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Full Length Research Paper

Exploration of Phenol Degradation Capabilities in Microbes from Olive Mill Waste: A Phenotypic Analysis

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The growth of the olive oil production in Saudi Arabia particularly in Al Jouf region in recent years has been accompanied by an increase in the discharge of associated processing waste. Olive mill waste is produced through the extraction of oil from the olive fruit using the traditional mill and press process. Deterioration of the environment due to olive mill wastes disposal is a serious problem. When olive mill waste is disposed into the soil, it affects soil quality, soil micro flora and also toxic to plants. The aim of this work is to isolate microorganism (bacterial or fungal strains) from OMW capable of degrading phenols. Olive mill wastewater, olive mill waste and soil (beside oil production mill) contaminated with olive waste were used for isolation of phenol tolerant microorganisms. Four strains (two fungal and two bacterial) were isolated from olive mill waste. The isolated strains were *Candida tropicalis* and *Phanerochaete chrysosporium* (fungal strains) and *Bacillus* sp. and *Rhodococcus* sp. (bacterial strains). These strains were able to degrade phenols and could be used for bioremediation of olive mill waste.

Key words: Bioremediation, bacteria, fungi, Sakaka.

INTRODUCTION

In the last few years, human activities have changed a large number of ecosystems. Protection of the environment against the damage caused by contamination is of great importance. During the previous

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Abbreviations: OMW, Olive mill waste; **OMWW,** olive mill wastewater; **SOMW,** solid olive mill waste; **COD,** chemical oxygen demand; **BOD,** biochemical oxygen demand; **TSS,** total suspended solids; **TKN,** total Kjeldahl nitrogen; **TP,** total phosphorus.

years, different researches were carried out to study the risks of OMW pollution. More than 30 million cubic meters of olive oil has been produced by Mediterranean countries (Roberta and Giuseppe, 2012) which account for 95% of the total olive oil production worldwide (European Commission, Directorate-General for Agriculture and Rural Development, 2012). Olive mill waste (OMW) is the by-product generated during olive oil production (Mekki et al., 2009). Disposal of OMW is an environmental issue facing the olive oil producing countries due to the generation of huge quantities in a short period of time.

The growth of the olive oil production in Saudi Arabia particularly in Al Jouf region in recent years has been accompanied by an increase in the volumes of associated processing waste. Olive fruits contain only 2% of phenol (Rodis et al., 2002). OMW is phytotoxic (Saravanakumar et al., 2009) and has disastrous effect on human, so it causes a number of ecological and acute environmental problems (Maria et al., 2013). Earlier studies showed that, OMW has several advantages. It has antibacterial, antiviral and antifungal activities due to its phenolic content (Vagelas et al., 2009; Thabet et al., 2008; Cristina, 2006; Anna et al., 2011).

Previous studies revealed chemical characterization of OMW (Niaounakis and Halvadakis, 2006) and application of biological treatments for the reduction of its high organic carbon contents (Diamadopoulos and Paraskeva, 2006). Most of these studies described treatments based on the use of yeasts (Papanikolaou, 2008) or white rot fungi (Laconi, 2007). The screening degrading microbial communities that proliferate on OMW is so far not done in Saudi Arabia. In this study, the isolation and phenotypic characterization of OMW degrading microorganisms generated from olive oil mills in Al Jouf was done.

MATERIALS AND METHODS

Sample collection

Al Jouf is located in the north-western part of Saudi Arabia, 29° 58′ 11″ N, 40° 12′ 0″ E. Field trips were carried out to collect OMW during the period of September to October, 2013. Soil and solid olive mill waste samples were collected in clean and sterile plastic bags. The bags were appropriately sealed, labeled and dated. The samples were transported to the laboratory in ice and processed within 24 h of collection.

Physico-chemical analyses of OMWW

Samples were collected to study the physico-chemical characteristics of the OMW. The physico-chemical analyses included: pH, chemical oxygen demand (COD), biochemical oxygen demand (BOD), total suspended solids (TSS), total Kjeldahl nitrogen (TKN) and total phosphorus (TP). The analyses were carried out according to the American Public Health Association for Examination of Water and Wastewater (2006).

