

with these compounds retard the treatment process (Fakharedine *et al.*, 2006), due to anaerobic consortia inhibition by the Zibar phenolic fraction (McNamara *et al.*, 2008).

With aerobic treatment, microorganisms convert organics into CO₂ in the presence of oxygen. Aerobic microorganism often-White rot fungi (WRF)-require oxygen so the air must be continuously circulated. Aerobic systems can act as stand-alone systems, or polish anaerobically pretreated wastewater by further removing. Although aerobic systems require higher amounts of energy for aeration and produce more sludge than anaerobic systems, which must be disposed of, they play a necessary role in the Zibar treatment (Hamdi 1996). Many researchers, such as Salman *et al.* (2014) have used *Ph. chrysosporium* in Zibar bioremediation. Where *Ph. chrysosporium* considered as the model white rot fungus, due to its specialized ability to degrade the abundant aromatic polymer lignin, while leaving the white cellulose nearly untouched. To break-up the complex three-dimensional structure of lignin into components that can be utilized by its metabolism, *Ph. chrysosporium* releases non-specific oxidizing agents extracellular enzymes (hydrogen peroxide, hydroxyl radicals) used to cleave the lignin bonds. *Ph. chrysosporium* specialized degradation abilities, directed extensive research toward understanding the mechanism in order to enhance

the bioremediation of a diverse range of pollutants. Therefore, *Ph. chrysosporium* is the first member of the Basidiomycetes to have its complete genome sequenced (Martinez *et al.*, 2004). The *Ph. chrysosporium* fungus is sustainable at moderate to higher temperatures, specifically 40°C. A main role of *Ph. chrysosporium* assumes is that of degradation of the complex lignin from various trees and plants. This process reduces lignin into less complex molecules, maintaining the cycle of the decomposer of plants (Janusz *et al.*, 2017).

Lignin biodegradation involves both depolymerization and aromatic ring cleavage. Extracellular enzyme brought about oxidation of lignin by Oxidation of B–O–4 linkages to arylglycerol compounds. Then cleavage the aromatic rings, mostly follows the B–keto adipate pathway. Finally, Cleaved aromatic rings coupled with B–O–4 oxidation leads to the formation of cyclic carbonate structures. The lignin first breaks down into smaller constituents, and later, these small constituents recombine to form more complex organic molecules (Janusz *et al.*, 2017)..

2.5 Production of Fertilizers

Zibar may be considered as an inexpensive source of organic and inorganic compounds to be recovered because of their potential economic interest or their ability to be transformed into products for use in biotechnology, agriculture, and the pharmaceuticals industry as well as in the food industry. Where its use in many processes that aim at the production of various products (e.g. recovery of antioxidants, composting, production of biopolymers, production of biogas and production of animal feed) (Fiorentino *et al.*, 2004).

Because of its phytotoxic properties, Zibar should not be directly applied on soil and crops. But with certain treatment it could be converted into a useful fertilizer and soil conditioner, due to its high content of organic matter, water, and plant nutrients (Chatjipavlidis *et al.*, 1996). In this study, the aerobic bioremediation process is adopted by using different fungal isolates including indigenous isolates, to reduce the phenols, phytotoxicity and COD of unsterilized and undiluted Zibar. Where the bioremediation process will be under the optimum growth condition for each isolate, without pre-treatment of Zibar as has been adopted in many researches.

The process of treating Zibar with an aerobic microbial population results in a nonphytotoxic liquid that could be characterized as an organic soil-conditioner biofertilizer with the many characteristics: (e.g. contains exopolysaccharides (microbial metabolites), contains all the major and trace plant nutrients that were originally present in Zibar, contains plant growth-promoting factors such as auxins and cytokinins produced by the microorganisms metabolic activity, it is a soil microbial inoculant that allows the establishment of favorable to plants rhizospheric microorganisms and enhances soil suppressiveness) (Chatjipavlidis *et al.*, 1996; Fiorentino *et al.*, 2004).

3 Materials and Methods

3.1 Collection of Zibar

Samples of Zibar were collected from Nablus city during the olive harvest season in 2018. The samples were taken directly from a three-phase decanter press, and stored in 20 L plastic containers in the dark at the laboratory of the kadoorie Agriculture Research Center (KARC) until use. Characteristics of Zibar including pH, (COD), total suspended solid (TSS), total phenols and Electrical Conductivity (EC) were determined. To remove suspended solids, Zibar was centrifuged at 5000 g for 20 min, filter-sterilized and stored at 20°C until use (Aytar *et al.*, 2011). The pH was measured using pH meter™, while EC meter™ was used to measure EC. The TSS was measured by filtering 100 ml of untreated Zibar by preweight Whatman #1 filter paper and then the difference in the weights of the paper were calculated, after drying at 65°C for 24 hours.

3.2 Fungal Isolates, maintenance and growth conditions

Four fungal isolates (*Ph. chrysosporium*, PTUK, OMWW1 and OMWW2) were obtained from the culture collection provided by Dr. Mazen Salman. The fungal isolates were maintained on potato dextrose agar (PDA), stored at 4°C until use and subcultured routinely every three weeks.

3.3 Molecular identification of fungal isolates

3.1.1 Fungal DNA Isolation

Fungal genomic material was isolated using CTAB method (Gardes and Bruns, 1993) with slight modification. Total DNA was extracted from fungal mycelia grown on PDA by collecting 50-100 mg mycelia of three days freshly grown fungi. The mycelia were placed in 1.5 ml microfuge tube containing sterile sea sand (*ca.* 100 mg) and 500ul of extraction buffer (100mM, Tris-HCl, 10mM EDTA, 2% SDS, 100 ug/ml proteinase K, and 1% B-mercaptoethanol). Samples were grind into slurry using pellet pestles homogenizer with sterilized tips and incubated at 60°C for 60 min in a water bath with shaking every 3-4 min. Salt concentration was adjusted to 1.4M by adding 200µl of 5M NaCl. After that, 70µl of 10% CTAB was added and further incubated for 10 min at 60°C.

One volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to each tube that were gently emulsified by inversion, then incubated on ice for 30 min to be spin after that at 4°C, 12000 rpm for 10 min. Top phase was transferred to new 1.5 ml microfuge tube and then half the transferred volume was added with 5M NH₄OAc and mixed gently, tubes then were incubated at 0°C or 60 min to be spun after that at 4°C, 12000 rpm for 15

min. The resulted supernatant was transferred to new 1.5 ml microfuge tube then RNase solution was added to have a final concentration of 0.02 Ug/ml and 0.55 of the resulted volume was added with cold isopropanol that was then mixed gently. The mixture was spun at 1000 rpm for 5min with discarding the resulted supernatant without disrupting pellet. Pellets were suspended in 200µl TE buffer and 20µl of 3M NaOAc pH 7 and then 2.5 of the resulted volume was added with 75% of cold ethanol that was mixed gently and then spun at 10000 rpm for 5 min for pellet washing that was repeated. Finally, the resulted supernatant was suspended in 50µl TE buffer after drying all samples were stored at -20°C for further use.

3.1.2 PCR Identification of the fungal isolates

Identification of the fungal isolates was carried out using PCR amplification program as mentioned in Gardes and Bruns (1993). Total DNA from each isolate was used as a template for amplification of the 18S rRNA genes. The 18S rRNA gene was amplified using ITS1 (forward primer) (5'-TCC GTA GGT GAA CCTTGC GG-3') and ITS4 as (reverse primer) (5'-TCC TCC GCT TAT TGA TAT GC-3').

Reaction mixtures were performed in final volume of 20µl containing 10µl GoTaq® Green (2X) Master Mix (Promega Corporation), 0.5µl of each primer (10 pmol), 1µl fungal DNA and 8µl nuclease-free water. PCR

amplification was carried out in thermal cycle (Verti™ Dx Thermo Fisher Scientific) according to the following program: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, then annealing at 55 °C for 1 min and primer extension at 72°C for 2 min, finally at 72°C for 10 min a final primers extension were carried.

3.1.3 Gel Electrophoresis

The total DNA fragment and PCR products were separated by electrophoresis according to their molecular weight using 1.2% agarose gel containing 1µl Gel Red DNA stain.

DNA fragments of total DNA and PCR products were visualized under UV light illuminator and documented using UVitec Gel Documentation Systems™ for estimating the size of the amplified DNA fragment, a 100bp DNA RTU ladder was used as a molecular size marker. After that sequence analysis of the PCR products were done at the Arab American University in Ramallah

3.5 Growth of fungal isolates under different conditions

3.5.1 Effect of temperature on fungal growth

The fungi were grown on PDA media and incubated at different temperatures of 10, 15, 25, 35°C. Colony diameters were measured daily for 5 days after incubation.

3.5.2 Effect of NaCl concentrations on fungal growth

The fungi were grown on PDA media of different NaCl concentrations (0, 50, 100, 150, 200, and 250mM). Cultures were incubated at the optimum temperature, and the growth rates of the fungi were determined daily for 5 days.

3.5.3 Effect of pH on fungal growth:

The effect of pH on fungal growth was studied after adjusting the pH values, to (5, 5.5, 6, 6.5, 7, 7.5 and 8) using 1M NaOH or HCl. Cultures were incubated at the optimum temperature, and the growth rates of the isolates were determined daily for 5 days.

3.6 Fungal Growth on Liquid Zibar

The fungi were grown in 125 ml Erlenmeyer flasks containing 25ml of 100% Zibar, by inoculating five discs of 7mm diameter PDA discs grown with

fungus isolates *Ph. chrysosporium*, PTUK, OMWW1 and OMWW2. The flasks were placed on a Rotary Shaker™ at 150 rpm for two weeks at room temperature and at optimum temperature for each isolate. The effect of Zibar on fungal growth was measured weekly by filtering the growth media on preweight Whatman #1 filter paper. Mycelia dry weights were then recorded after drying at 65°C for 24 h.

3.7 Measurements of Total Phenolic Compounds

Total phenols extraction was done as mentioned in Lesage- Meesen *et al.* (2001). Samples of Zibar (25 ml) were inoculated each with 5 discs of 7mm diameter PDA discs grown with fungus isolates *Ph. chrysosporium*, PTUK, OMWW1 and OMWW2. The cultures were incubated at room temperature and optimum temperature. After that, the Zibar was centrifuged at 3000 g for 5 min to remove fungal biomass and 2.5 ml were diluted to 50%, acidified to pH 2.0 with 5M HCl and extracted with ethyl acetate (1:1, v/v). The extraction was repeated twice and the solvent layers were combined in 100 ml beakers and allowed to evaporate under fuming hood.

The residual materials were dissolved in 2.5 ml methanol and the volume was completed to 25 ml with distilled water. Total phenol concentrations were determined using Folin-Ciocalteu reagent® (Sigma Aldrich) to Makkar

et al. (1993) using HACH™ DR6000 Laboratory Spectrophotometer at 725 nm wavelength

The results were expressed as tannic acid equivalent using the standard curve prepared by dissolving tannic acid in distilled water (Table 2).

Table 2. Preparation of calibration curve.

Treatments	Tannic acid solution (0.1 mg/ml)	Distilled water	Folin reagent	Sodium carbonate solution (20%)
	(μ l)	(μ l)	(μ l)	(μ l)
1	0	1000	250	1250
2	20	980	250	1250
3	40	960	250	1250
4	60	940	250	1250
5	80	920	250	1250
6	100	900	250	1250
7	200	800	250	1250
8	400	600	250	1250
6	600	400	250	1250
10	1000	0	250	1250

3.8 Characterization of treated Zibar

Characteristics of treated Zibar including pH, (COD), (EC) and total phenols were determined after (3,5,7 and 14 day) of treatment with each isolates which were grown at optimum and room temperature.

3.9 Phytotoxicity studies

Phytotoxicity of treated Zibar was tested on tomato seeds and seedlings. About 30 seeds were spread on Whatman No 1 filter paper in 9 cm petri dishes. Three ml of 25, 50, 75 and 100% Zibar (treated and untreated) were added and the plates were incubated at room temperature for 7 days. Tap water was used in the control experiment. Germinated seeds were then counted and the percent of germination was calculated.

Pot experiment was also conducted on tomato seedlings. The seedlings were planted in 9 cm diameter pots filled with peat moss vermiculite (2:1 v/v). The pots were irrigated with 100 ml T Zibar (25, 50, 75 and 100% diluted using tap water). The pots were kept in growth room at 16:8 h light dark photoperiod and 25°C.

Control pots were irrigated with tap water. The effect of Zibar was recorded by measuring the fresh and dry weights of roots and stems as well as the length of the seedlings after one month of the experiment.

3.10 Effect of Zibar on microflora and soil properties

To study the effect of Zibar on soil microflora, 10 g of soil from tomato pots were suspended in 100 ml sterile deionized water, placed on the shaker for 30 min and then left for one hour to still down. After that, 10 μ l of the top layer of the mixture was diluted serially in 90 μ l of sterile deionized water. Then 10 μ l of different dilution were cultured on Nutrient agar (NA) media plates. The number of colonies were calculated after 24 h. In addition to that, soil properties including pH and EC were measured.

