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ORIGINAL ARTICLE

Assessment of biotechnological potentials of strains isolated from repasso olive pomace in Tunisia

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Abstract

Purpose The agri-food industry generates significant amounts of byproducts, among them repasso olive pomace (ROP) which are rejected and can constitute a serious environmental problem. Our study aimed to investigate the diversity of microbiota isolated from ROP and screen for their biotechnological potentials.

Methods A collection of 102 strains (88 bacteria and 14 fungi) was obtained from a ROP sample. The diversity of the bacterial collection was evaluated by amplification of the internal transcribed spacers between the 16S and the 23S rRNA genes (ITS-PCR) and by 16S rRNA sequencing. Fungal identification was performed by polymerase chain reaction (PCR) amplification of the ITS1–5.8S–ITS2 ribosomal DNA region. The specific enzymatic screening of the detected microorganisms was tested.

Result Partial 16S rRNA gene sequencing performed on 44 isolates showed a high level of identity with known strains. Fungal strains identification showed that they belong to four families: *Trichocomaceae*, *Pleurostomataceae*, *Mucoraceae*, and *Bionectriaceae*. Our results demonstrated that Gram-positive bacteria were mostly active, particularly for protease, lipase, amylase, cellulase laccase, and for biosurfactant production. From the 88 isolated bacteria, *Firmicutes* were the most prevalent and microdiverse. *Bacillus* and *Paenibacillus*, together with some other Gram-negative bacteria such as *Pseudocitrobacter anthropi*, and *Acinetobacter johnsonii* showed significant hydrolytic activities and biosurfactant production. The 14 isolated fungi showed a high capacity of enzyme production. This is the first study in Tunisia describing the microbial diversity in ROP as well as the isolation of *Bacillus mojavensis* producing lipase.

Conclusion Microorganisms especially fungi present in the repasso olive cake produce diverse hydrolytic enzymes of industrial interest.

Keywords Bacteria · Fungi · ROP · Biotechnological activities

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Introduction

The use of by-products and agricultural waste for recovery has benefited in recent years from a renewed interest in economic and environmental reasons (Orlando et al. 2002). The value of these agricultural by-products resides in their abundance and low cost. However, today, the global industries use less than 10% of the plant biomass produced (Liu et al. 2008) and significant amounts of agricultural residues are burned every year (Sun et al. 2002).

In Tunisia, the olive oil sector is a major component of the agriculture and food sectors. It contributes to the achievement of national goals of economic growth, food security, and job creation, and increases export earnings, and preservation and enhancement of natural resources. Due to the existence of modern techniques using a three-phase system for extraction,

the increase in olive oil production is placed in a delicate position of potential polluter (Molina-Alcaide and Yáñez-Ruiz 2008). Indeed, the olive industries in Tunisia leave two residues, the liquid (the vegetable) and the solid (olive pomace). This requires a specific management of these wastes to minimize and attenuate their impact on the environment. In the microbiological field, the solid residues (olive pomace) are characterized by a high organic content. The latter may inhibit seed germination and hamper the growth of methanogenic bacteria. In addition, it may halt the conventional secondary and anaerobic treatments in wastewater treatment plants (Sassi et al. 2006).

The solid residues are generally used for oil production by organic solvent extraction (hexane) (Amarni and Kadi 2010), as fertilizer or combustible or as supplement for animal feed (Fadel and El Ghonemy 2015).

Recent studies shed light on the diversity of the microorganisms (bacteria and fungi) isolated from olive pomace. This includes species of the genera *Agrococcus*, *Pseudomonas*, *Cellulosimicrobium*, *Streptococcus*, *Sinorhizobium*, *Olivibacter*, *Ochrobactrum*, *Rhizobium*, *Pleomorphomonas*, *Azoarcus*, *Starkeya*, and other bacteria (Dashti et al. 2015), whereas fungi are mainly affiliated to the genera *Phellodon*, *Lachancea*, *Saccharomyces*, and *Candida* (Romo-Sanchez et al. 2010). In addition, several reports describe the production of various enzymes using olive pomace as a substrate in solid-state fermentation (SSF) or as a supplement in the production medium (Anvari and Khayati 2011). Olive pomace is an ideally suited nutrient support in SSF that provides carbon and nitrogen sources and may constitute the appropriate substrate for enzyme production by fungal species. Therefore, the optimization of physiological and biological conditions could enhance enzyme production (Anvari and Khayati 2011). Moreover, recent studies reported the use of the olive pomace for (1) the production of biofuels such as biochar and biodiesel (Lama Munoz et al. 2014), (2) as a substrate for SSF (Anvari and Khayati 2011), and (3) for the recovery of phenolic compounds (Cardoso et al. 2005).

In Tunisia, during the last 10 years, the repasso units of three phases of olive pomace were developed. They re-extract the oil from these residues by the establishment of a second decanter. This technique is less expensive than the extraction of the oil pomace with hexane. The repasso process allows recovering 40% to 50% of the olive oil remaining in the pomace, thus increasing profitability. The process is based on several steps: (1) cleaning the olive pomace, (2) separation between stones and pulp, (3) malaxation, (4) separating the oil from the water and solids by the decanter, and (5) centrifugation of the oil pomace.

The repasso system also produces two wastes, the solid (repasso olive pomace, ROP) and the liquid. It is interesting to note that the quantity of the by-product produced varied between 60,000 and 80,000 tons/year in Tunisia. It was used for the production of oil by hexane extraction after drying and the final solid residue (ROP) was an excellent fuel solution for biomass boilers. ROPO is a by-product resulting from the re-extraction of pomace olive oil produced by three-phase-system oil mills. The originality of this work comes from the fact that we have put evidence of the existence of microorganisms in this ROP with enzymatic activities that have never been demonstrated before. In fact, the physicochemical properties and microbiological diversity of the ROP have been performed for the first time in this study, in addition to the evaluation of the biotechnological potential of these strains by screening and quantifying their abilities to produce extracellular enzymes and biosurfactants.

Materials and methods

Sample collection

The ROP (one sample) collected from the mixture of three olive cake samples was obtained from repasso units in a Tunisian coastal city, Mahdia. Samples were collected into sterile bags and kept aseptically at 4 °C until analyzed.

Chemical analysis

Dry content: in order to determine the water content, the sample (45 g) was dried for 12 h in an oven at 105 °C. The loss of weight gave the amount (%) of water and volatile matter in the sample. Fat content: the dried sample was then used to measure the oil content by Soxhlet extraction. The oil was extracted with hexane for 6 h using the Soxhlet extractor and the oil content was then determined gravimetrically (Neifar et al. 2013). Ash content: the fiber samples were ignited and incinerated in a muffle furnace at 550 °C for 16 h to remove carbon. The total ash content was expressed as percentage of dry matter. The determination of heavy metals was carried out by atomic absorption spectrophotometry (Alburquerque et al. 2004).

Molecular analysis of bacterial and fungal strains

Isolation of bacteria and fungi

Isolation of bacteria was performed by serial dilution of sample (ROP) using minimal medium (Mm) containing (%) 0.07 K_2HPO_4, 0.03 KH_2PO_4, 0.05 $MgSO_4$, 0.01 $MnCl_2$, 0.025 $(NH_4)_2SO_4$, 0.001 $FeSO_4$ $7H_2O$, and 0.01 $CaCl_2$ and supplemented with 1% olive oil as the sole carbon source for the isolation of lipid-degrading bacteria. The pH was adjusted to 7.0. For the enrichment medium, 10 g of each sample were

added to Erlenmeyer flasks containing 100 ml of Mm in the presence of 1% olive oil. Flasks were then incubated for 3 days at 30 °C on a rotary shaker. After a serial dilution in Mm agar with 1% olive oil, plates were incubated at 30 °C for 3 days. Colonies growing on the plates were selected based on their morphological features, considering pigmentation and size. Each isolate was subjected to successive streak plating until a pure colony was obtained. The isolates were stored in glycerol stocks (25% *v*/*v*) at − 80 °C.

