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Olive-pomace harbors bacteria with the potential for hydrocarbon-biodegradation, nitrogen-fixation and mercury-resistance: Promising material for waste-oil-bioremediation

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ABSTRACT

Olive-pomace, a waste by-product of olive oil industry, took up >40% of its weight crude oil. Meanwhile, this material harbored a rich and diverse hydrocarbonoclastic bacterial population in the magnitude of 10^6 to 10^7 cells g^{-1}. Using this material for bioaugmentation of batch cultures in crude oil-containing mineral medium, resulted in the consumption of 12.9, 21.5, 28.3, and 43% oil after 2, 4, 6 and 8 months, respectively. Similar oil-consumption values, namely 11.0, 29.3, 34.7 and 43.9%, respectively, were recorded when a $NaNO_3$-free medium was used instead of the complete medium. Hydrocarbonoclastic bacteria involved in those bioremediation processes, as characterized by their 16S rRNA-gene sequences, belonged to the genera *Agrococcus*, *Pseudomonas*, *Cellulosimicrobium*, *Streptococcus*, *Sinorhizobium*, *Olivibacter*, *Ochrobactrum*, *Rhizobium*, *Pleomorphomonas*, *Azoarcus*, *Starkeya* and others. Many of the bacterial species belonging to those genera were diazotrophic; they proved to contain the *nifH*-genes in their genomes. Still other bacterial species could tolerate the heavy metal mercury. The dynamic changes of the proportions of various species during 8 months of incubation were recorded. The culture-independent, phylogenetic analysis of the bacterioflora gave lists different from those recorded by the culture-dependent method. Nevertheless, those lists comprised among others, several genera known for their hydrocarbonoclastic potential, e.g. *Pseudomonas*, *Mycobacterium*, *Sphingobium*, and *Citrobacter*. It was concluded that olive-pomace could be applied in oil-remediation, not only as a physical sorbent, but also for bioaugmentation purposes as a biological source of hydrocarbonoclastic bacteria.

1. Introduction

Oil spills have become frequent all over the globe; they result in serious pollution of the marine and terrestrial ecosystems. Such spills are associated with conventional activities, e.g. oil production, oil transport and oil use as energy source, and accidents and illegal activities, e.g. waste disposal, military conflicts and others. To record but one estimate, alone the marine environment becomes charged yearly with 10 Mt of hydrocarbon pollutants (Banerjee et al., 2006).

Physical and microbiological approaches may be used to remediate oil-polluted sites. Physical remediation involves using oil-blooms, dispersants, skimmers, sorbents and others (Wardley-Smith, 1983). Physical techniques are not always environmentally friendly. For example, most of the dispersants are inflammable, toxic and lead to fouling of coastal areas (National Research Council, 1989). On the other hand, removing oil physically by using sorbents seems to be environmentally friendlier (Banerjee et al., 2006; Jong-Sik et al., 2002; Husseien et al., 2009).

Commercially, there are effective synthetic sorbents made of polypropylene (Teas et al., 2001), yet their resistance to biodegradation poses an environmental problem (Choi and Cloud, 1992; Deschamps et al., 2003). Natural sorbents e.g. barley straw (Husseien et al., 2009) and sawdust (Ali et al., 2011) are environmentally more friendly. These natural sorbents are rich in lignin and cellulose (Erikson et al., 1990; Alexander, 1997). Lignin is a complex polymer based on phenyl propane i.e. aromatic and aliphatic residues. In an earlier study, our group found that such lignified sorbents harbor communities of hydrocarbonoclastic bacteria (Ali et al., 2011).

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Bioremediation is the biotechnology in which microorganisms are used to degrade xenobiotic compounds including hydrocarbon pollutants (Atlas and Pramer, 1990; Radwan, 2009). This biotechnology essentially involves two approaches; inoculating oily environments with hydrocarbonoclastic bacteria (seeding or bioaugmentation), and enhancing activities of the indigenous, hydrocarbonoclastic microflora using fertilizers (biostimulation). For bioaugmentation, commercial consortia of hydrocarbonoclastic microorganisms are available (Radwan, 2009; Applied Biotreatment Association, 1989, 1990).

In this study, we show that olive-pomace, a natural by-product of olive oil industry, is an effective crude oil sorbent. Meanwhile, this natural product harbors a rich and diverse bacterioflora making it in addition, a suitable inoculum for waste-oil-removal via bioaugmentation.

2. Material and methods

2.1. Olive-pomace

In definition, Olive-pomace is the brown solid residue resulting from the olive oil production process by mechanical pressing of the olive fruits without any chemical treatment. It consists of olive skin, mainly hydrocarbons and waxes, seed and other solid parts of the olives, mainly lignins, celluloses, and glycans, in addition to 5–8% of residual oil, proteins and carbohydrates (La Rubia-García et al., 2012; Ferhat et al., 2014). The material sample used in this study was obtained from South Lebanon. In this context, olive pomace has been suggested for fish and animal feeds; its environmental and nutritional implications for aquaculture and agriculture industries are recognized (for review see Nasopoulou and Zabetakis, 2013).

To determine the oil-sorbing capacity of olive-pomace, known weights of this material were submerged in excess crude oil (Kuwaiti light crude) for 15 min. The material was filtered using cellulose filter papers until no crude oil drained down. The olive-pomace samples with the associated crude were removed and weighed. The oil holding capacity was expressed as % increase in weight based on the olive-pomace weight. Triplicates were done and mean values were calculated.