4 RESULTS

4.1 Fungal Isolates, maintenance and growth under different conditions

The study began by maintenance and determining the optimum growth conditions of all fungi isolates (Figure 2) by growth and development of them under different conditions of NaCl concentrations, pH and temperature values on PDA media pleats, and tracking the increase in growth diameters (growth rate).

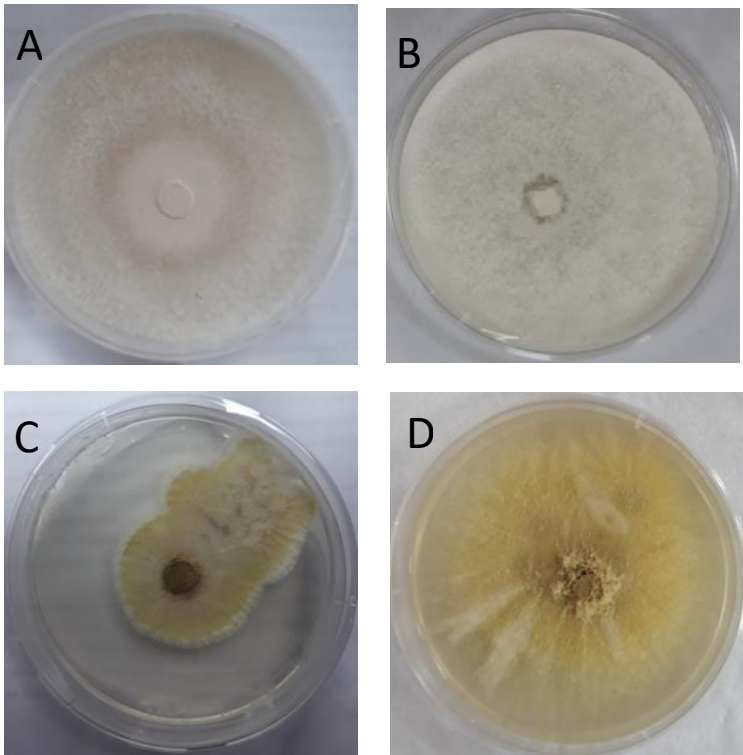


Figure 2. Four different types of isolates were used in the experiment: *Ph. chrysosporium* (A), PTUK (B), OMWW1 (C) and OMWW2 (D).

The optimum temperature was determined by the highest increase in the growth diameter (growth rate). The optimum temperature for *Ph. chrysosporium* and PTUK was approximately 35°C (Figure 3 and Figure4) and 25°C for OMWW1 and OMWW2 (Figure 7 and Figure8).

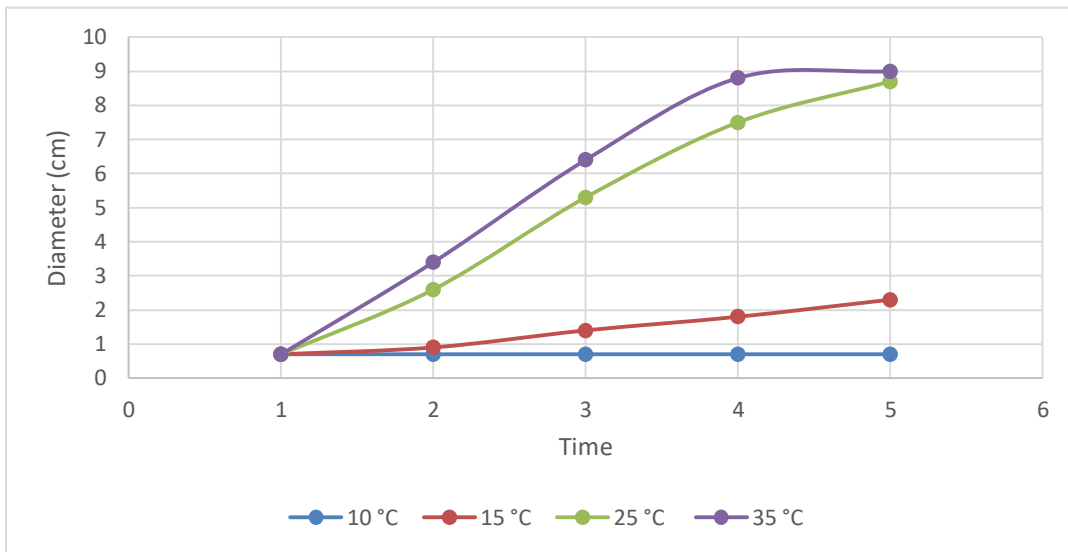


Figure 3. Growth diameter of *Ph. chrysosporium* under different temperatures.

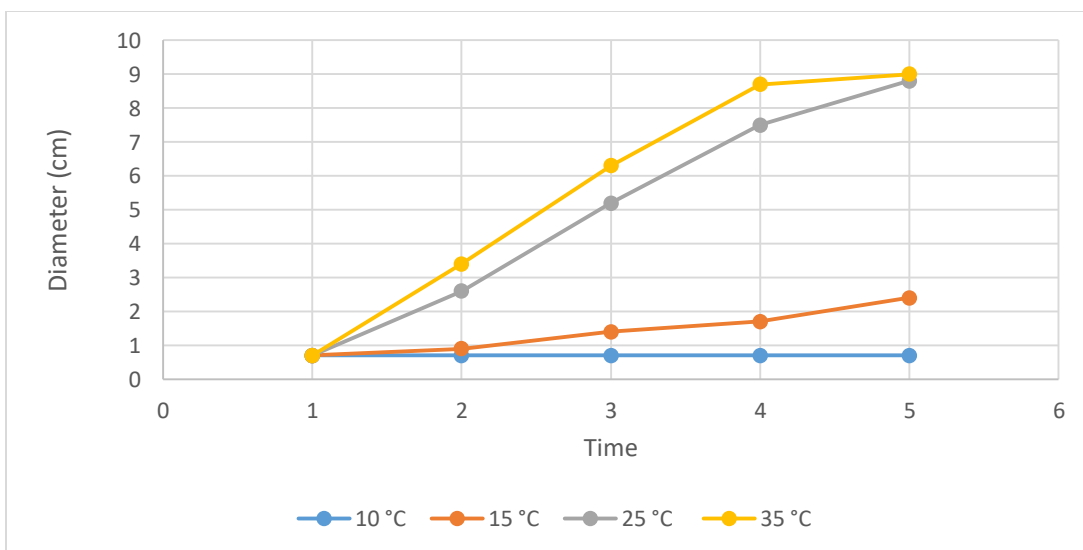


Figure 4. Growth diameter of PTUK under different temperatures.

It was observed that on the fifth day, the growth intensity in both isolates *Ph. chrysosporium* and PTUK at 35°C was significantly higher than 25°C (Figure 5 and Figure 6).

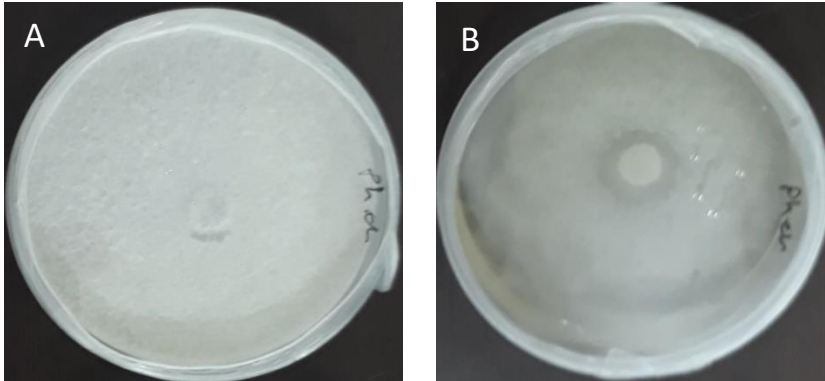


Figure 5. *Ph. chrysosporium* growth intensity at: 35°C (A) and 25°C (B).

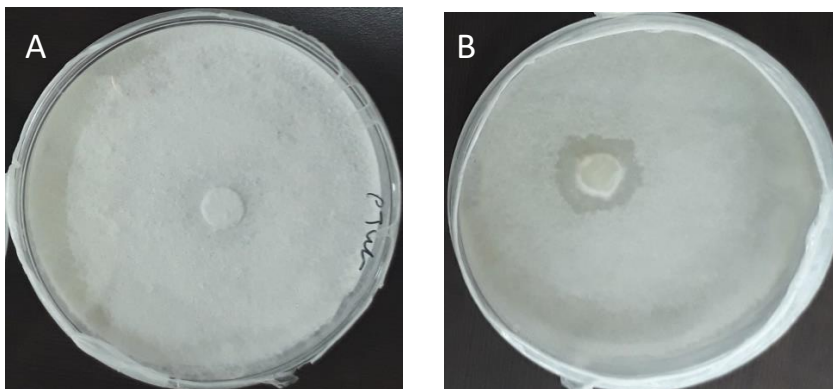


Figure 6. PTUK growth intensity at: 35°C (A) and 25°C (B).

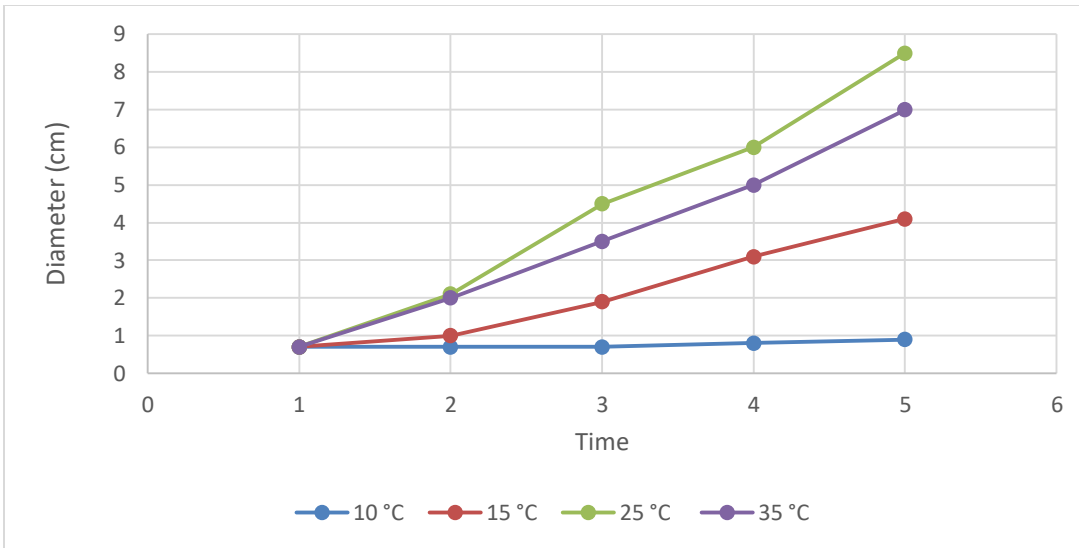


Figure 7. Growth diameter of OMWW1 under different temperatures.

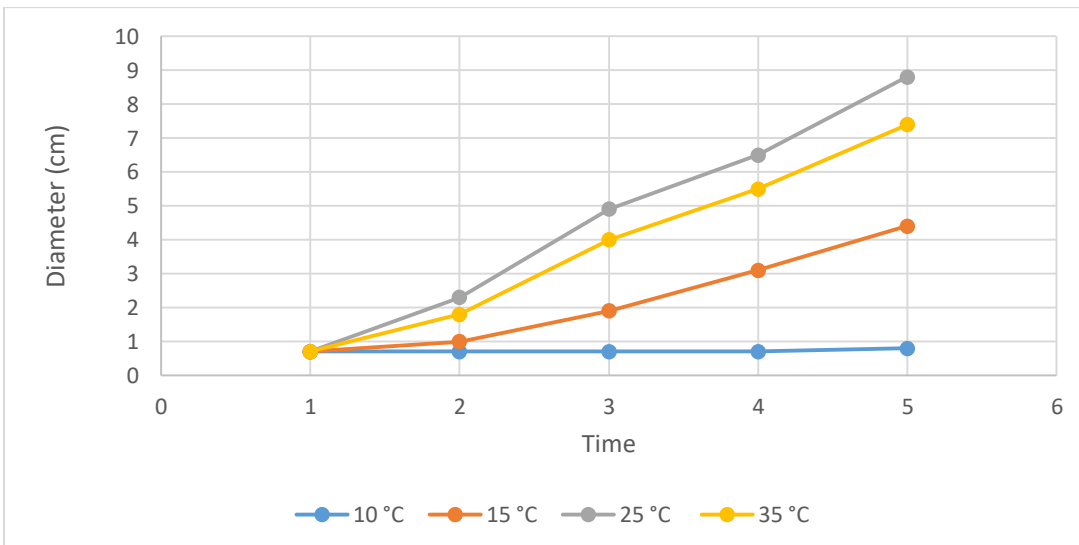


Figure 8. Growth diameter of OMWW2 under different temperatures.