Fungal colonies were isolated form ROP by serial dilution method. One milliliter of sample diluted up to 10^{-5} was spread on respective solidified PDA (potato dextrose agar). The inoculated Petri plates were incubated at 30 °C for 3–4 days. The isolates were grouped based on their morphological structures such as color, texture of the mycelia, and spore formation.

DNA extraction and PCR conditions

Genomic DNA of bacteria was extracted by sodium dodecyl sulfate–proteinase K treatment. Amplification of the 16S–23S ITS region and the 16S rRNA gene was performed by using for ITS the universal primers S-D-Bact-1494-a-20, L-D-Bact-0035-a-15 and for 16S: 50-S-D-Bact-0008-a-S-20- 3′ and 5′-S-D-Bact-1495-a-S-20-3′ (Mahjoubi et al. 2013).

The reaction mixture consisted of 1× PCR reaction buffer, 2.5 mM $MgCl_2$, 0.12 mM deoxynucleoside triphosphate, 0.2 mM of each primer, 1 U Taq DNA polymerase, and 1 μl of total DNA. The PCR program consisted of an initial step at 94 °C for 3 min, 35 cycles (94 °C for 45 s, 55 °C for 1 min, and 72 °C for 2 min), followed by final step at 72 °C for 8 min. The ITS-PCR amplification patterns and 16S products were visualized on standard 2% and 1.5% agarose gels in 0.5× Tris–borate–EDTA buffer, respectively.

The genomic DNA of fungi was recovered from scrapped mycelia using i-Genomic BYF DNA Extraction Mini Kit (Intron Biotechnology, Inc.) according to the manufacturer's instructions. The quality of genomic DNA was evaluated using a NanoDrop spectrophotometer (Thermo Scientific) and followed by 1% (*v*/*v*) agarose gel electrophoresis. The polymerase chain reaction (PCR) amplification was conducted using primers for internal transcribed spacer ribosomal DNA (ITS rDNA): ITS 1F (forward primer 5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS 4 (reverse primer 5′-TCC TCC GCT TAT TGA TAT GC-3′). The 20-μl PCR reaction mixture contained 4 μl of buffer, 1.2 μl of $MgCl_2$, 2 μl of dNTP, 0.6 μl of each primer, 10.4 μl of distilled water, and 1 μl of DNA sample. The PCR was performed using PCR Thermo Cycler as follows: initial denaturation at 94 °C for 1 min followed by 35 cycles for each denaturation (94 °C for 1 min), annealing (51 °C for 1 min), and extension (72 °C for 1 min). The last stage was the final extension at 72 °C for 8 min and cooling to 10 °C. Then, these PCR products were analyzed on gel electrophoresis using 1% (*v*/*v*) agarose gel that was run at 100 V for 30 min. The gel was stained with ethidium bromide and visualized on a UV light transilluminator.

DNA sequencing and phylogenetic analysis

DNA sequencing of bacterial 16S rRNA and the amplified product of fungi has been performed using the automated capillary ABI Biosystems 3130.

The obtained sequences were compared with the available sequences at the National Center for Biotechnology Information database (NCBI) (www.ncbi.nlm.nih.gov) using the basic local alignment search tool (BLAST). Phylogenetic tree was constructed by the neighbor-joining method and branch support was evaluated by non-parametric bootstrap using MEGA software v.6 (Tamura et al. 2011).

Screening for extracellular hydrolytic activities

For bacterial strains, the extracellular protease production was performed by streaking colonies onto Mm agar plates supplemented with 1% skim milk. A clear zone around the colony after 3 days of incubation was taken as evidence of proteolytic activity (Pérez Borla et al. 2009). Amylase activity was performed according to the method described by Amoozegar et al. (2003). The strains were screened for their ability to hydrolyze starch on Mm supplemented with 1% soluble starch as substrate. After incubation at 30 °C for 5 days, the plates were flooded with 1% Lugol solution. A clear zone around the colony indicated the hydrolysis of starch. Cellulase production was determined by plating colonies onto Mm plates containing 1% carboxymethylcellulose (CMC). The plates were incubated for 3 days at 30 °C. The colonies were washed with distilled water before staining step using 0.2% Congo Red, followed by destaining with 1 M NaCl (Cojoc et al. 2009). A positive cellulase activity was detected by the formation of a yellow halo against a red background. To detect laccase activity, one colony was spread on minimal agar medium supplemented with 0.01% of 2,6-dimethoxyphenol (DMP) (Wan et al. 2008). The pH was adjusted to 7.4 before incubation at 121 °C for 15 min. The plates were then incubated at 30 °C for 3–4 days. The presence of brick color around the colonies was considered as DMP oxidizing laccase-secreting organism (Neifar et al. 2016).

Qualitative lipase activity assay was evaluated on rhodamine B agar (Mazzucotelli et al. 2013). The plate assay was used to detect bacterial lipase in a Mm containing olive oil and the florescent dye rhodamine B. Growth medium was adjusted to pH 7.0, incubated, and cooled at 60 °C. Then 1% of olive oil and 10 ml of Rhodamine B solution (0.001% *w*/*v*) were

added with vigorous stirring and emulsified by mixing for 1 min. The medium was poured into a Petri dish. The overnight culture was spotted in the center of the plate and incubated for 72 h at 30 °C. Lipase production was monitored by irradiating plates with UV light at 350 nm. The bacterial culture with "lipases" presented an orange fluorescence (Mazzucotelli et al. 2013).

For fungal strains, the hydrolytic activities were evaluated using plate assay method as described previously (Namasivayam and Nirmala 2013). CMC, DMP, olive oil, starch, and skim milk were used as substrates for cellulase, laccase, lipase, amylase, and protease, respectively.

For all extracellular hydrolytic activities, the diameter of the halo was measured in millimeters.

Screening of biosurfactant production

Biosurfactant production was screened by three distinct methods: CTAB agar method, drop collapse test, and oil spreading test.

CTAB agar method

Mineral Salts Agar was supplemented with 2% (*w*/*v*) glucose as carbon source, 0.5 mg/ml cetyltrimethylammonium bromide, and 0.2 mg/ml methylene blue (Siegmund and Wagner 1991). Fifty microliters of the culture supernatant was poured into a well and punctured into the plate using a clean cork borer. The agar plates were incubated for 48–72 h at 30 °C. The presence of dark blue halo around the well indicates the probable secretion of biosurfactant.

Drop collapse test

The second biosurfactant screening method is the drop collapse test. In order to carry out this test, 7 μl of mineral oil was added to each well of a 96-well microtiter plate. Covered plates were equilibrated for 24 h at room temperature before adding culture supernatant. To induce biosurfactant production, bacterial isolates were cultivated on liquid minimal medium supplemented with 2% glucose. To test the presence of biosurfactant, 20 μl of the culture supernatant was added on the oil surface in the microtiter well. SDS with the sterile distilled water was used as a control suspension (Tugrul and Cansunar 2005).

Oil spreading test

The selected strains were compared by measuring the diameter of the clear zones that occurred when a drop of a biosurfactant-containing solution is placed on an oil–water surface. Fifty milliliters of distilled water was added to a large Petri dish followed by the addition of 20 μl of crude oil to the surface of water and 10 μl of supernatant of culture broth. The diameters of clear zones of triplicate assays from the same sample were determined (Anandaraj and Thivakaran 2010).

Results and discussion

This recent study of ROP provides new results for physicochemical and microbiological characterization.