Sample culturing

Isolation of microorganisms was carried out by using three olive mill wastewater (OMWW), two soil mixed with olive mill waste and three solid olive mill waste (SOMW). 3 g of SOMW was inoculated into 45 ml on chemically defined medium (CDM). The contents of the CDM per liter were NH_4NO_3 (2.0 g), KH_2PO_4 (0.5 g), K_2HPO_4 (1.0 g), $MgSO_4.7H_2O$ (0.5 g), $CaCl_2.2H_2O$ (0.5 g), KCl (0.1 g), yeast extract (0.06 g), NaCl (5.0 g), Resazurin (0.0001 g), cysteine hydrochloride (0.5 g), trace element solution (10 ml), vitamin solution (10 ml) and phenol (3 mM), pH 7.2 ± 0.1 at 25°C. All components were added to distilled water and volume was brought up to 1.0 L except phenol. The medium was mixed thoroughly and gently heated until it dissolved and then autoclaved. After autoclaving, 3 mM phenol was added using Whitman's filter paper. After 5 days of incubation, the sample was subjected to serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) of SOMW made in sterile saline (0.85% w/v). Soil samples were incubated for five days before isolation of microorganisms for the possible isolation of a wide variety of microorganisms from soil. As nutrients become depleted or are made less available by the drying out of the soil, vegetative cells of some of the microorganisms could produce reproductive spores which can withstand a wider variety of deleterious conditions such as radiation and lack of nutrients and water. Like reproductive spores, endospores will germinate when growth conditions return, and generations of vegetative cells will again thrive as long as appropriate nutrients are available. So, our motive was to provide the favorable environment for the microorganisms to get enough nutrition and sufficient growth of microorganisms that are present in the soil. A 5 ml aliquot of each diluted sample was spread on chemically defined medium (CDM) agar (15-20% w/v) plates. Dilution of each soil sample was analyzed in triplicate. The plates were inverted and incubated for 3 days at 35°C. Results were recorded as colony forming unit (CFU). Colonies growing on the plates were counted and the density of microorganisms in the original sample was estimated by multiplying the colony count times the dilution. The single colonies were streaked onto nutrient agar plates, incubated at 35°C overnight and then the pure isolates were stored on Luria broth (LB) agar. Slant supplemented with phenol was used as sole carbon source at 4°C for future use. The same procedures were carried out using 5 ml of OMWW and soil mixed with olive mill waste. Malt extract media (Sigma-Aldrich) was used for isolation of fungi.

Characterization of isolates

Selected isolated colony samples were characterized by Gram-stain and by observed cell morphology (shape and size) using a Leica DMD108 digital microimaging, Leica Microsystems, Germany.

Characterization of isolates

Selected isolated colonies were characterized by Gram-stain and by observing cell morphology (shape) using a light microscope. To obtain various groups of isolates, colonies that showed different color plate morphologies, different colony size, shape and textures were chosen. Isolates were tested for the presence of the enzyme catalase by aseptically transferring a small amount of cells onto a glass slide and adding 2-3 drops of 3% H_2O_2. The observed production of bubbles was considered as positive test for catalase.

Table 1. Physico-chemical characteristics of OMWW.

Parameter	Unit	Minimum	Maximum	Average
pH		4.2	4.9	
COD	mgO_2/L	112,980	187,600	167,500
BOD	mgO_2/L	35,490	69,530	57,576
BOD/COD		0.31	0.37	0.34
TSS	mg/L	18,930	25,640	21,768
TKN	mgN/L	465	786	564
TP	mgP/L	118	154	134
Oil and grease	mg/L	4,532	6,435	5,213
Phenol	mg/L	2,143	2,879	2,543

COD, chemical oxygen demand; **BOD,** biochemical oxygen demand; **TSS,** total suspended solids; **TKN,** total Kjeldahl nitrogen; **TP,** total phosphorus

The phenotypic characteristics of all isolates studied were determined and compared with phenotypic data of known organisms described in the Bergey's Manual of Systematic Bacteriology. Varieties of biochemical tests were conducted according to the standard determinative bacteriology procedure (Beishier, 1991; Smibert and Krieg, 1994). Biochemical profiles for isolates include tests for fermentation of some carbohydrates and H_2S acid production. The number and types of positive tests were tabulated for the isolates and used to construct biochemical phenotype profiles of the cultures which were compared amongst the isolates.

RESULTS AND DISCUSSION

OMWW characteristics

Table 1 shows the characteristics of OMWW discharged during the production of oil. The pH of OMWW was found to be slightly acidic ranging from 4.2 to 4.9. The average concentration of organic load represented by COD, BOD and TSS was 167,500, 57,576 and 21,768 mg/l, respectively. The nutrient concentration of TKN and TP ranged from 465 to 786 and 188 to 154 mg/l with average of 564 and 134 mg/l, respectively. The average concentration of oil and grease and phenol was 5,213 and 2,543 mg/l, respectively.