It was noticed that the color and morphology of OMWW2 isolates were changed to brownish color, when it was grown at 35°C as shown in figure 9.

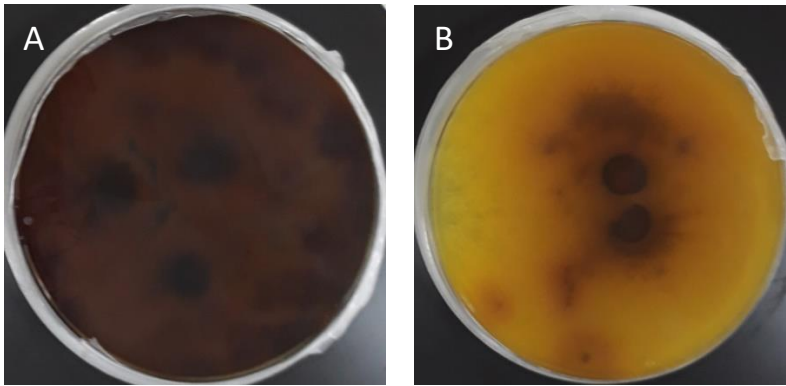


Figure 9. Effects of temperature on OMWW2 color and morphology: 35°C (A) and 25°C (B).

In Other hand, there were no effects of different pH or NaCl concentrations, on increasing the growth diameter (growth rate) and the intensity of all isolates.

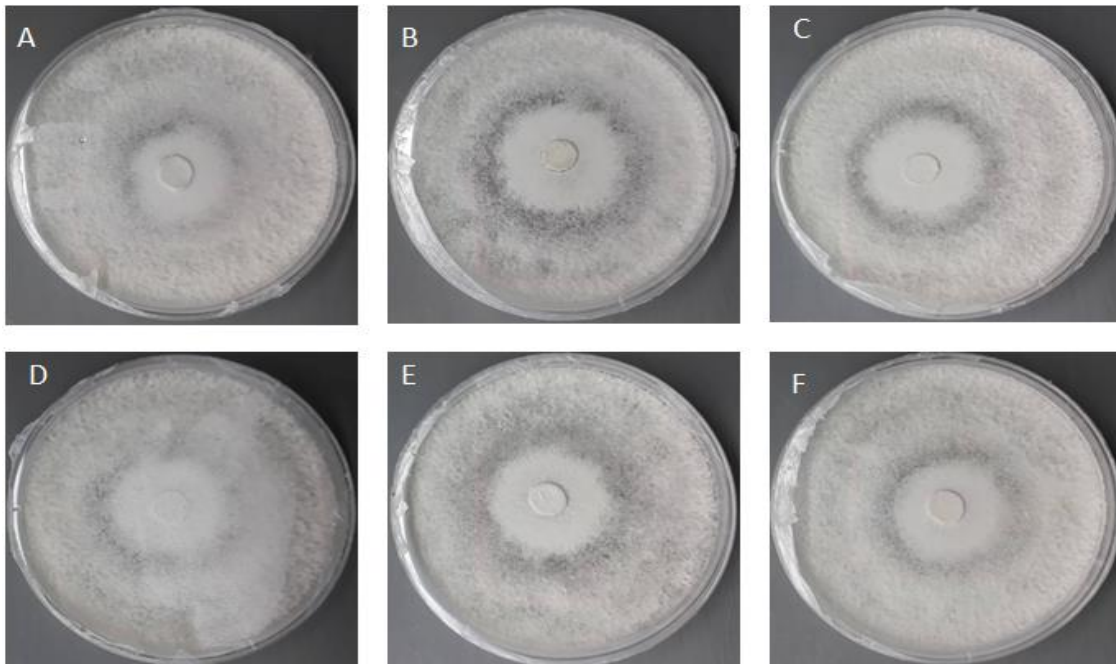


Figure 10. The effects of different NaCl concentration on *Ph. chrysosporium* growth, [0 mM] NaCl (A), [50 mM] NaCl (B), [100 mM] NaCl (C), [150 mM] NaCl (D), [200 mM] NaCl (E), and [250 mM] NaCl (F).

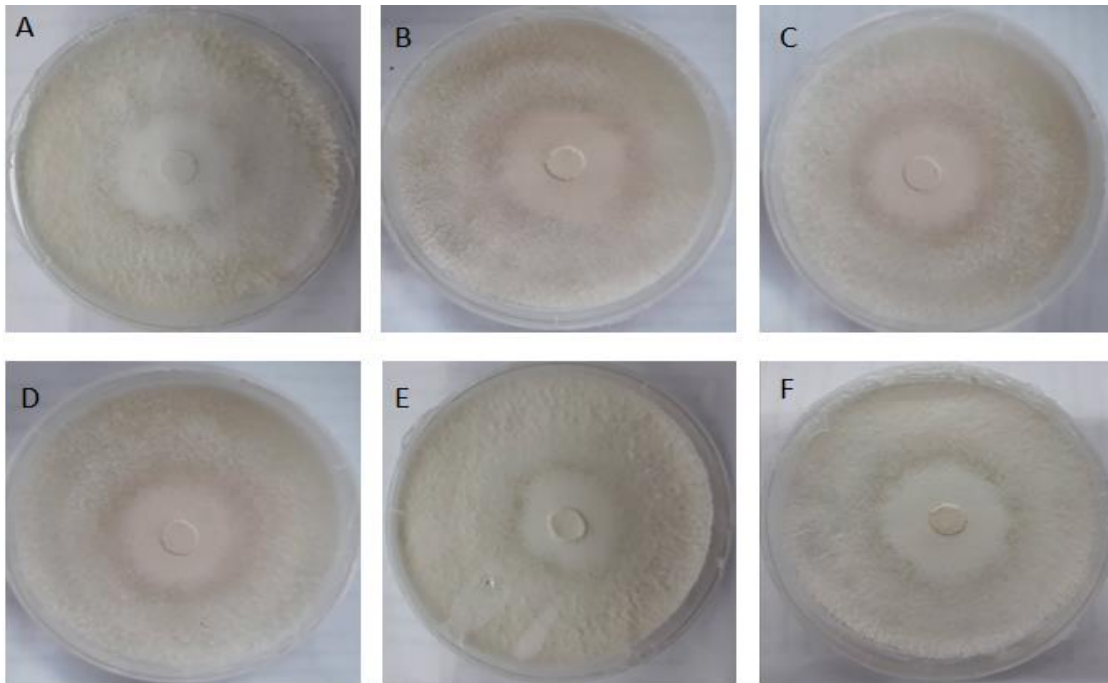


Figure 11. The effects of different NaCl concentration on PTUK growth, [0 mM] NaCl (A), [50 mM] NaCl (B), [100 mM] NaCl (C), [150 mM] NaCl (D), [200 mM] NaCl (E), and [250 mM] NaCl (F).

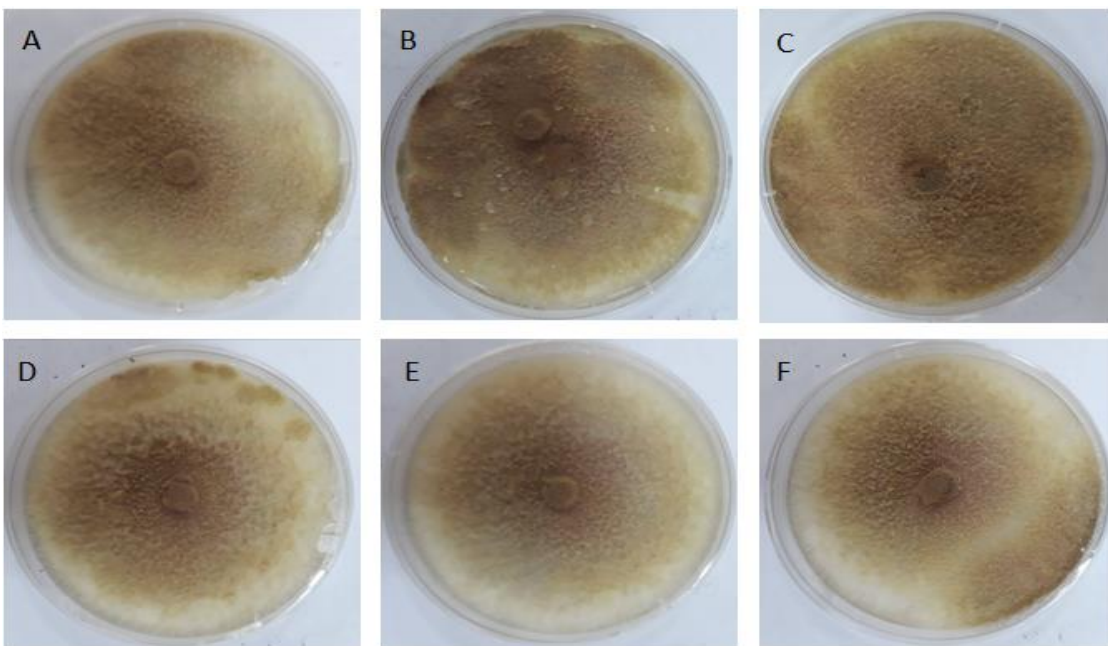


Figure 12. The effects of different NaCl concentration on OMWW1 growth, [0 mM] NaCl (A), [50 mM] NaCl (B), [100 mM] NaCl (C), [150 mM] NaCl (D), [200 mM] NaCl (E), and [250 mM] NaCl (F).

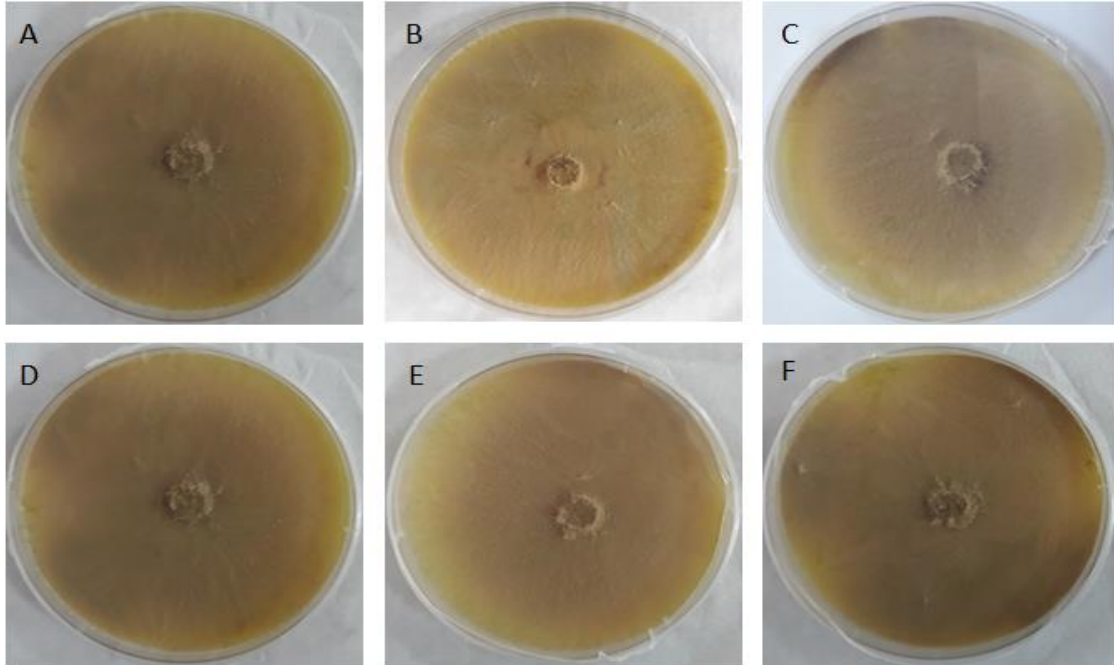


Figure 13. The effect of different NaCl concentration on OMWW2 growth, [0 mM] NaCl (A), [50 mM] NaCl (B), [100 mM] NaCl (C), [150 mM] NaCl (D), [200 mM] NaCl (E), and [250 mM] NaCl (F).

4.2 Fungal Growth on Liquid Zibar

The effect of Zibar on the fungal growth were measured by filtering the growth media on pre weight Whattman #1 filter paper. Mycelia dry weight was then recorded after drying at 65°C for 24 hours (Table 3). As can be seen in the picture the biomass of all isolates are increasing until they reach to stationary phase. As the fungi population continues to grow, all the nutrients in the growth medium are used up by the microorganism for their rapid multiplication. This result in the accumulation of waste materials, toxic metabolites and inhibitory compounds in the medium. This condition create

an unfavorable environment for the growth. The cell number is not increased and thus the growth rates were stabilized.