Data presented in Table 1 revealed that the crude ROP is characterized by a low protein content (6.56%), relatively high fat content (5.90%), and high ash content (13%). This chemical composition is different from what has been previously reported by Neifar et al. (2013) in which the fat and ash contents represent 3.3% and 7%, respectively. In addition, metallic ion content (< 0.01 mg/kg) was lower than what has been described in the studies of Alburquerque et al. (2004) with metallic ion content of > 1 mg/kg.

ROP represents an important source of nitrogen and carbon which can be used as a culture medium or as a substrate for the production of enzymes, such as lipase production, due to its elevated fat content (5.90%). It could also serve as a good conductor for the removal of heavy metals (Khiari et al. 2019; Oliveira et al. 2018).

Isolation and identification of bacterial isolates

The results of the present study show the identification of 88 isolated bacteria from ROP. The diversity of this collection was evaluated by amplification of the ITS between the 16S and the 23S rRNA genes (ITS-PCR) followed by 16S rRNA sequencing.

ITS regions represent highly conserved sequences in the areas encompassing the rRNA genes and relatively stable regions located at the ends of the spacer. Within the whole bacterial collection, ITS-PCR fingerprinting revealed 44 distinct haplotypes (H1–H44) (Fig. 1). Each ITS type was represented

Table 1 Chemical composition of the repasso olive pomace (ROP)

Component	Contents (% dry matter)
Dry matter	42.62
pH	5.14
Crude protein	6.56
Ash	13
Crude fat	5.90
Heavy metals: Cu, Fe, Ni, Cd, Cr, Pb, Hg, and Zn	< 0.01 (mg/kg)

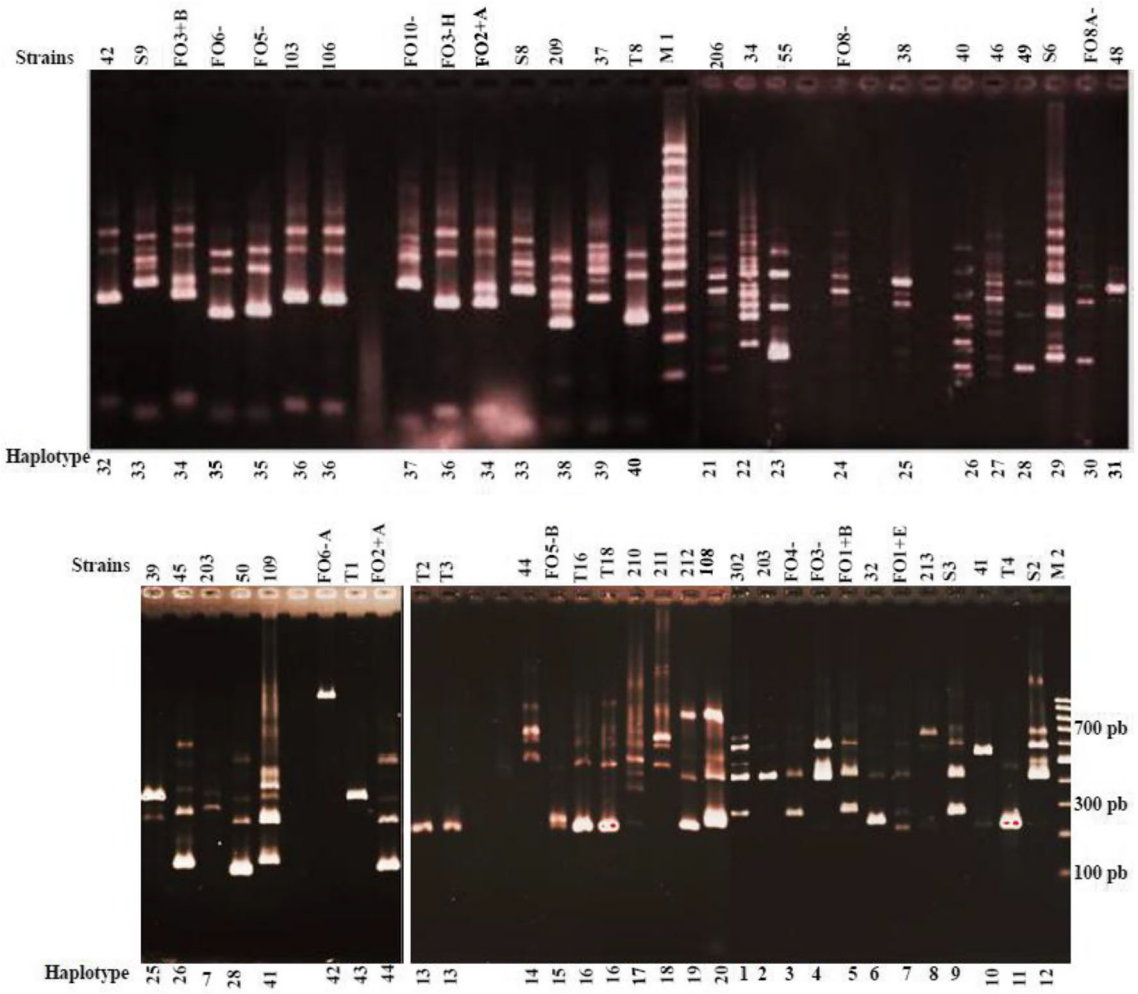


Fig. 1 Results of the amplified internal transcribed spacers 16S–23S rDNA: ITS-PCR analysis; M1: 1 kb; M2: 100 bp

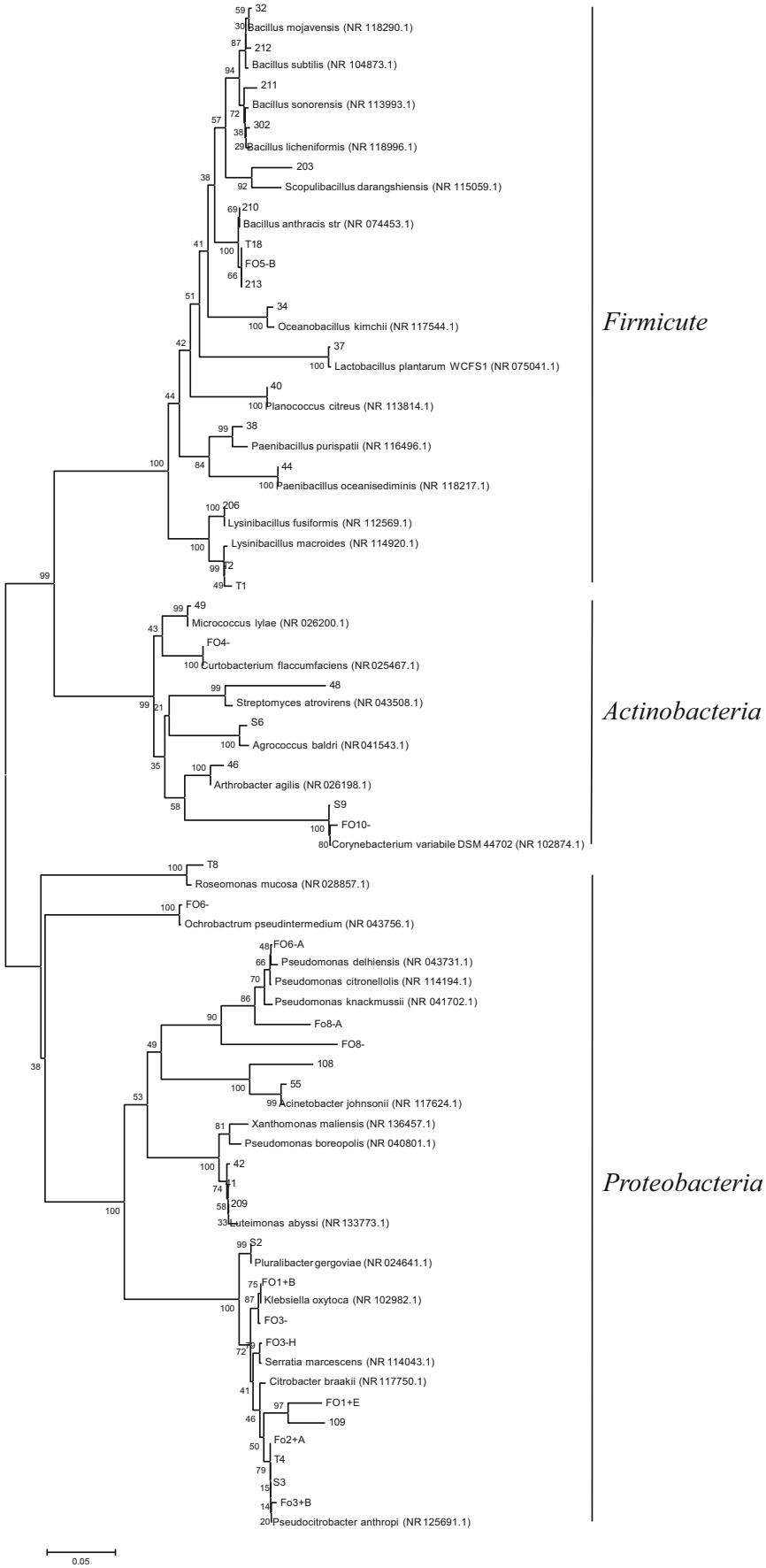
by 1 up to 14 reproducible bands showing an apparent molecular weight (MW) ranging from 150 base pairs (bp) to about 2000 bp. The most abundant pattern was haplotype H1, found in six isolates and composed by five reproducible bands of 300 to 1500 bp. The remaining ITS types are presented by one or two isolates.