The BOD/COD ratio of OMWW ranged from 0.31 to 0.37 with an average of 0.34. This result indicates that the biodegradability of OMWW was very poor. This may be attributed to the presence of phenolic compounds which hinder the activity of microorganisms.

Isolation and identification of microorganisms

Four strains of microorganisms were isolated from OMW and were screened for phenol degradation in Ramsay modified medium with 3 mM phenol at 35°C. The result for the screening of phenol degrading microorganisms is presented in Table 2.

The 4 strains which were able to degrade phenol are LW1A, OMSW2B, SW1A and SW2B. Out of the four strains, two were bacteria and two were fungi. LWI, OMSW2B, SWIA and SW2B were identified as *Candida tropicalis*, *Phanerochaete chrysoporium*, *Bacillus* sp. and *Rhodococcus* sp., respectively (Figures 1-4). *Rhodococcus* sp. (Figure 1) is non-motile, non-spore forming, aerobic Gram-positive filamentous rod bacteria.

P. chrysosporium (Figure 2) shows a pattern of septate hyphae, has some branching. At the end of the hyphae rests chlamydospores, the conidiophore gave rise to round asexual blastoconidia.

Candida tropicalis (Figure 3) shows the absence of terminal chlamydospores.

Bacillus sp. belong to genus Bacillus, Gram positive, rod shaped member of Firmicutes, catalase positive and oxidase negative bacteria.

It has been shown that the concentration of phenol is very important in order to determine the efficiency of phenol biodegradation. High concentration of phenol may contribute to toxic effects thus reduced biodegradation rates. However, low concentration of phenol below the threshold cannot support growth and degradation will not occur (Cornelissen and Sijm, 1996). Therefore, we recommended that, the study of different initial phenol concentration is very important in the determination of phenol biodegradation efficiency. According to Maria et al. (2013), phenol may act as a substrate and may also act as an inhibitor. Self-inhibition was also reported by Saéz and Rittmann (1991) where high concentration of substrate inhibits its own degradation. The utilization of phenol as sole carbon sources by microorganisms found in this study may probably be due to the presence of enzymes which is able to degrade phenol. The enzymes which are responsible for phenol degradation can also be studied by isolation, identification and characterization for further information. Continuous and fed-batch culture system can be used to study the performance of phenol

Table 2. Putative main characteristics of phenol degrading microorganisms isolated from Sakaka Olive Mill.

Feature	*Fungi*		*Bacteria*	
	Candida tropicalis	***Phanerochaete chrysosporium***	***Bacillus* sp.**	***Rhodococcus* sp.**
Morphology	*Oval*	septate hyphae	Rods	filamentous rods
Pigmentation	*None*	*None*	*None*	*None*
Gram stain	ND	ND	+	+
Motility	-	-	+	-
Oxidase	ND	ND	-	-
Catalase	ND	ND	+	+
Temp range (°C)	30-45	30-45	30-45	30-45
pH range	5-9	5-9	5-9	5-9
Acid production				
Sucrose	-	ND	-	-
Glucose	+	ND	+	+
Lactose	ND	ND	-	-
Maltose	+	ND	+	-
Mannitol	+	ND	-	+
Casein	+	ND	-	+
Starch	+	ND	-	-
Esculin	-	ND	+	+
H_2S production	ND	ND	ND	+

+ Positive, - negative, ND not determined.

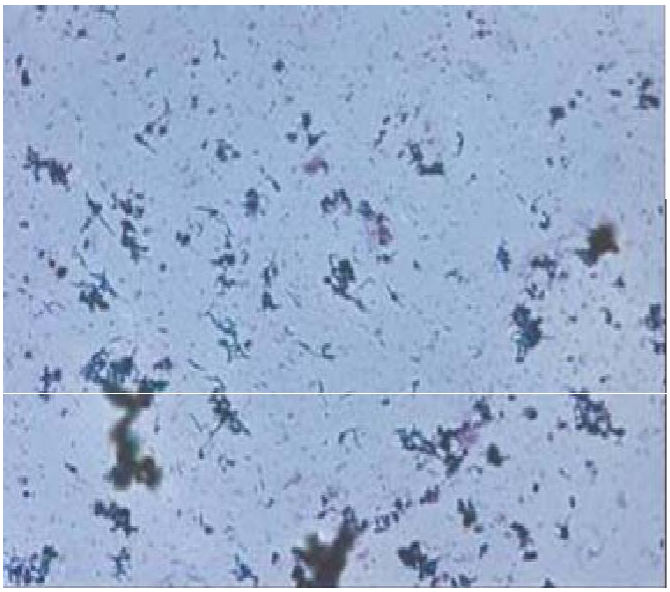

Figure 1. *Rhodococcus* sp. isolated from SW2B.