Table 3. Growth of different fungal at crude OMWW.

Time (D)	<i>Ph. chrysosporium</i> mass (mg/L)	PTUK mass (mg/L)	OMWW1 mass (mg/L)	OMWW2 mass (mg/L)
0	0.52	0.52	0.56	0.6
3	13.8	13.76	12.32	15.12
5	17.96	17.96	16.08	19.12
7	19.24	19.32	17.96	20.48
14	19.4	19.44	18.08	20.6

As shown in figure 14, isolation OMWW2 was the highest increase in biomass, while isolation OMWW1 had the lowest increase in biomass.

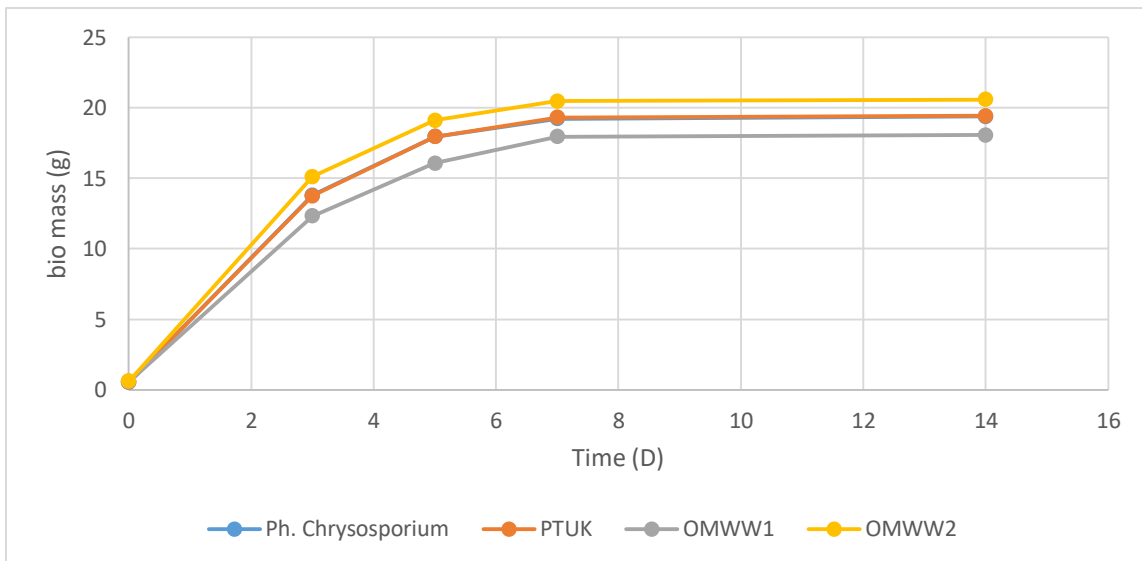


Figure 14. Experimental and calculated values of biomass of different isolates incubation in crude Zibar.

4.3 Determination of Zibar characteristics

After collecting fresh Zibar from Press located in the West Bank in Palestine and stored at the laboratory until use, characteristics of Zibar including pH, (EC), (TSM), (COD), and total phenols were determined and were compared with treated Zibar characteristics (Table 4). The resulting readings by HACH™ DR6000 Laboratory Spectrophotometer were expressed as tannic acid equivalent (Figure 15).

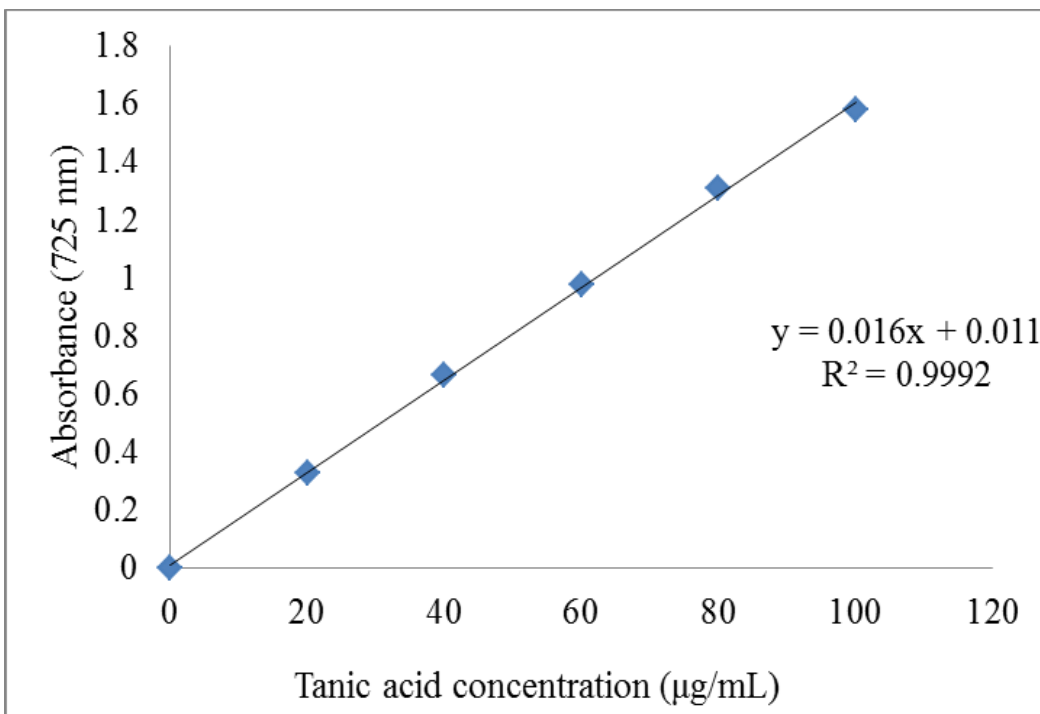


Figure 15. Standard curve of the phenolic compounds in Zibar.

Table 4. Composition of untreated and treated Zibar after 14 days.

	Untreated OMWW	Treated Zibar with <i>Ph.</i> <i>chryso sporium</i>	Treated Zibar with PTUK	Treated Zibar with OMWW1	Treated Zibar with OMWW2
pH	4.96	7.8	7.79	7.84	7.84
EC (ds/m)	9.73	10.03	10.03	10.67	10.55
COD (mg/L)	62566.02	19621	19621.8	26223.9	22621.8
Total polyphenol (mg/ml)	145	60.03	60.01	106.575	52.64
TSM	1.465g/100 ml				

Interestingly, as noted from the previous values that at the end of second week of incubation, OMWW2 is the best isolate for crude Zibar bioremediation and decreasing the polyphenol concentration. Where the decrease in phenols was approximately 63.7%, as shown in (Figure 16 and Figure 18). The fungus was also able to reduce COD about 63.89%. Although the best isolates to reduce COD are, *Ph. chryso sporium* and PTUK in decreasing reach about 68.69% (Figure 17).

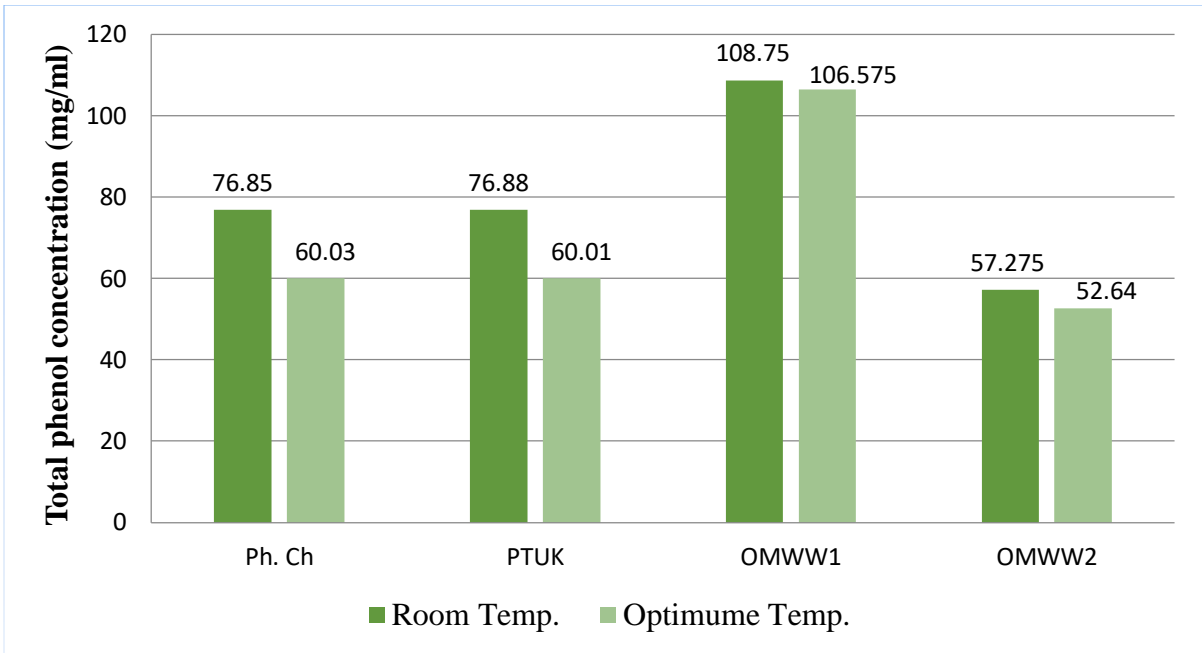


Figure 16. Removal of total phenol from Zibar by different isolates.

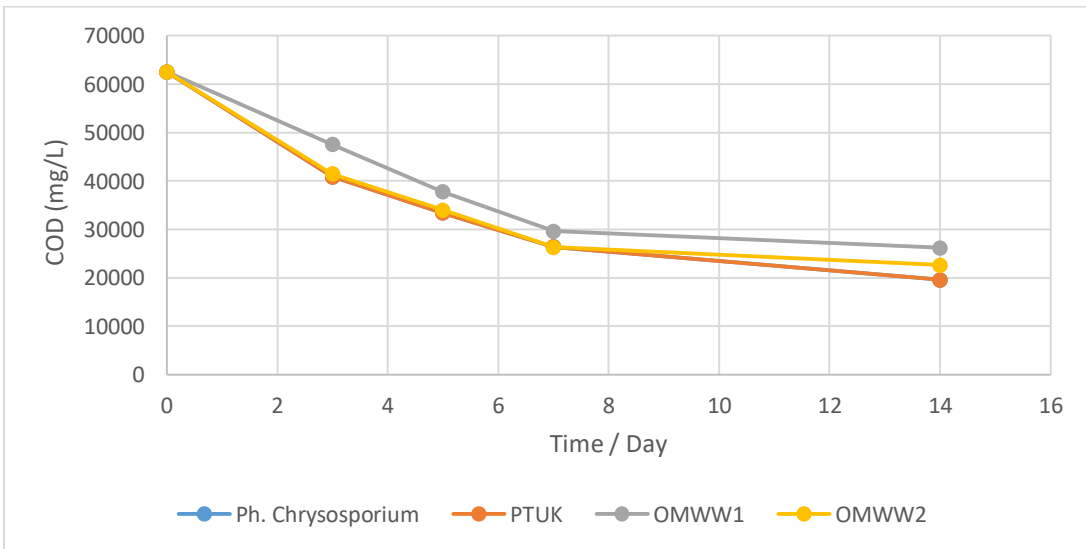


Figure 17. Experimental and calculated values of COD concentration of treated Zibar.

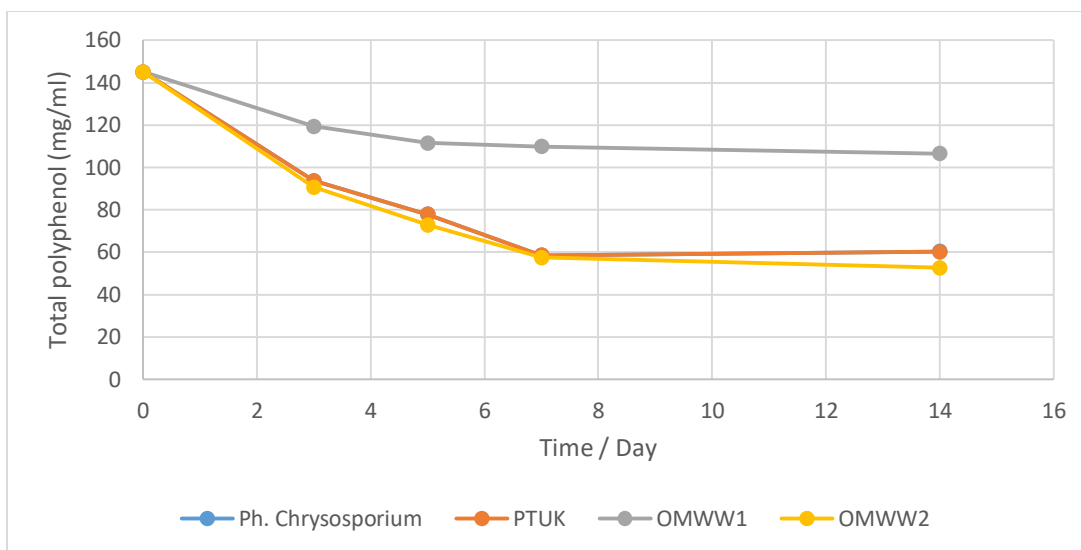


Figure 18. Experimental and calculated values of total polyphenol concentration of treated Zibar.