The phylogenetic analysis highlighted the predominance of Gram-positive bacteria (54.54%) belonging essentially to *Firmicutes* (38.63%) and *Actinobacteria* (15.9%) classes. The remaining isolates were affiliated to the *Gammaproteobacteria* (40.9%) and *Alphaproteobacteria* (4.54%) subclasses. Previous studies with olive-mill waste have also reported high bacterial diversity consisting of members of *Firmicutes* and *Proteobacteria* followed by *Actinobacteria* and *Bacteroidetes* (Tsiamis et al. 2012; Ntougias et al. 2013).

More specifically, *Firmicutes* were dominated by representatives from the families of *Bacillaceae* (12), *Paenibacillaceae* (2), *Sporolactobacillaceae* (1), *Lactobacillaceae* (1), and Planococcaceae (1). *Actinobacteria* was represented exclusively by members of the order Actinomycetales and dominated mainly by the *Micrococcaceae* (2), *Microbacteriaceae* (2), *Corynebacteriaceae* (2), and *Streptomycetaceae* (1) families (Fig. 2).

The strains were allocated into 23 different genera of variable occurrence (Fig. 2), showing a high genetic diversity in the olive cake sample. *Bacillus* was the most dominant genera in Gram-positive isolates and *Bacillus anthracis* showed significant diversity represented by four ITS haplotypes (H8, H15, H17, and H18) (Fig. 1). Five different ITS fingerprint patterns (H2, H9, H11, H34, and H44) were also detected in *Pseudocitrobacter anthropi* showing an important microdiversity. According to Li et al. (2019), this bacterium is capable of degrading lignin components and rapidly growing on various lignin analog dyes. Also, it had the ability to produce laccase and lignin peroxidase (Lip) enzymes (Li et al. 2019). This microdiversity needs to be further highlighted if we consider the additional complexity generated by the heteroduplex formation (Ettoumi et al. 2013).

The detection of *Acinetobacter*, *Pseudomonas*, *Arthrobacter*, *Klebsiella*, and *Serratia* strains has been


32
Bacillus mojavensis (NR 118290.1)
212
Bacillus subtilis (NR 104873.1)
211
Bacillus sonorensis (NR 113993.1)
302
Bacillus licheniformis (NR 118996.1)
203
Scopulibacillus darangshiensis (NR 115059.1)
210
Bacillus anthracis str (NR 074453.1)
T18
FO5-B
213
34
Oceanobacillus kimchii (NR 117544.1)
37
Lactobacillus plantarum WCFS1 (NR 075041.1)
40
Planococcus citreus (NR 113814.1)
38
Paenibacillus purispatii (NR 116496.1)
44
Paenibacillus oceanisediminis (NR 118217.1)
206
Lysinibacillus fusiformis (NR 112569.1)
Lysinibacillus macroides (NR 114920.1)
T2
T1
Firmicute
49
Micrococcus lylae (NR 026200.1)
FO4-
Curtobacterium flaccumfaciens (NR 025467.1)
48
Streptomyces atrovirens (NR 043508.1)
S6
Agrococcus baldri (NR 041543.1)
46
Arthrobacter agilis (NR 026198.1)
S9
FO10-
Corynebacterium variabile DSM 44702 (NR 102874.1)
Actinobacteria
T8
Roseomonas mucosa (NR 028857.1)
FO6-
Ochrobactrum pseudintermedium (NR 043756.1)
FO6-A
Pseudomonas delhiensis (NR 043731.1)
Pseudomonas citronellolis (NR 114194.1)
Pseudomonas knackmussii (NR 041702.1)
Fo8-A
FO8-
108
55
Acinetobacter johnsonii (NR 117624.1)
Xanthomonas maliensis (NR 136457.1)
Pseudomonas boreopolis (NR 040801.1)
42
41
209
Luteimonas abyssi (NR 133773.1)
S2
Pluralibacter gergoviae (NR 024641.1)
FO1+B
Klebsiella oxytoca (NR 102982.1)
FO3-
FO3-H
Serratia marcescens (NR 114043.1)
Citrobacter braakii (NR 117750.1)
FO1+E
109
Fo2+A
T4
S3
Fo3+B
Pseudocitrobacter anthropi (NR 125691.1)
Proteobacteria
0.05

◀ **Fig. 2** Phylogenetic analyses of bacterial isolates based on 16S rDNA partial sequences. Branch length support was evaluated using non-parametric bootstrap of 1000 data sets using MEGA 6. Accession numbers of the reference strains are shown in parentheses

also reported in previous studies (Di Gioia et al. 2002; Yaoa et al. 2006). Bacterial strains isolated from olive pomace and olive-mill wastewater such as *Serratia marcescens* and *Bacillus* spp. could have strong biotechnological potential since they have been reported to be able to degrade aromatic compounds and pesticides (Yaoa et al. 2006). In addition, Purkayastha et al. (2018) showed that *Serratia marcescens* strain exhibited biocontrol traits such as broad-spectrum antifungal activity and production of lytic enzymes, HCN, IAA, siderophore, and antibiotics. Also, *Bacillus* spp. has been reported to have the ability to inhibit growth of pathogenic fungi (Muleta et al. 2009).

In this work, we described for the first time in Tunisia the diversity of the microorganisms bacteria and fungi isolated from ROP. This waste could be used as biofertilizer thanks to its bacterial diversity which has a modulating effect on the growth of the plants. In fact, some bacteria isolated from ROP, for instance, *Bacillus mojavensis*, have been reported to produce volatile organic compounds like 2,3-butanediol that promote plant growth (Rath et al. 2018). Many bacterial endophytes such as *B. mojavensis* and *Bacillus subtilis* are being used as biological control agents to control plant pathogens and promote plant growth (De Almeida Lopes et al. 2016).