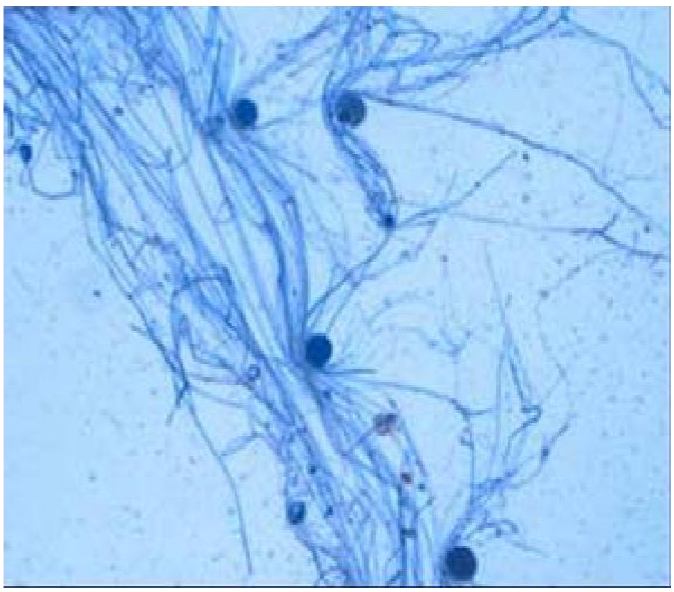

Figure 2. *Phanerochaete chrysosporium* isolated from OMSW2B

biodegradation in different fermentation modes. The incubation temperature of microorganisms is an important factor to determine the biodegradation efficiency of phenol. This is because at high temperature which is above optimum temperature, the microbial activity declined due to enzyme denaturation (Hiba et al., 2014). However, at low temperature below optimum, the microbial activity is relatively slow or no microbial activity because the movement of molecules is slower and there is not enough energy to start a chemical reaction.

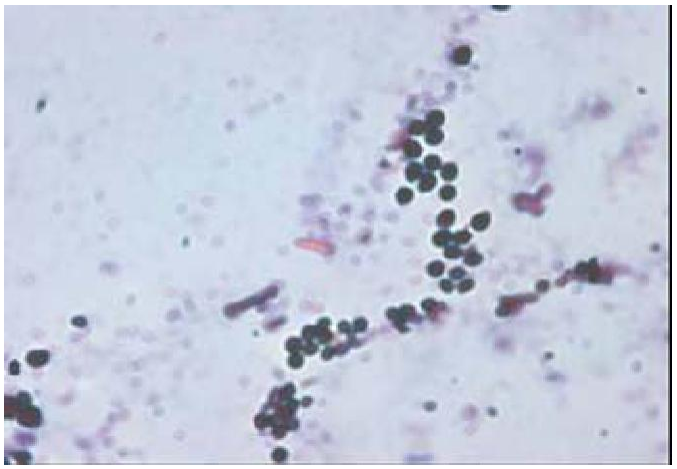

Figure 3. *Candida tropicalis* isolated from LW1A.

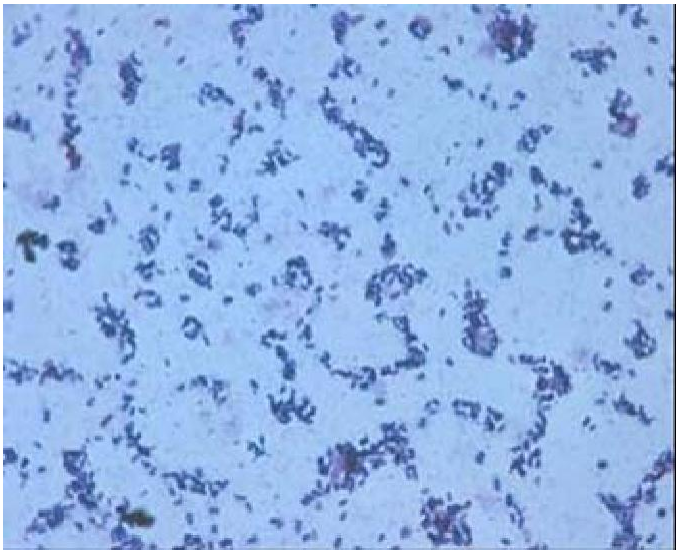

Figure 4. *Bacillus* sp. isolated from SW1A.