Some elements in Zibar which treated with OMWW2 (T Zibar) and untreated Zibar were analyzed spectrophotometrically using HACH™ DR6000 Laboratory Spectrophotometer, as shown in the (Table 5), to compare their components. Where it appears that T Zibar contains many important elements for plant growth.

Table 5. Important element found in Zibar and T Zibar.

Kits Type	Sample volume	T Zibar (mg/ml)	Zibar (mg/ml)	Cat. #
CuVer® 1 copper Reagent	10 ml	15	2.8	2105869
Sodium Periodate	10 ml	238	121	2107769
Ferro Ver® Iron Reagent	10 ml	10.4	4.4	2105769
ZincoVer® 5 Zinc Reagent	20 ml	1.14	1.06	2106669
Potassium 1 Reagent Powder Pillows		12.7	9	1432198
PhosVer® Phosphate reagent	10 ml	3600	1750	2106069

4.4 Effects of Zibar on seeds germination and seedling growth

The effects of T Zibar on tomato seeds were studied and several concentrations of T Zibar were used (Table 6). Interestingly, there was no significant effect of 100% treated Zibar compared to tap water on seeds germination after 7 days of incubation (Figure19). However, there was no germination of seeds in untreated Zibar (Figure 20).

Table 6. Seed germination under different treatments.

	Average # of seeds germination	Average % of seeds germination
Control	27.3	91.1%
Untreated Zibar	0	0.0%
100% T Zibar	25.0	83.3%
75%	25.7	85.6%
50%	26.7	88.9%
25%	27.0	90.0%

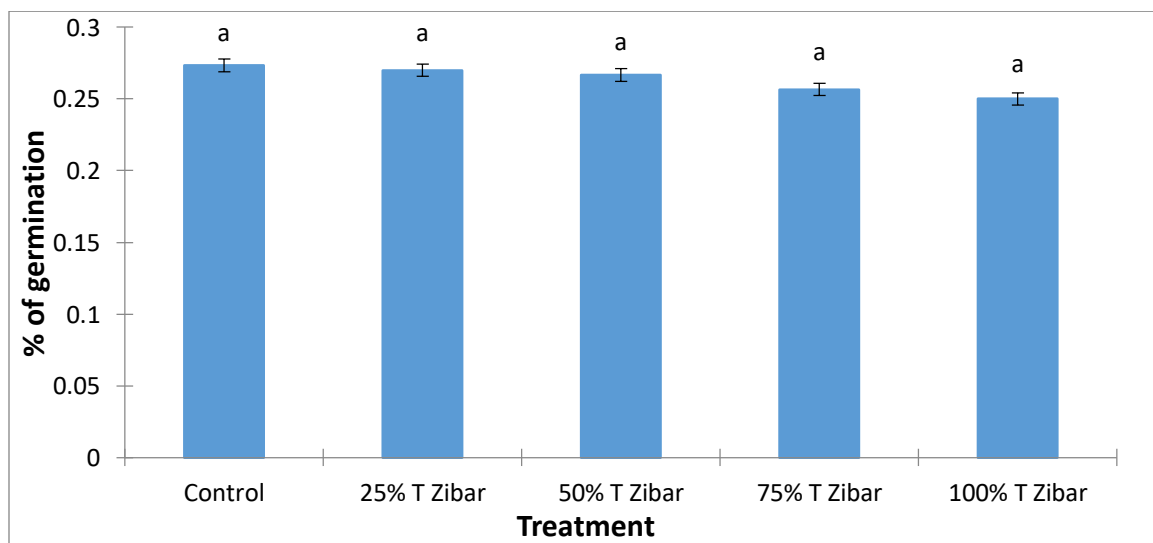


Figure 19. Tomato seed germination after 7 days under different Zibar concentration. Data of different letters are significantly different after Tukeys HSD test using ANOVA at $p < 0.05$.

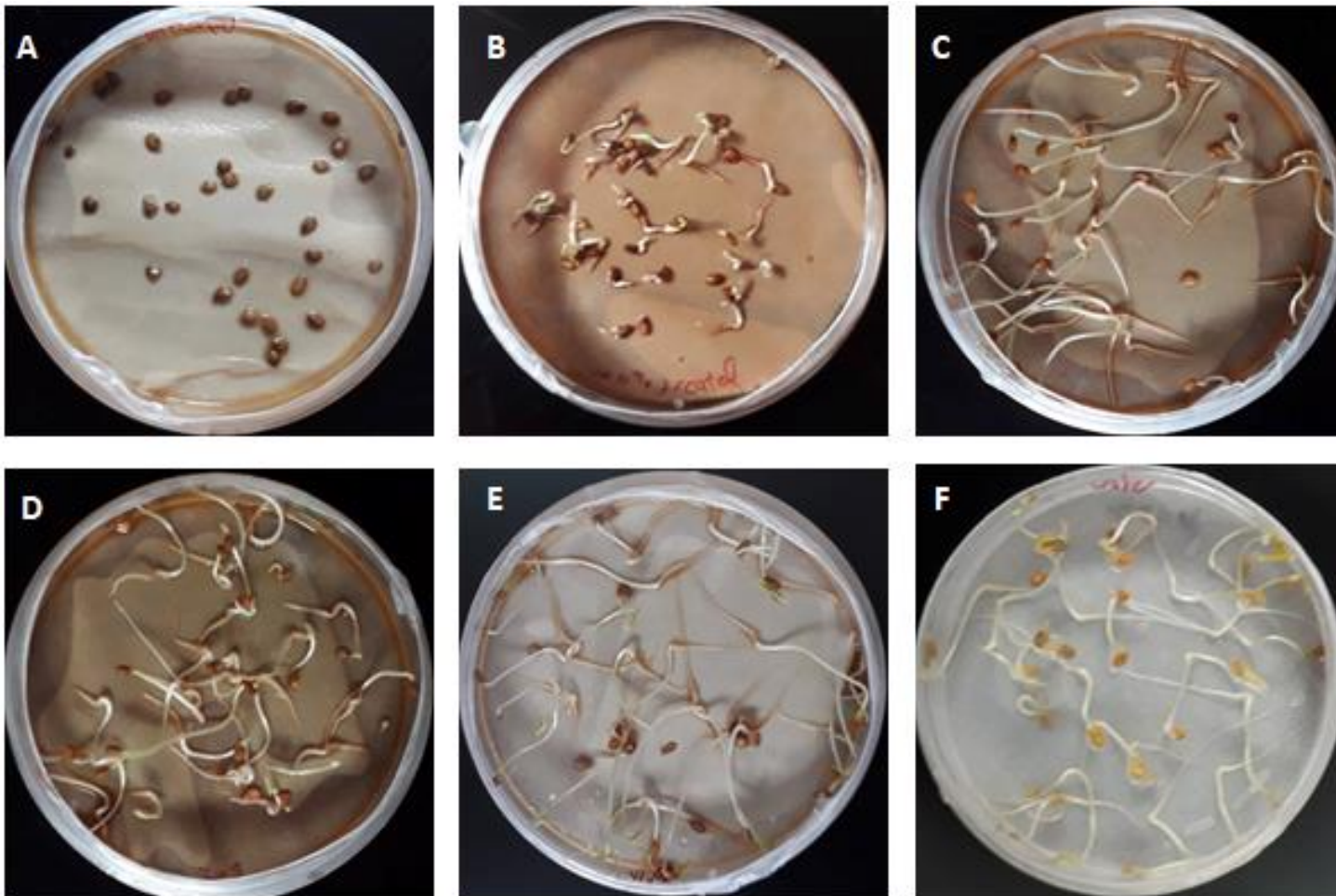


Figure 20. Tomato seeds germination under different condition, untreated Zibar (A), 100% T Zibar (B), 75% T Zibar(C), 50% T Zibar (D), 25% T Zibar (E) and Tap water (F).

As for pot experiments on tomato seedlings (Figure 21), T Zibar application did not show any morphological or physiological inhibition effect. The positive effects of the T Zibar fertilization seemed evident. After one month, the maximum height of the treated plants with 25% T Zibar was significantly better than that of the control ones (Figure 22). The positive effect of the addition of the OMWW2 discs on the seedlings length was observed. In addition, the root and shoot dry weights for tomato seedlings were significantly more in the treated ones compared to the control (Table 7). As shown in figure 23 and figure 24 the seedlings which were irrigated with 25% concentration were the best, where they had after one month the highest dry weights of the roots and shoots compared with control seedlings.

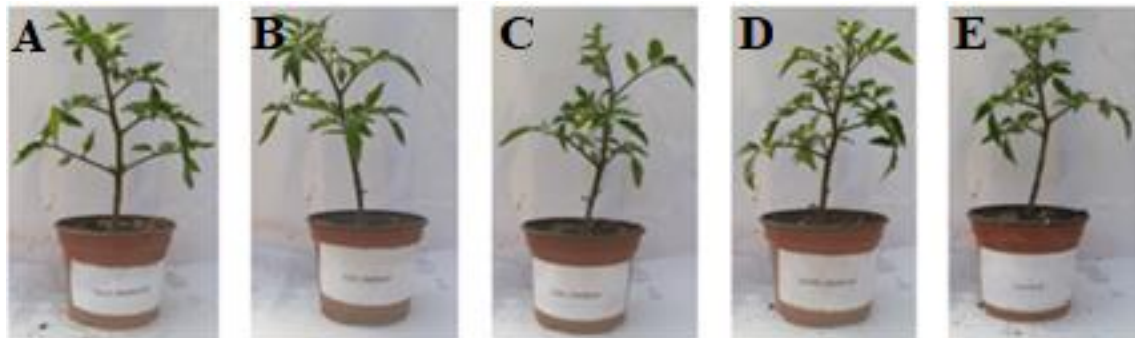


Figure 21 Tomato seedling irrigated with different Zibar concentration: Pot with 5 disc OMWW2 (A), Pot fertilized with 25% Zibar (B), Pot fertilized with 50% Zibar (C), Pot fertilized with 100% Zibar (D), Control pot (E).

Table 7. The effects of T Zibar on tomato seedlings.

Treatment	Length (cm)	Fresh weight for root (g)	Fresh weight for top (g)	Dry weight for root (g)	Dry weight for top (g)
100% T Zibar	32.3	3.6	16.9	0.4	3.7
50% T Zibar	32.3	3.6	16.9	0.4	3.7
25% T Zibar	34.5	3.6	17.0	0.4	3.7
water	30.8	3.4	16.3	0.4	2.8
Water + OMWW2	31.5	3.4	16.3	0.4	3.1

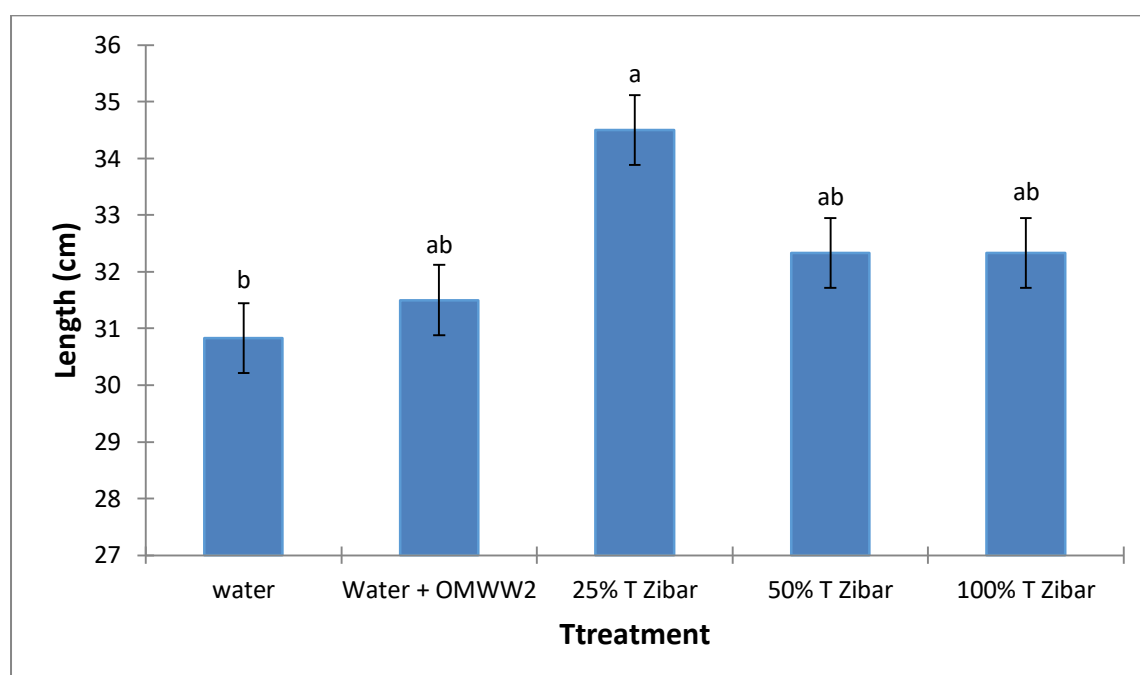


Figure 22. Length of tomato seedlings after one month of growing under Zibar irrigation. Data of different letters are significantly different after Tukeys HSD test using ANOVA at $p < 0.05$.