Isolation and identification of fungal isolates

A total of 14 fungal isolates were characterized phenotypically (Fig. 3) and phylogenetically using 18S rRNA sequences.

Representatives of four fungal families were isolated from the olive pomace sample; two isolates were closely related to the *Trichocomaceae*, represented by *Aspergillus* and *Penicillium* species. The three other isolates clustered with *Pleurostomophora*, *Lichtheimia*, and *Bionectria* in the *Pleurostomataceae*, *Mucoraceae*, and *Bionectriaceae* families, respectively. The reconstructed phylogenic tree of these 14 strains is shown in Fig. 4. Baffi et al. (2012) reported the detection of these strains from fresh olive fruits (*Olea europaea* L.), olive paste (crushed olives), and olive pomace (solid waste).

The diversity of fungi is well described. Among these species, *Aspergillus fumigatus* is known to play an important role in the recycling of carbon and nitrogen in the environment. *A. fumigatus* produces secondary metabolites of medical importance, such as "fumagiline," an antibiotic which is promising for treating certain types of cancer (Lamrani et al. 2008).

Biotechnological activities for bacteria

Hydrolytic enzymes

All the 44 representative strains were examined for their ability to produce extracellular enzymes. Lipase and laccase were produced by all bacterial strains ($n = 44$), whereas protease was produced by 40 strains. Cellulase and amylase were produced by 18 and 14 strains, respectively (Figs. 5 and 6).

Several enzymes are detected in this study such as lipase (100%), laccase (100%), and protease (90.9%) (Fig. 5). Phylogenetic analysis shows high diversity of lipase- and laccase-producing bacterial strains. These strains belong to the phylum *Firmicutes*, *Actinobacteria*, and *Proteobacteria.* These strains were assigned to 23 different genera (*Pseudocitrobacter*, *Serratia*, *Klebsiella*, *Pluralibacter*, *Xanthomonas*, *Pseudomonas*, *Acinetobacter*, *Ochrobactrum*, *Roseomonas*, *Citrobacter*, *Corynebacterium*, *Arthrobacter*, *Streptomyces*, *Micrococcus*, *Agrococcus*, *Curtobacterium*, *Lysinibacillus*, *Paenibacillus*, *Planococcus*, *Oceanobacillus*, *Lactobacillus*, *Bacillus*, and *Scopulibacillus*).

Prominent hydrolytic activity observed among Gram-positive isolates showed higher amylolytic, proteolytic, and cellulolytic activities than Gram-negative bacteria. The lipase and laccase activities are similar for both Gram-positive and Gram-negative bacteria. As reported previously, hydrolytic enzymes including amylase, protease, lipase, and cellulase were also produced by different bacteria under extreme conditions (Mukhtar et al. 2019).

It is worth noting that our bacterial collection can be active and produce different enzymes even in extreme conditions of pH, temperature, and salinity. This specific feature facilitates their biotechnological application. In fact, they are isolated from the ROP which is characterized by an acidic pH (Table 1).

It is interesting to emphasize that the combined hydrolytic activity was frequently detected in these isolates. Ten strains affiliated to *B. mojavensis* (32), *Bacillus sonorensis* (211), *B. subtilis* (212), *Bacillus licheniformis* (302), *B. anthracis* str. Ames (T18 and 210), *Paenibacillus oceanisediminis* (44), *Paenibacillus purispatii* (38), *Streptomyces atrovirens* (48), and *P. anthropi* (FO1 + E) showed all the five enzyme activities (amylase, cellulase, protease, lipase, and laccase).

To our knowledge, the present study constitutes the first report in Tunisia showing the detection of lipase and laccase activities in *B. mojavensis*. Previous studies have described protease, amylase, and cellulase activities in this strain. In fact, *B. mojavensis* A21 has been identified as a producer of extracellular bleaching-stable alkaline proteases and thermostable α-amylases (Hmidet et al. 2010; Mhamdi et al. 2014).

Four hydrolytic activities (amylase, cellulase, protease, and lipase) were demonstrated by 10 isolates affiliated to *Bacillus*, *Curtobacterium*, *Agrococcus*, *Arthrobacter*,

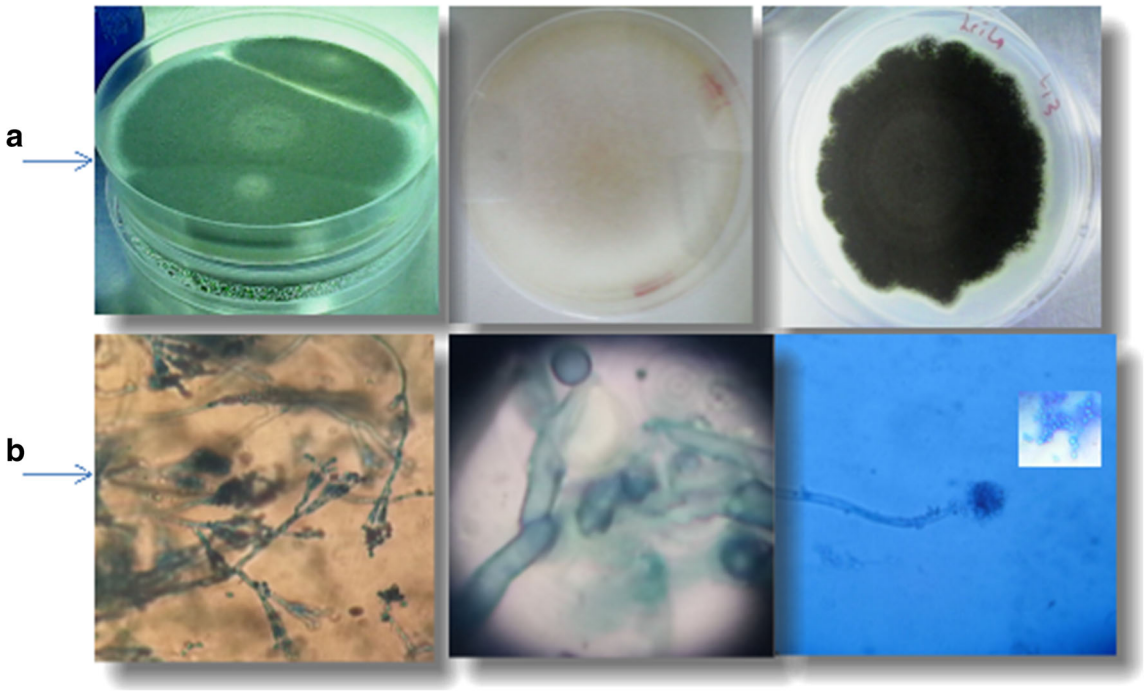


Fig. 3 Morphological appearances of fungi isolated from repasso olive pomace. **a** Macroscopic appearance. **b** Microscopic appearance