2.2. Experimental set-up

Oil-consumption and microbiological analysis were recorded for pristine, i.e. fresh, untreated olive-pomace samples, as well as for oily samples. The latter were prepared by submerging the material in excess oil and subjecting it to filtration until no crude oil drained down, as described above.

To determine oil-consumption by microorganisms associated with the pristine samples, 1 g aliquots of fresh olive-pomace were inoculated into 100 ml portions (dispensed in 250 ml conical flasks) of a mineral medium containing 1.0%, w/v, crude oil, as a sole source of carbon and energy (Sorkhoh et al., 1990). The mineral medium had the following composition (g l^{-1}): 5.0 $NaNO_3$, 0.56 KH_2PO_4, 0.86 Na_2HPO_4, 0.17 K_2SO_4, 0.37 $MgSO_4.7H_2O$, 0.007 $CaCl_2.H_2O$, 0.004 Fe (III) EDTA; and trace element solution (25 ml l^{-1}) consisting of (g l^{-1}): 2.32 $CuSO_4.5H_2O$, 0.39 Na_2MoO_4. $2H_2O$, 0.66 KI, 1.0 EDTA, 0.4 $FeSO_4.7H_2O$, 0.004 $NiCl_2.6H_2O$. The medium pH was adjusted to 7. Triplicate flasks were prepared throughout. The flasks were sealed to prevent oil volatilization, and incubated onto an electrical shaker, 120 rpm, at 30 °C for 8 months. Triplicate flasks were collected at time zero and in 2 month-intervals for residual oil determination and microbiological analysis. To study the effect of nitrogenous compounds on oil-removal, this experiment was repeated using the above mineral medium after removing $NaNO_3$, the only nitrogen source available.

To determine oil-consumption by the oily olive-pomace sample the latter was incubated aseptically sealed at 30 °C for 8 months. Triplicate samples were collected at time zero and in 2-month determination intervals for oil and microbiological analysis.

2.3. Oil-consumed by the olive-pomace associated microflora

The residual oil in the culture media and in association with the oily olive-pomace samples was recovered by extraction using three 15 ml aliquots of pentane. The combined pentane extract was completed to 50 ml using pure pentane and 1 μl was analyzed by gas liquid chromatography (GLC). The GLC analysis of hydrocarbons was done using a Varian 3900 (USA) instrument equipped with a FID, a WCOT-fused silica CP-SIL-5 CB capillary column (Varian, USA) and a temperature program 45–310 °C with temperature rising 10 °C min^{-1}, using N_2 as a carrier gas. The detector temperature was 300 °C and injector temperature 270 °C. The percentage of hydrocarbon-consumption was calculated as the percentage reduction in total hydrocarbon peak areas based on the total areas of peaks of hydrocarbons recovered from the time zero aliquots (controls).

2.4. Culture-dependent analysis of the hydrocarbonoclastic bacterioflora

To count and analyze the hydrocarbonoclastic bacteria in the medium and olive-pomace aliquots, the conventional dilution plating method was used. For this, the mineral medium (Sorkhoh et al., 1990) with crude oil vapor as sole source of carbon and energy, was used. To prepare the nitrogen-free medium, $NaNO_3$ was omitted, and to study the effect of the heavy metal mercury, 10 mg/l $HgCl_2$ was added to the complete medium as a source of mercury ions.

The media were solidified with 2.0%, w/v, agar and the pH was adjusted to 7. Crude Oil-impregnated sterile filter papers placed in the lids of the dish covers made the volatile hydrocarbon phase available to the developing colonies. The plates were sealed and incubated at 30 °C for 10 days. Three replicate plates were prepared for each dilution. The total colony numbers were counted, and numbers of colony-forming units (CFU) per g of sample were calculated. The three replicate plates of dilutions were pooled and identical bacterial colonies therein were recognized according to their colony characteristics, cell shapes, sizes and Gram stain reaction. Identical colonies were counted, their proportions of the total CFU calculated and representative colonies were subcultured, purified and maintained for further study.

The purified bacterial isolates were characterized according to their 16S rRNA-gene sequences. For this, the total genomic DNA was extracted from fresh (24–48 h) cultures using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, USA) and the 16S rRNA-genes therein were amplified by polymerase chain reaction (PCR). The PCR mixture contained puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, UK), 1 μl (25 ng) of DNA template, 1 μl each of the universal primers GM5F (5′-CCT ACG GGA GGC AGC AG-3′) and 907R (5′-CCG TCA ATT CMT TTG AGT TT-3′) (Santegoeds et al., 1998). The reaction volume was completed to 25 μl with molecular water (Sigma). Amplification was done in the Veriti Thermal Cycler (Applied Biosystems, USA) using a touch-down PCR in which the annealing temperature started at 65 °C and decreased by 1 °C every cycle to 55 °C, at which additional 12 cycles were carried out, denaturation was at 94 °C for 1 min, and primer extension at 72 °C for 1 min. The initial denaturation was at 95 °C for 5 min and the final extension was at 72 °C for 7 min. The PCR products were purified using the QIA quick PCR purification kit (Qiagen, USA). Partial sequencing of the 16S rRNA-genes was done using the