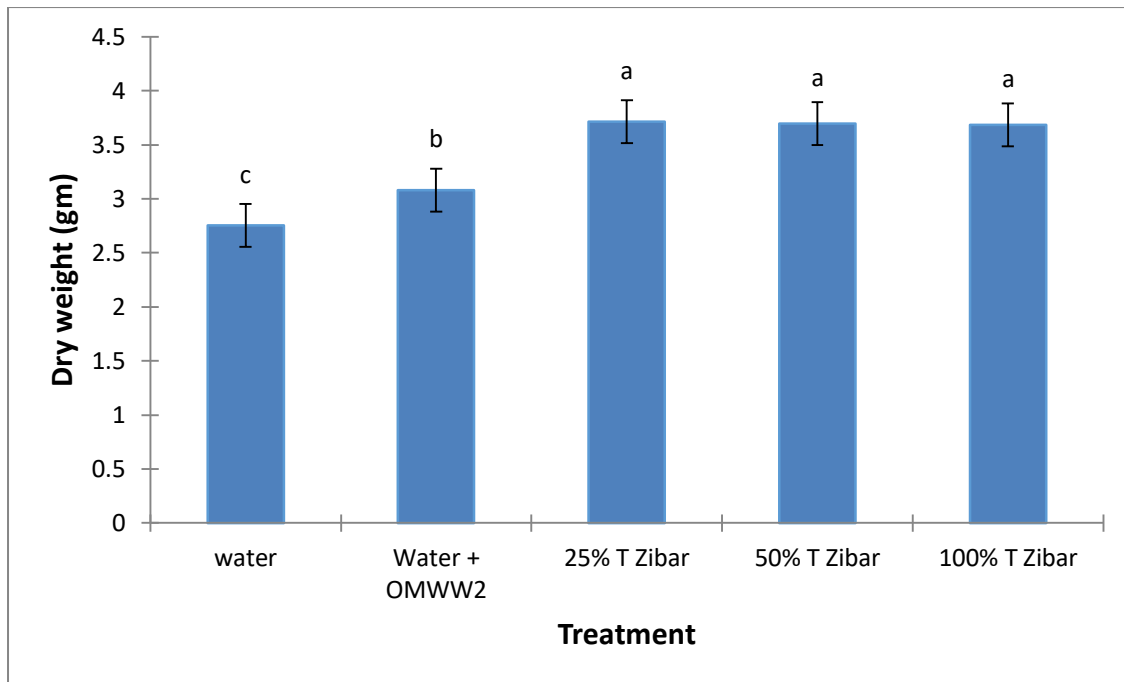


Figure 23. Dry weight of tomato seedlings shoot after one month of growing under Zibar irrigation. Data of different letters are significantly different after Tukeys HSD test using ANOVA at $p < 0.05$.

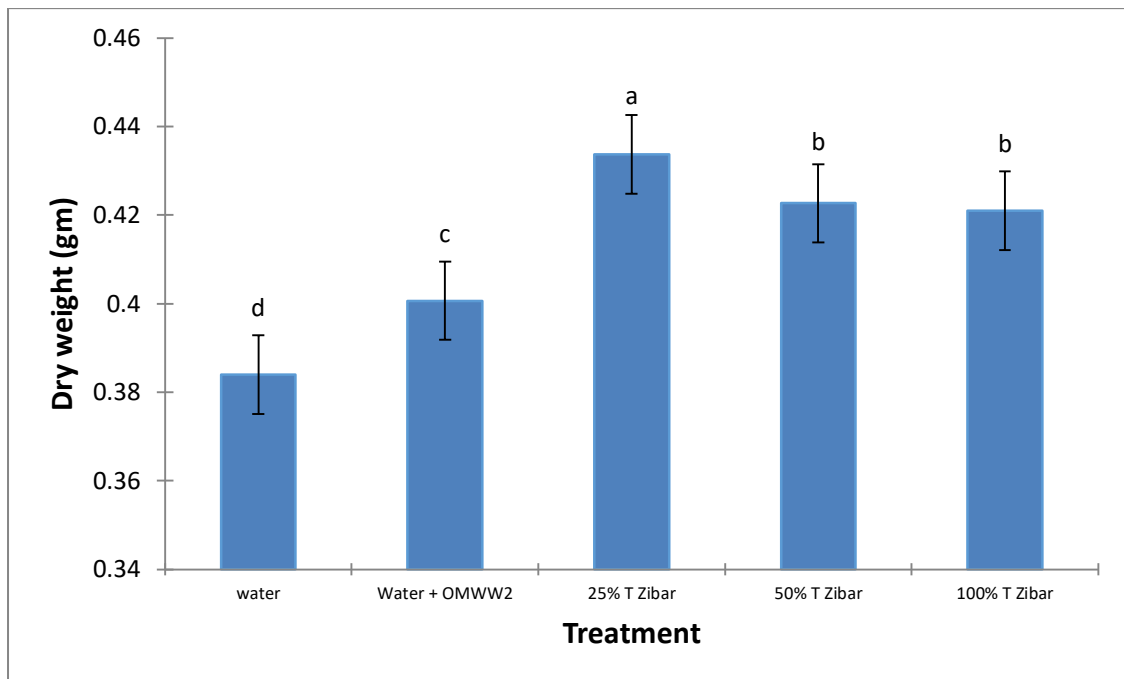


Figure 24. Dry weight of tomato seedlings roots after one month of growing under Zibar irrigation. Data of different letters are significantly different after Tukeys HSD test using ANOVA at $p < 0.05$.

4.5 Effects of Zibar on microflora and soil properties

Morphological differences between the control soil and the T Zibar fertilized soil in tomato experiment were not observed. While pH and salinity were investigated as well as the effect of Zibar on microorganisms present in the soil (Table 8). In addition, there were a significant increase in the soil microflora of the soil that irrigated with T Zibar compared with control soil (Figure 25). On other hand, there were significant effect of addition of OMWW2 discs into the soil on increasing the soil microflora (Figure 26).

Table 8. The effects of different treatment on soil microflora.

Treatment	Average # of colonies in 10 μ l	Average # of colonies in 10 g soil	Average # of colonies in 1ml (CFU/ml)	Average CfU/g
100% T Zibar	21	2.133×10^3	2.133×10^5	2.133×10^4
50% T Zibar	22	2.2×10^3	2.2×10^5	2.2×10^4
25% T Zibar	23	2.267×10^3	2.267×10^5	2.267×10^4
water	11	1.067×10^3	1.067×10^5	1.067×10^4
Water + OMWW2	48	4.833×10^3	4.833×10^5	4.833×10^4

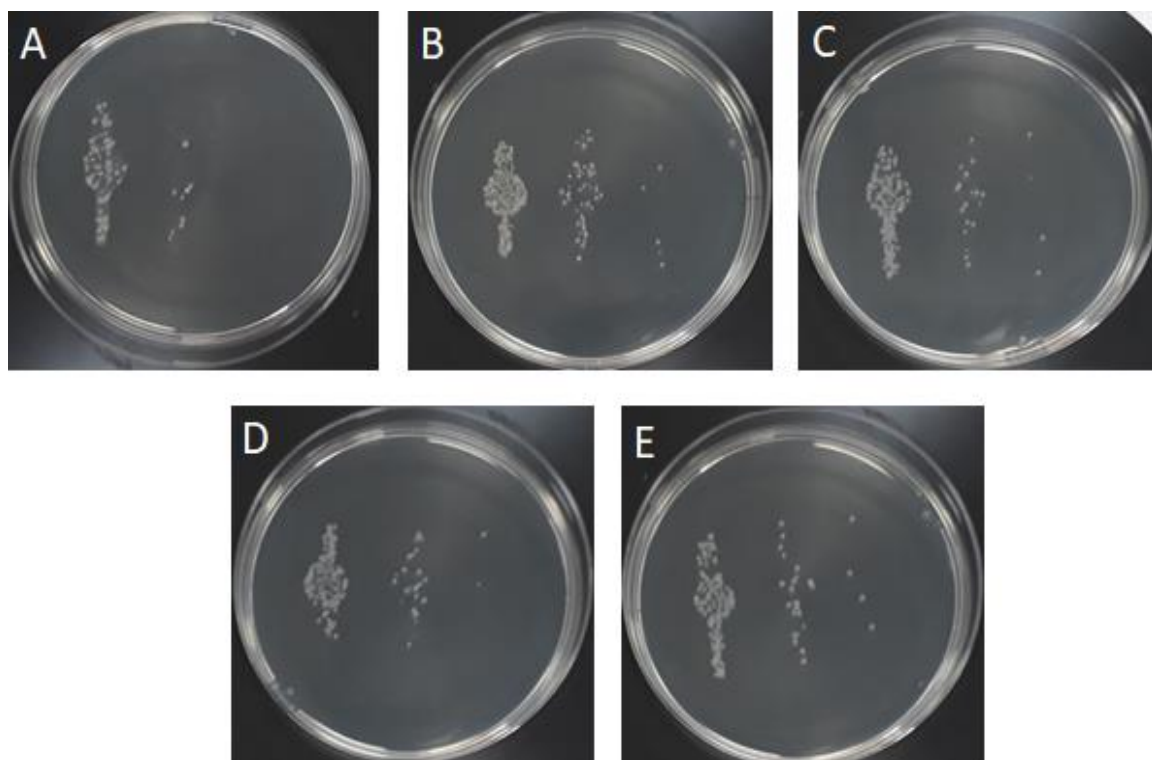


Figure 25. Microflora colonies in soil treated with: water (A), water + five disc OMWW2 (B), 100% T Zibar (C), 50% T Zibar (D) and 25% T Zibar (E).

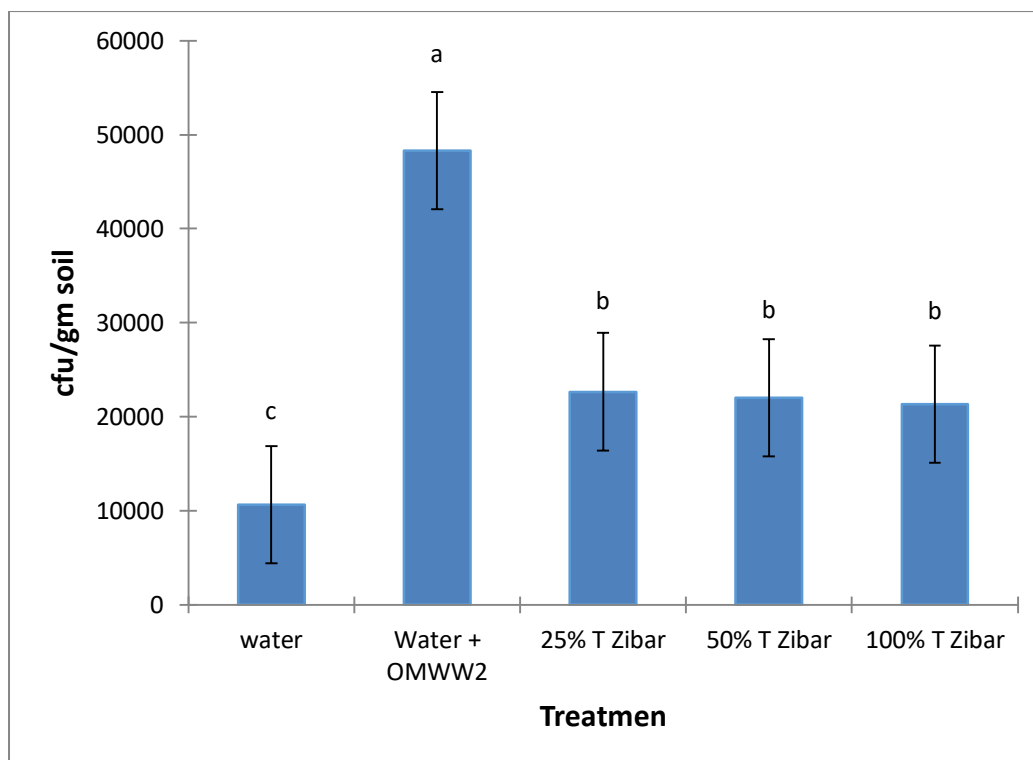


Figure 26. Effect of different treatment on soil microflora. Data of different letters are significantly different after Tukeys HSD test using ANOVA at $p < 0.05$.