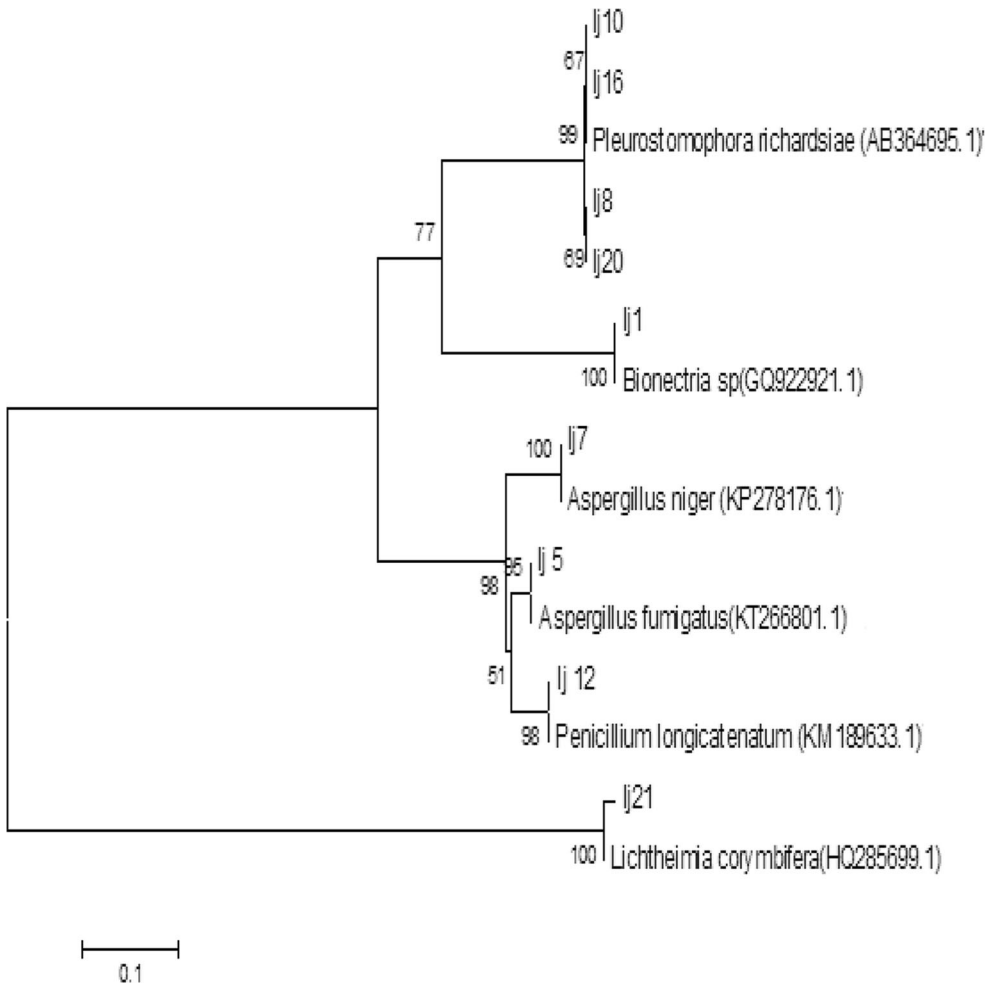


Fig. 4 Neighbor-joining tree of all fungi isolated from olive cake which was produced in MEGA 6 and branch support was evaluated by non-parametric bootstrap. Accession numbers of the reference strains are indicated in parentheses

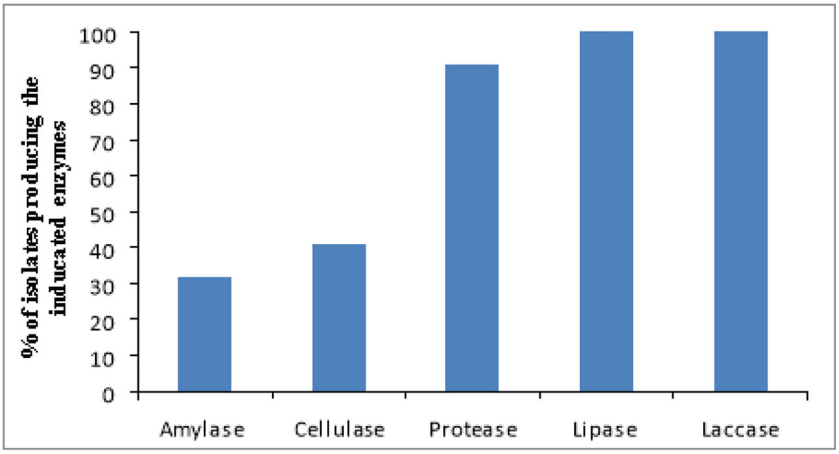


Fig. 5 Hydrolytic enzymes production profiles of the bacterial isolates

Roseomonas, *Xanthomonas*, *Pseudomonas*, *Klebsiella*, *Serratia*, and *Pseudocitrobacter* genera. In addition, 22 isolates have been reported to show three enzymatic activities, and two isolate members of *Pseudomonas* and *Citrobacter* genera showed at least two activities (Table 2). Although the same results have been reported previously for bacterial strains detected in different habitats, our work described higher number of strains showing combined hydrolase activity (Rohban et al. 2009). Microorganisms that exhibit two or more hydrolytic activities constitute an important resource for the recycling or reprocessing of industrial waste and by-products.

As reported previously (Rohban et al. 2009), most environmental isolates able to produce hydrolytic enzymes were Gram-positive bacteria. In fact, among the Gram-positive hydrolase-producing isolates in this study, representatives of the genera *Bacillus* and *Paenibacillus* were the most abundant. *Bacillus* is well known as an extracellular enzyme producer and many industrial processes use several species of this genus for commercial production of enzymes (Moreno et al. 2012).

The genus *Bacillus* produces a large variety of extracellular enzymes, of which amylase presents a particular interest in industry. The present study clearly recorded the occurrence of bacterial strain able to produce amylase (31.82%). Most amylase producers were affiliated to the *Bacillus* and *Paenibacillus* genera. Similar results were reported for *Bacillus* species isolated from spoiled food waste in India (Sudharhsan et al. 2007) and soil samples in India and Bangladesh (Dash et al. 2015).

Our findings describe for the first time the presence of *B. mojavensis* in ROP. In this work, this strain is characterized by its ability to produce five enzymes. According to the literature, *B. mojavensis* has other biotechnological potentialities (Rath et al. 2018; Souii et al. 2018).

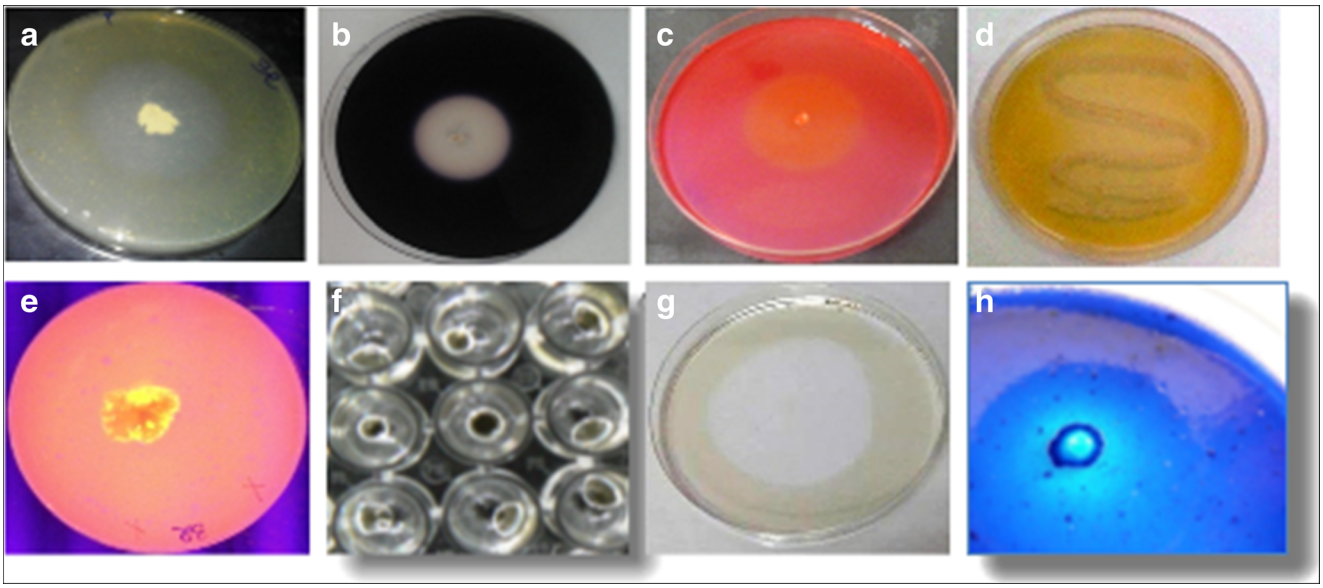


Fig. 6 Plate incorporated media screening of hydrolytic enzymes in bacterial isolates (I), protease (**a**), amylase (**b**), cellulase (**c**), laccase (**d**), lipase (**e**), and biosurfactant (II), drop collapse test (**f**), oil spreading test (**g**), and CTAB test (**h**)