Conclusions

Bioremediation system of OMWW was conducted using microorganisms and phenol degrading bacteria grown in OMWW at the expense of its constituents and transformed into an organic liquid of high fertilizing value. Results from screening showed that 6 strains were able to grow in Ramsay medium and have capability to degrade phenol and its compounds. Phenol and its derivatives are organic pollutants which pollute the environment considerably. Therefore, the study of new approaches should be continued for this research in order to find out a suitable condition to degrade phenol.

The reaction of the degradation mechanism can also be studied in order to increase the degradation activity. Besides, the enzymes which is responsible for phenol degradation can also be studied by isolation, identification and characterization for further information.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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REFERENCES

Roberta S, Giuseppe I (2012). Environmental impacts of olive oil production: a Life Cycle Assessment case study in the province of Messina (Sicily). J. Clean. Prod. 28:88-100.

European Commission, Directorate-General for Agriculture and Rural Development (2012). Economic analysis of the olive sector, July.

Mekki A, Dhouib A, Sayadi S (2009) . Evolution of several soil properties following amendment with olive mill wastewater. Progr. Nat. Sci. 19:1515-1521.

Rodis PS, Karathanos VT, Mantzavinou A (2002). Partitioning of olive oil antioxidants between oil and water phases. J. Agric. Food Chem. 50(3):596-601.

Saravanakumar A, Venkateshwaran K, Vanitha J, Ganesh M, Vasudevan M, Sivakumar T (2009). Evaluation of antibacterial activity, phenol and flavonoid contents of Thespesia populnea flower extracts. Pak. J. Pharm. Sci. 22(3): 282-286.

Maria CC, Anna MG, Angela V (2013). Soil amendment with olive mill wastes: Impact on groundwater. J. Environ. Manage. 131:216-221.

Vagelas I, Kalorizou H, Papachatzis A, Botu M (2009). Bioactivity of olive oil mill wastewater against plant pathogens and post harvest diseases. Biotechnol. Biotechnol. Equip. 23(2):1217-1219.

Thabet Y, Ali R, Mohamed AT, Kamel G, Jalel B (2008). Control of damping- off caused by *Rhizoctonia solani* and *Fusarium solani* using olive mill waste water and some of its indigenous bacterial strains. Crop Prot. 27:189-197.

Cristina SR, Ana GR, Isabel P, Gemma JB, Francisco RM, Harry JW (2006). Microbiological effects of olive mill waste addition to substrates for Pleurotus pulmonarius cultivation. Int. Biodeterior. Biodegradation 57:37-44.

Anna P, Maria AR, Riccardo S, Liliana G (2011) Changes in soil chemical and biochemical properties following amendment with crude and dephenolized olive mill waste water (OMW). Geoderma 161:8-17..

Niaounakis M, Halvadakis CP (2006) . Olive processing waste management: literature review and patent survey. Elsevier, Amsterdam, 2nd ed.

Diamadopoulos E, Paraskeva P (2006). Technologies for olive mill wastewater (OMW) treatment: A review. J. Chem. Technol. Biotechnol. 81:1475-1485.

Papanikolaou SF (2008). Biotechnological valorisation of raw glycerol discharged after bio-diesel (fatty acid methyl esters) manufacturing process: Production of 1, 3- propanediol. citric acid and single cell oil,

Biomass Bioenergy. 32: 60-71.
Laconi RM (2007). Bioremediation of olive mill wastewaters and production of microbial biomas. Biodegradation. 18: 559-566.
APHA, AWWA, WEF (2006). Standard Methods for the Examination of Water and Wastewater, 21st ed., American Public Health Association, Washington, DC.
Beishier L (1991). Microbiology in Practice (5th ed.)", New York: Harper Collins.
Smibert RM, Krieg NR (1994). Phenotypic characterization, In: Manual of Methods for General Bacteriology, P. Gerhardt, R.G.E. Murray, W.A. Wood and N.R. Krieg (Eds.), Washington, D.C.: Am. Soc. Microbiol.
Cornelissen G, Sijm DTHM (1996). An energy budget model for the biodegradation and catabolism of organic substances. Chemosp. 33(5):817-830.
Saéz PB, Rittmann BE (1991). Biodegradation kinetics of 4-chlorophenol, an inhibitory co-metabolite. Wat. Pollut. Contr. Fed. 63:838-847.
Hiba AT, Naim N, Carlos D, Ahmed T, Hassan A (2014). Potential of bioethanol production from olive mill solid wastes. Bioresour. Technol. 152:24-30.