The control soil pH was 7.9. However, in soil fertilized with T Zibar, this value increased to 8.2, 8.4 and 8.8 in 25%, 50% and 100% T Zibar respectively, and 8.06 in soil with OMWW2 discs. The soil EC increase was more pronounced in T Zibar irrigated soil compared to control or which contained discs of OMWW2. Where there were 8.82, 9.01, 9.4, 8.06, 8.09 ds/m, in soil treated with 25% T Zibar, 50% T Zibar, 100% T Zibar, water, addition 5 discs of OMWW2 respectively.

4.6 Molecular identification of fungal isolates

Identification of fungi is increasingly reliant on DNA sequencing rather than on morphological or breeding characteristics. After growth of the four candidate fungi and extraction of the DNA. Electrophoresis of Total

DNA and the PCR products (Figure 27 and Figure 28) were carried and showed the presence of intact DNA of expected size for each of the four candidate fungi.

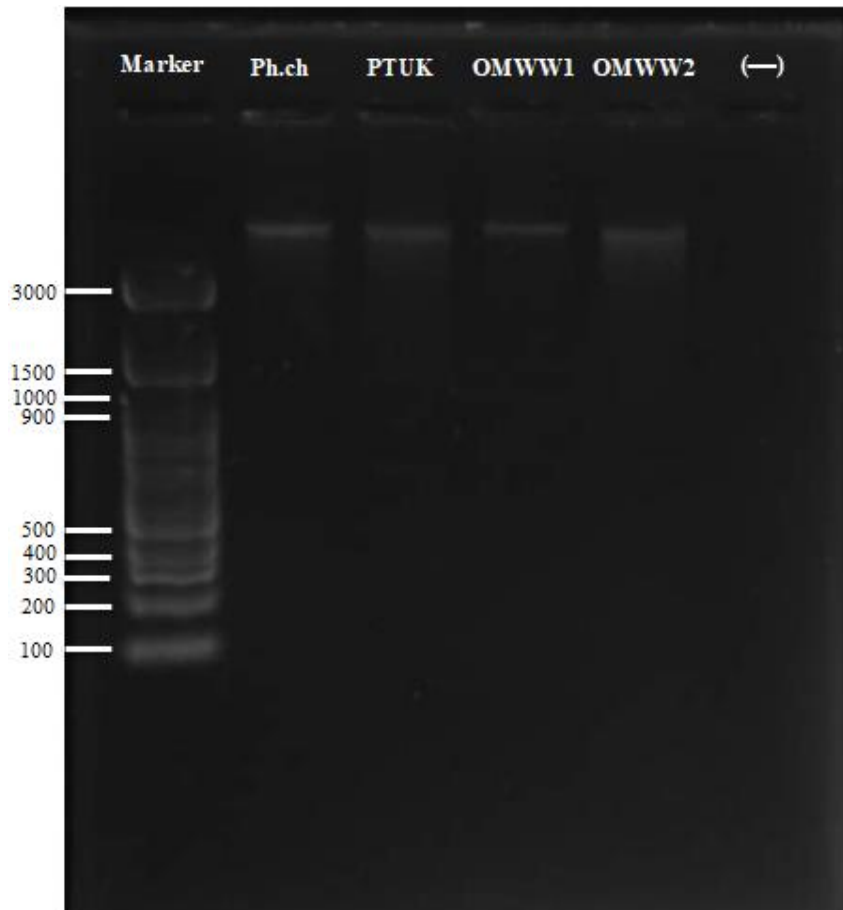


Figure 27. Total DNA fragment separated by electrophoresis according to their molecular weight using 1.2% agarose gel.

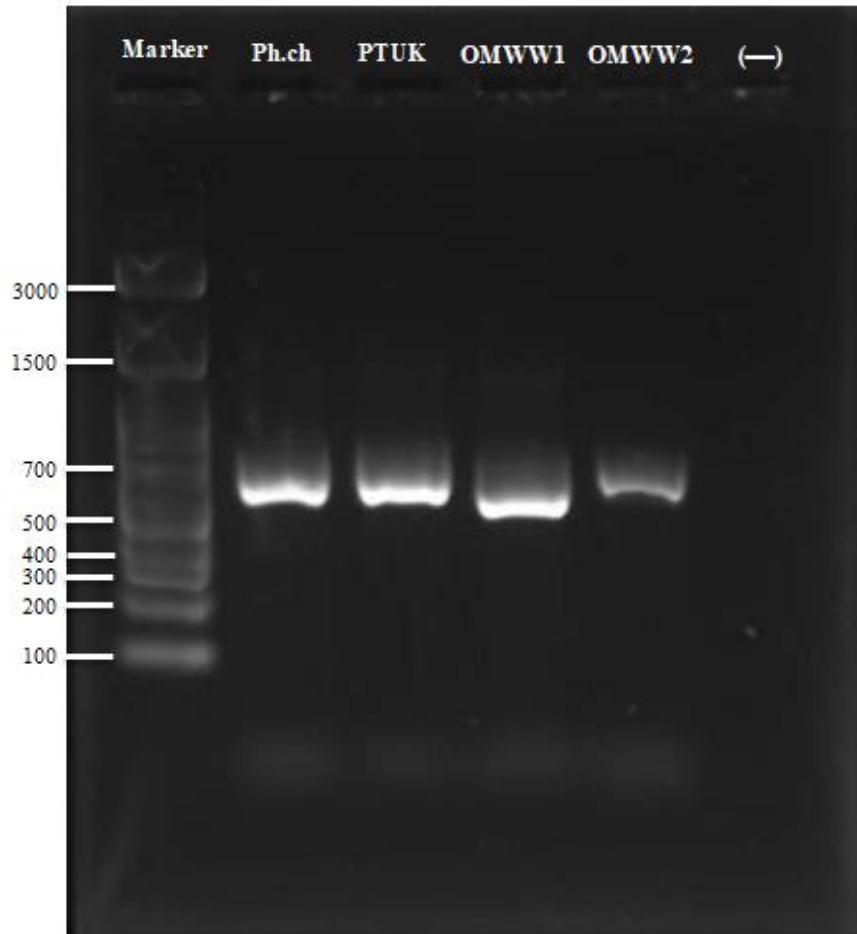


Figure 28. PCR product fragment separated by electrophoresis according to their molecular weight using 1.2% agarose gel.

After electrophoresis the PCR products of PTUK, OMWW1 and OMWW2 were sent to the Graduate Department of the Arab American University in Ramallah for sequencing the component. Two primers, ITS1 and ITS4 were used for this process. The PTUK isolate was identified as *Phanerochaete chrysosporium* strains W1-2 by ITS1 primer in partial sequence of 18S ribosomal RNA gene. In addition to partial sequence of 18S ribosomal RNA gene; complete sequence of internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed

spacer; and partial sequence of 28S ribosomal RNA gene by ITS4 primer (Figure 29).

Phanerochaete chrysosporium strain W1-2 18S ribosomal RNA gene, partial sequence
 Sequence ID: [KY401430.1](#) Length: 627 Number of Matches: 1

Range 1: 14 to 383 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
651 bits(352)	0.0	363/371(98%)	1/371(0%)	Plus/Plus
Query 16	CTGAMMGGTTGTAGCTGGCCTCTCKKGGTYWGTGACGCGCTGGCTCATCCACTCTTCAA	75		
Sbjct 14	CTGACAGGTTGTAGCTGGCCTCTC-GGGGCATGTGACGCGCTGGCTCATCCACTCTTCAA	72		
Query 76	CCTCTGTGCACTTGTGTAGGTCGGTAGAAGAGCGAGCATCCTCTGATGCTTTGCTTGA	135		
Sbjct 73	CCTCTGTGCACTTGTGTAGGTCGGTAGAAGAGCGAGCATCCTCTGATGCTTTGCTTGA	132		
Query 136	AGCCTTCCTATGTTTTACTACAAACGCTTCAGITTAAGAATGTCTACCTGCGTAYAACGC	195		
Sbjct 133	AGCCTTCCTATGTTTTACTACAAACGCTTCAGITTAAGAATGTCTACCTGCGTACAAACGC	192		
Query 196	ATCTATATACAACITTCAGCAACGGATCTCTGGCTCTCGCATCGATGAAGAACGCAGCG	255		
Sbjct 193	ATCTATATACAACITTCAGCAACGGATCTCTGGCTCTCGCATCGATGAAGAACGCAGCG	252		
Query 256	AAATGCGATAAGTAAATGTGAATTGCAGAAATTCAGTGAATCATCGAATCTTTGAACGCACC	315		
Sbjct 253	AAATGCGATAAGTAAATGTGAATTGCAGAAATTCAGTGAATCATCGAATCTTTGAACGCACC	312		
Query 316	TTGCGCTCCCTGGTATTCCGGGGAGCATGCCTGTTTGAGTGTGATGGTATCCTCAACCTT	375		
Sbjct 313	TTGCGCTCCCTGGTATTCCGGGGAGCATGCCTGTTTGAGTGTGATGGTATCCTCAACCTT	372		
Query 376	CATAACTTTTT 386			
Sbjct 373	CATAACTTTTT 383			

Figure 29. PTUK sequencing data by ITS1 primer

The indigenous fungus OMWW1 was identified as *Paecilomyces* sp. in Eurotiomycetes class of fungi in the phylum Ascomycota (sac fungi) within the kingdom Fungi, through partial and complete sequence of JCM 28097 genes for 18S ribosomal RNA, ITS1, 5.8S ribosomal RNA, ITS2 and 28S ribosomal RNA by ITS1 (Figure 30). And it was determined that this isolates was B6 isolate, through partial sequence of transcribed spacer 1; complete sequencing of 5.8S ribosomal RNA gene and internal transcribed spacer 2; and partial sequence of large subunit ribosomal RNA gene.

Paecilomyces sp. JCM 28097 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA, partial and complete sequence
 Sequence ID: [LC133789.1](#) Length: 590 Number of Matches: 1

Range 1: 42 to 416 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
614 bits(332)	1e-171	360/378(95%)	5/378(1%)	Plus/Plus
Query 18	GGTCCCTCG-RG-CCMACCTCCCATCCGGGTTTGTAAACACCTGTTGCTTCGGCGGGC	75		
Sbjct 42	GGTCCCTCGAGGCCAACCTCCCA-TCCGTG-TTGTAAACACCTGTTGCTTCGGCGGGC	99		
Query 76	CCKYKYGTTTACGCGCGTGSCCGCCGGGGGCACTCGCCCCGGGGCCCGCGCCCGC	135		
Sbjct 100	CCGCCGTGGTTACGCGCGTGGCCGCCGGGGGCACTCGCCCCGGGGCCCGCGCCCGC	159		
Query 136	AAGACCCCTCGAACGCTGCCTTGAAGGTTGCCGCTGAGTATGAAATCAATCGTTAAA	195		
Sbjct 160	AAGACCCCTCGAACGCTGCCTTGAAGGTTGCCGCTGAGTATGAAATCAATCGTTAAA	219		
Query 196	CTTCAACAACGGATCTCTTGGTTCGGCATCGTYGAARAACGAGCGAAATGCGATAAG	255		
Sbjct 220	CTTCAACAACGGATCTCTTGGTTCGGCATCGATGAAGAACGAGCGAAATGCGATAAG	279		
Query 256	TAATGTGAATTCAGAAATCCGTGAATCATCGAATCTTTGACGCACATTGCGCCCTG	315		
Sbjct 280	TAATGTGAATTCAGAAATCCGTGAATCATCGAATCTTTGACGCACATTGCGCCCTG	339		
Query 316	GCATTCGGGGGCAATGCCTGTCCSAGCGTCAITGCTAACCCCTCCMGCYCCGGCTGGTGT	375		
Sbjct 340	GCATTCGGGGGCAATGCCTGTCCGAGCGTCAITGCTAACCCCTCCAGC-CCGGCTGGTGT	398		
Query 376	GTTGGGTCGACGTCCTCC 393			
Sbjct 399	GTTGGGTCGACGTCCTCC 416			

Figure 30. OMWW1 sequencing data by ITS1 primer.

As for the third isolate OMWW2, it was identified as *Paecilomyces* sp., F-BTUL-E1 isolate, in Eurotiomycetes class of fungi, by ITS1 and ITS4 through partial sequencing of small ribosomal RNA gene; complete sequencing of 5.8S ribosomal RNA gene and internal transcribed spacer 2; and partial sequencing of large subunit ribosomal RNA gene (Figure 31).