Table 2 Identification of bacterial strains detected in repasso olive pomace and characterization of their biotechnological activities

Isolate	ITS haplotype	Accession number	Closest relative	Sequence similarity (%)	A	B	C	D	E	F	G
32	H6	NR_118290.1	*Bacillus mojavensis*	98	+++	+++	+++	++	+++	+++	+
212	H19	NR_104873.1	*Bacillus subtilis*	98	+++	++	+++	+	−	++	−
302	H1	NR_118996.1	*Bacillus licheniformis*	98	+++	+++	+++	+++	−	−	+
211	H18	NR_113993.1	*Bacillus sonorensis*	98	+++	+++	+++	++	++	−	−
203	H7	NR_115059.1	*Scopulibacillus darangshiensis*	98	−	−	+++	+	−	−	+
T18	H16	NR_074453.1	*Bacillus anthracis* str. Ames	99	+++	+++	+++	++	−	−	−
Fo5-B	H15	NR_074453.1	*Bacillus anthracis* str. Ames	99	+++	−	+++	+	−	++	+
213	H8	NR_074453.1	*Bacillus anthracis* str. Ames	99	−	−	+	++	−	−	+
210	H17	NR_074453.1	*Bacillus anthracis* str. Ames	99	+++	+	+++	+++	−	++	−
37	H39	NR_075041.1	*Lactobacillus plantarum* WCFS1	99	−	−	+	+	−	−	−
34	H22	NR_117544.1	*Oceanobacillus kimchii*	98	−	−	+	+	−	−	+
40	H26	NR_113814.1	*Planococcus citreus*	99	−	−	++	+	−	−	+
38	H25	NR_116496.1	*Paenibacillus purispatii*	98	+++	+++	+	+++	−	++	+
44	H14	NR_118217.1	*Paenibacillus oceanisediminis*	99	+++	+++	+++	+++	−	−	+
206	H21	NR_112569.1	*Lysinibacillus fusiformis*	99	−	−	+	+++	−	+	+
T2	H13	NR_114920.1	*Lysinibacillus macroides*	98	−	−	+	+	−	+	−
T1	H43	NR_114920.1	*Lysinibacillus macroides*	98	−	−	+	+	−	+	−
Fo4-	H3	NR_025467.1	*Curtobacterium flaccumfaciens*	100	−	++	+++	++	+	−	+
S6	H29	NR_041543.1	*Agrococcus baldri*	99	+	−	+	+	−	−	−
49	H28	NR_026200.1	*Micrococcus lylae*	99	−	−	+	++	−	−	−
48	H31	NR_043508.1	*Streptomyces atrovirens*	98	+++	+++	+	++	−	−	−
46	H27	NR_026198.1	*Arthrobacter agilis*	98	+	−	++	++	−	+	+
S9	H33	NR_102874.1	*Corynebacterium variabile*	98	−	−	+	++	−	−	+
FO10-	H37	NR_102874.1	*Corynebacterium variabile*	98	−	−	+	+++	−	−	−
109	H41	NR_117750.1	*Citrobacter braakii*	99	−	−	−	+	+	−	−
T8	H40	NR_028857.1	*Roseomonas mucosa*	98	−	+++	+	+	+	−	+
FO6-	H35	NR_043756.1	*Ochrobactrum pseudintermedium*	98	−	−	+	+	−	+	−
55	H23	NR_117624.1	*Acinetobacter johnsonii*	99	−	−	+	+++	−	+++	+
108	H20	NR_117624.1	*Acinetobacter johnsonii*	98	−	−	+	+++	−	−	+
FO8-	H24	NR_041702.1	*Pseudomonas knackmussii*	99	−	−	−	+	−	−	+
FO8-A	H30	NR_114194.1	*Pseudomonas citronellolis*	98	−	−	+	+	+	−	+
FO6-A	H42	NR_043731.1	*Pseudomonas delhiensis*	99	−	−	++	+	−	−	−
209	H38	NR_133773.1	*Luteimonas abyssi*	98	−	+	−	++	−	−	+
42	H32	NR_040801.1	*Pseudomonas boreopolis*	98	−	++	++	+	−	−	−
41	H10	NR_136457.1	*Xanthomonas maliensis*	98	−	++	++	+	−	+	−
S2	H12	NR_024641.1	*Pluralibacter gergoviae*	99	−	+	−	+	−	−	+
Fo3-	H4	NR_102982.1	*Klebsiella oxytoca KCTC 1686*	98	−	−	+	+	+	−	+
Fo1 + B	H5	NR_102982.1	*Klebsiella oxytoca KCTC 1686*	99	−	+	+	+	+	−	−
FO3-H	H36	NR_114043.1	*Serratia marcescens*	99	−	+	+++	+++	+	−	+
FO1 + E	H2	NR_125691.1	*Pseudocitrobacter anthropi*	98	++	+++	+	++	+	+	+
FO2 + A	H44	NR_125691.1	*Pseudocitrobacter anthropi*	98	+	−	+	+	+	−	−
T4	H11	NR_125691.1	*Pseudocitrobacter anthropi*	99	−	−	+	++	−	+	−
S3	H9	NR_125691.1	*Pseudocitrobacter anthropi*	99	−	−	+	+	+	+	+
FO3 + B	H34	NR_125691.1	*Pseudocitrobacter anthropi*	98	−	−	+	+	+	−	+

+ Positive/− negative (*A* amylase; *B* cellulase; *C* protease; *D* lipase; *E* CTAB; *F* drop collapse test; *G* oil spreading test)

+, weak activity; ++, moderate activity; +++, strong activity

Biosurfactant production

Microbial molecules exhibiting high surface and emulsifying activities are classified as biosurfactants. These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures making them potential agents for bioremediation (Banat et al. 2000). In this study, we tested the production of biosurfactant using different methods: the drop collapse test, the oil spreading test, and the CTAB agar plate.

Drop collapse test showed that 34.1% of the isolates were positive for biosurfactant production belonging to the following classes: *Firmicutes* (18.19%), *Gammaproteobacteria*

(13.64%), and *Alphaproteobacteria* and *Actinobacteria* (2.27%) (Table 2).

The oil spreading method was used to detect the low biosurfactant production. The results revealed that more than half of the isolates were positive (56.82%). Most of them were members of *Gammaproteobacteria* (27.27%) and *Firmicutes* (22.73%) phylum (Table 2). In fact, several isolates, not detected by the drop collapse test, were revealed positive by oil spreading method such as *Pseudomonas*, *Klebsiella*, and *Roseomonas*. These results are in agreement with those of Bodour et al. (2003) reporting that biosurfactant could have different structures and therefore could be used in various fields, such as in food processing industry, in cosmetic industry, and in medicine.