Paecilomyces sp. (in Eurotiomycetes) isolate F-BETUL-E1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
 Sequence ID: [MG664272.1](#) Length: 589 Number of Matches: 1

Range 1: 30 to 407 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
656 bits(355)	0.0	369/379(97%)	2/379(0%)	Plus/Plus
Query 17	GTGCGGTCCTCGTGGCC-HAOCCTTYTYWTCGGTGTGACCGACMCCCTGTTGCTTgggc	75		
Sbjct 30	GTGCGGTCCTCGTGGCCAACT-CCCATCCGTTGTGACCGACACCTGTTGCTTGGCC	88		
Query 76	ggggccggccggggctccggccggggccggggggggggatggggccggggccggggccggc	135		
Sbjct 89	GGGCCCCCAGGGCTCCCGCCCCGGCCCGGGGGGCACTGGCCCCGGGGCCCGCGCCCGC	148		
Query 136	cgAAGACCCCTCGAACGCTGCCTTGAAGGTTGCCGCTGAGTATCAAATCAATCGTTAAA	195		
Sbjct 149	CGAAGACCCCTCGAACGCTGCCTTGAAGGTTGCCGCTGAGTATCAAATCAATCGTTAAA	208		
Query 196	ACTTTCACAACGGATCTCTTGGTTCGGCATCGATGAAGAACGAGCGAAATGCGATAA	255		
Sbjct 209	ACTTTCACAACGGATCTCTTGGTTCGGCATCGATGAAGAACGAGCGAAATGCGATAA	268		
Query 256	GTAATGTGAATTCAGAAATCCGTGAATCATCGAATCTTTGAAACGCACATTGCGCCCTG	315		
Sbjct 269	GTAATGTGAATTCAGAAATCCGTGAATCATCGAATCTTTGAAACGCACATTGCGCCCTG	328		
Query 316	GGCATTCCGGGGGCAATGCCTGTCCGAGCGTCAITGCTAACCCCTCCAGCCCGCTGGTGT	375		
Sbjct 329	GGCATTCCGGGGGCAATGCCTGTCCGAGCGTCAITGCTAACCCCTCCAGCCCGCTGGTGT	388		
Query 376	GTTGGGCTCTTCCCTC 394			
Sbjct 389	GTTGGGCTCTTCCCTC 407			

Figure 31. OMWW2 sequencing data by ITS1 primer.

5 Discussion

Processing of liquid olive waste is considered as key environmental issue for olive industry (Nair and Markham 2008). The bioremediation process was applied in this study to solve this environmental problem, as the most environmentally compatible and least expensive method. However, the selection of the microorganisms employed and in their adaptation to treating Zibar, as phenolic substances are inhibitory to microorganisms must be considered. Fungi were used because they are notably aerobic and can also grow under environmentally stressed conditions such as low pH and poor nutrient status, where bacterial growth might be limited. In addition to that the fungi are easy to transport, genetically engineered, and produce in large quantities (Aissam et al., 2007)

During the study, two approaches were adopted to select microorganisms to bioremediate Zibar include either bioprospecting for fungi known to produce relevant quantities of LME since these degrade phenolic compounds like *Ph. chrysosporium* and PTUK; which was isolated from the soil and was suspected of being *Ph. chrysosporium*, and was confirmed after sequencing; or the selection of indigenous Zibar microbes since these will be acclimated to Zibar, like OMWW1 and OMWW2. Because the Indigenous Zibar microorganisms are interesting because

they are likely to be tolerant to the toxic components of Zibar and may be able to degrade it, while they may or may not produce LME.

In turn, Aggelis et al. (2003) showed that the problem with the use of a single culture to remediate Zibar is the competitive exclusion of the introduced strain by a population of well-acclimated indigenous flora. Because of that, these indigenous microorganisms were given great attention during the work because the orientation was to treat unsterilized Zibar, because sterilization results in significant changes to the chemical profile of Zibar. After sequencing these fungi were identified *Paecilomyces sp.* This species has been found to be useful in the bioremediation of dry olive residue (Sampedro et al., 2004), but they have not been used in many studies in the bioremediation of Zibar.

Many studies have resorted to adopting a sterilization Zibar before introducing microorganisms, utilize inputs as dilution, or expensive nutrients, which make the proposed treatment less industrially relevant. A 100% unsterilized Zibar was bioremediated during the study by four isolates *Ph. chrysosporium*, PTUK, OMWW1 and OMWW2. The biological degradation of polyphenol in Zibar was 58.6, 58.6, 26.63 and 63.69% respectively. On other hand the biomass of the isolates after 14 days of growth in Zibar were 19.4, 19.44, 18.08 and 20.6 g/L. This shows that the OMWW2 isolates were the best in the treatment and reduction of

polyphenol substances. However, we can't relate the increase of isolates biomass with the ability to remove toxic substances. Because it depends on their ability to produce byproducts that break down these substances. This requires a detailed study of the byproducts of each isolation separately.

Presently, various origins and natural organic wastes are widely used as amendments to increase soil organic matter and crop productivity. Therefore, this work was aimed to use the Zibar management strategy, which combined of detoxifying Zibar and utilizing it, at the same time, for producing valuable by-products. Where T Zibar has a very small amount of phenolic compounds and a significant amount of fertilizing elements such as P, K and Fe. T Zibar application in tomato fertilization showed very encouraging results, which may make its use in agriculture a potential solution for Zibar management and valorization.

The finding in this work confirmed that T Zibar did not show any significant inhibitory effect on seed germination where the germination percent of the seeds presented a high germination ratio (>83), with no significant difference between the germination ratio in control and 100% T Zibar. On other hand, the germination percent in untreated Zibar was 0%. Therefore, it can be suggested, that the germination inhibition is principally due to the phenolic compounds present in untreated Zibar.

An interesting result was born in this work, which confirmed that T Zibar can be used in the fertilization. The results of T Zibar application on tomato seedlings at different concentrations and compared with the control results encourage further study and research for the production of alternative fertilizers for chemical fertilizers. On the other hand, the result shows that the concentration 25% was the best in increasing the dry weights of seedlings shoot and root, in addition, the length of seedlings and the soil microflora. Therefore, it can be said that the remaining polyphenols in T Zibar have an unfavorable effect on the growth of the tomato seedlings and the soil microflora and these effects were decreased with dilution. In line with this finding, Peredes et al. (2000) reported also an increase in the total viable counts in the soil that was fertilized with T Zibar. Due to the virtue of considerable powers and ability to break down complex organic molecules, Actinomycetes play a significant role in the organic matter cycle in nature. Actinomycetes counts were strongly enhanced by T Zibar. The addition of organic pollutants, which can potentially act as nutrient sources and toxic substances, was shown to preferentially stimulate specific populations (Atlas et al. 1991). It was noted also that the addition of OMWW2 disks had a positive effect on the increase of microorganisms in the soil, contributed to increase the dry mass of the shoot, root and length in tomato seedlings. This is due to the fact that the fungus may analyze some of the substances present in the

soil to turn them into useful substances for bacteria. These fungi may in themselves be food for bacteria.

6 Conclusion

The Zibar produced during the extraction of olive oil constitutes a serious environmental problem, due to its phytotoxic, high in phenolic compounds, highly colored and malodorous. Aerobic bioremediation process was used in this study by using different fungal isolates to reduce the Zibar phenols, phytotoxicity and COD. It was noted that the bioremediation efficiency was increased, through the provision of the optimum temperature for each isolate. The optimum temperature for *Ph. chrysosporium* and PTUK was approximately 35°C, and 25°C for OMWW1 and OMWW2.

during the culturing in Zibar. Where the total phenol concentrations were reduced by (58.6, 58.61, 26.5, 63.69%) for *Ph. chrysosporium*, PTUK, OMWW1 and OMWW2 respectively. Also the COD reducing percent were (68.89, 68.89, 58, 63.89 %) for *Ph. chrysosporium*, PTUK, OMWW1 and OMWW2 respectively.

Several experiments were carried out on the fertilization with T Zibar in different concentration on tomato seed and seedlings, which established that this wastewater has a high fertilizer value when applied to the soil. Consequently, the controlled spreading of Zibar on agricultural soil can be considered as an alternative technique to the use of chemical fertilizers,

since it provides the soil with fertilizing substances, due to its high content of water, plant nutrients and organic matter.

7 Recommendations for further work

1. For consistency, the experimental work was necessarily carried out using a single batch of Zibar, considerable inter-batch compositional variability is expected with Zibar, and the ability of the organism to achieve similar reductions in other batches needs to be tested
2. It is recommended that Zibar be evaluated as an economic foundation for the production of organic fertilizers. By comparing it with available chemical fertilizer or by using it as a secondary fertilizer. In addition to study the ability to applied Zibar in many biotechnological applications. This is due to its important components that can be exploited in many fields rather than an environmental problem.
3. It is also important to conduct a detailed study on how these isolates can adapt in the Zibar environment, and how to bioremediate it. In order to enhance the bioremediation process and access to 100% polyphenol degradation.
4. In the end, it is important to conduct detailed studies on the possibility of applying the bioremediation processes in large economic and commercial quantities. In addition to finding ways to save the T Zibar as fertilizer and marketing it.

المعالجة البيولوجية للمياه العادمة لمعاصر الزيتون (الزيبار) باستخدام فطر
Phanerochaete chrysosporium وإمكانية استخدامه في الأغراض الزراعية

الطالبة: ساجدة حمدالله عويصات

المشرف: الدكتور مازن السلطان

ينتج عن عمليات إنتاج زيت الزيتون نفايات سائلة سوداء اللون، تسمى " المياه العادمة لمعاصر الزيتون " أو " الزيبار " ، والتي تخلق مشكلة بيئية كبيرة؛ وذلك لاحتوائها مستويات عالية من المركبات السامة للنباتات ومضادات الميكروبات، مثل: الفينولات أحادية البوليمرات، والأحماض المتطايرة، والكحوليات السكرية. لذا يعتبر الزيبار مادة سامة للنباتات والكائنات الدقيقة المتواجدة في التربة، بالإضافة الى أنه يؤثر على جودة التربة. تم اقتراح العديد من العمليات الفيزيائية والكيميائية لحل هذه المشكلة البيئية، مثل أحواض التبخر لكنها لم تكن فعالة في تقليل سميته العالية، وبالتالي التقليل من أثره السلبي على البيئة ، وتعود عدم فعالية العديد من الاقتراحات لأسباب اقتصادية وفنية. ولكن من ناحية اخرى تعتبر المعالجة البيولوجية باستخدام الكائنات الحية الدقيقة وسيلة بديلة متوافقة مع البيئة وأقل تكلفة.

في هذا العمل ، تم اختبار فعالية أربع عزلات فطرية في الحد من متعددات الفينول الكلية المتواجدة في الزيبار، حيث تم تلقيح دوارق مخروطية بحجم 125 مليلتر، يحتوي كل منها على 25 مليلتر

من الزيبار بخمس أقراص بقطر 7 ملم لكل منها، كل على حدة، ومن ثم وضعت الدوارق الملقحة على جهاز دوار (Rotary shaker) على سرعة 150 دورة في الدقيقة لمدة اسبوعين.

ومن اجل استخراج متعددات الفينول الكلية بعد اسبوعين من المعالجة، تمت إزالة الكتلة الحيوية الفطرية عن طريق الترشيح. ومن ثم، تم تخفيف 2.5 مليلتر من الزيبار المعالج إلى 50 % بالماء المقطر، ومن ثم ضبطت درجة الحموضة إلى 2، بواسطة استخدام حمض الهيدروكلوريك (HCL) 5M، واخيرا تم استخلاص متعددات الفينول من خلال الإيثيل (Ethyl acetate)، (1:1 ، ح/ح)، ووضع المستخلص بعد ذلك في كمة الدخان؛ ليتبخر الإيثيل لمدة 24 ساعة.

وقد تم إذابة البقايا في 2.5 مل من الميثانول وإضافة الماء المقطر للوصول إلى الحجم النهائي 25 مل، من ثم تم تحديد تركيز الفينولات الكلي طيفياً على طول موجي 725 نانوميتر، باستخدام كاشف فولين، وأظهرت نتائج هذا العمل أن جميع العزلات الفطرية كانت قادرة على النمو في الزيبار غير المخفف. الا ان عزلة واحدة فقط أظهرت انخفاضاً كبيراً في الفينولات الكلية في الزيبار المعالج، حيث كان تركيز الفينولات في الزيبار المعالج ب العزلة OMWW2، يساوي 57.75 ملغ / مل بالمقارنة مع 159.27 ملغ / مل في الزيبار غير المعالج. وكانت نسبة الانبات لبذور البندورة المنقوعة في الزيبار المعالج بهذه العزلة أعلى من 83% بدون وجود فروقات معنوية في نسبة الانبات بالمقارنة مع البذور المنقوعة بالماء. بينما كانت نسبة الانبات في الزيبار غير المعالج 0%. وقد تم التعرف على هذه العزلة عن طريق تحديد تسلسل الحمض النووي على أنها من نوع *Paecilomyces* ويتبع لعزلة OF-BTU-E1 وصنف العفونيات.

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