The CTAB agar plate method is a semi-quantitative assay for the detection of extracellular glycolipids or other anionic surfactants. Siegmund and Wagner (1991) revealed that about 29.55% of isolates were positive. These strains belong to three phyla: *Actinobacteria* (2.27%), *Firmicutes* (4.55%), and isolates belonging largely to the group of *Proteobacteria* (22.73%) (Table 2). Investigating the rhamnolipids, typical biosurfactants produced by *Pseudomonas aeruginosa* consist of either one or two rhamnose molecules, linked to one or two fatty acids of saturated or unsaturated alkyl chain between C8 and C12. The *P. aeruginosa* 47T2 produced two main rhamnolipid homologs (Rha-C10-C10 and Rha-Rha-C10-C10) when grown in olive oil wastewater or in waste frying oils consisting from olive/sunflower (Pantazaki et al. 2010).

These results showed the adequacy of the drop collapse test and the sensitivity of the oil spreading method toward low amount of biosurfactant, confirming the results reported by Tugrul and Cansunar (2005). *Acinetobacter*, *Bacillus*, *Corynebacterium*, *Pseudomonas*, *Rhodococcus*, and *Serratia* are the common genera known as biosurfactant-producing bacteria which are normally isolated from hydrophobic substrate of contaminated samples either from terrestrial or marine sites (Saimmai et al. 2012). The detection of different bacterial genera in olive pomace sample indicates that there is a wide biodiversity of biosurfactant-producing bacteria in such environments.

The conjugation of both activities (the drop collapse test and the oil spreading test) endows these strains with good prerequisite for hydrocarbon degradation, making them potential candidates, as pure cultures or in consortia, for bioremediation experiments. Furthermore, the production of biosurfactant is of great importance because it facilitates the design of the process of bioremediation. It constitutes the primary mechanism for the elimination of hydrocarbons from contaminated sites by naturally existing populations of microorganisms (Mahjoubi et al. 2013). Therefore, ROP which is rich in microorganisms producing biosurfactants could be applied in petroleum-contaminated sites.

Biotechnological activities for fungi

All the five enzymes (cellulase, laccase, lipase, amylase, and protease) were produced by the 14 fungal strains (Table 3). There are several studies describing the production of various enzymes using olive cake as substrate in SSF or as supplement to the production medium. Olive pomace are an ideally suited nutrient support in SSF rendering both carbon and nitrogen sources and reported to be a good substrate for enzyme production using fungal species (Ramachandran et al. 2007).

As far as we know, *Aspergillus niger* and *A. fumigatus* are endowed with the enzymatic activities (lipase, amylase, protease, cellulase, and laccase) in both solid and liquid fermentations and with different agricultural substrates (Salihua et al. 2016). For the other species, *Pleurostomophora richardsiae*, *Bionectraia* sp., *Penicillium longicatenatum*, and *Lichtheimia corymbifera*, no enzymatic activity has been reported and therefore they can be useful for biotechnological applications.

In our study, the best activities for cellulase and laccase were observed for the isolates of *A. fumigatus*, *A. niger*, and *P. longicatenatum* (Table 3). These enzymes enhance the olive oil quality and it could be used in some countries because they considerably increase antioxidant phenol compound levels, conferring a protective effect and thus prolonging oil shelf life. In addition, they are also important from a technological standpoint because they increase the oil yield by hydrolyzing olive cell-wall polysaccharides (De Faveri et al. 2008).

Table 3 Enzymatic activities of the fungi isolated from repasso olive pomace

Species	Number of isolates	Enzymes level of activity				
		Protease	Amylase	Lipase	Cellulase	Laccase
A. niger	2	+++	++	++	+++	++
A. fumigatus	3	+++	+++	+++	+++	+
P. longicatenatum	3	++	++	++	++	+++
P. richardsiae	4	+	+	++	+	+
L. corymbifera	1	+	+	+++	++	+
Bionectria sp.	1	++	+	+	+	+

+, weak activity; ++, moderate activity; +++, strong activity

Cellulase and laccase from *Aspergillus* with biotechnological applications in various industries, e.g., textile, food, paper, detergents, and biofuels, have been reported by other authors (Lin et al. 2016). In fact, Paul et al. (2017) demonstrated a high alkaline cellulase-producing *A. fumigatus* strain obtained from the Peruvian Amazon rainforest.

In addition, all the isolated species displayed strong lipase activity (Table 3). The presence of lipase-producing microorganisms in olive substrates could have a side effect as it could negatively influence the shelf life of the product through increasing the acidity of the olive oil from damaged fruits. However, it may decrease the fat content and then the olive pomace could be used as fertilizer or feed (Fadel and El Ghonemy 2015).

Previous studies have described that lipases of *Penicillium* and *Aspergillus* are among the most well-known lipase producers and their enzymes are suitable for use in many industrial applications, mainly in the dairy industry (Mehta et al. 2018). The lipase-producing isolates described in our study need to be further investigated to consider their role in increasing the acidity of olive oil and their possible use in olive waste treatment.

For amylase and protease enzymes, the best activities were also observed for *A. fumigatus, A. niger*, and *P. longicatenatum* (Table 3). Although amylase may be obtained from several sources, such as plants and animals, the enzymes from microbial sources generally meet industrial demand. *Aspergillus niger* produced more amylase yield from agricultural waste. This is because agricultural waste is very cheap, easily available from mill factories, and is more suitable for solid-state cultivation of *A. niger* for amylase production (Suganthi et al. 2011). Various agro-industrial residues (wheat bran, rice husk, rice bran, spent brewing grain, coconut oil cake, palm kernel cake, sesame oil cake, jackfruit seed powder, and olive oil cake) were screened for protease production in SmF and SSF (Sandhya et al. 2005).

To the best of our knowledge, this is the first study describing the detection of filamentous fungi from Tunisian olive ecosystem (ROP) with enzymatic potential of biotechnological interest. In addition, the present work showed the potential of a rich microflora isolated from ROP suggesting that these olive wastes could be used to take advantage of biotechnology and enzyme production in different sectors. The identified species and their enzymes could be applied not only in the olive oil industry but also in other industries which could use biotechnological processes for agricultural by-product bioconversion.

Conclusion

The present study described for the first time the microbial diversity obtained from ROP in a Tunisian coastal city, Mahdia. Besides, rapid screening of some specific enzymes being the first reported in these strains showed highly variable activity profiles.

The overall data indicate that *Proteobacteria* group constitutes an important part of the microbiota of ROP in Tunisia. However, from the 88 isolated bacteria, *Firmicutes* were the most microdiverse group and *Bacillus* was the most dominant genera. For the fungal representatives of four families that were recovered from the olive pomace sample, the isolates were closely related to the *Trichocomaceae*, *Pleurostomataceae*, *Mucoraceae*, and *Bionectriaceae* families.

For enzyme screening, *Bacillus* and *Paenibacillus*, together with some Gram-negative representatives (*P. anthropi* and *Acinetobacter johnsonii*), showed significant activities (amylase, cellulase protease, lipase, laccase). The lipase and laccase activity of *B. mojavensis* has been described for the first time in this microorganism.

In addition, we tested the production of biosurfactant using different methods: the drop collapse test, the oil spreading test, and the CTAB agar plate. The detection of different bacterial genera in ROP sample indicates that there is a wide biodiversity of bacteria producing different types of biosurfactants.

Fourteen fungal isolates showed high production of some specific enzymes (amylase, cellulase, protease, lipase, and laccase). In particular, in *Pleurostomophora richardsiae*, *Bionectraia* sp., *Penicillium longicatenatum*, and *L. corymbifera*, no enzymatic activities have been reported and therefore they can be useful for biotechnological applications of industrial importance.

Finally, the phenotypic and phylogenetic diversities in bacterial and fungal isolates point out that ROP could represent a valuable source of new lineages and metabolites. Moreover, it could be used as a substrate to produce microorganisms and enzymes applicable in several fields.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication of this paper.

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