

Microorganisms Isolated from Moroccan Olive-Mill Wastes: Screening of Their Enzymatic Activities for Biotechnological Use

*Asmae Misbah,
Mouna Aouine,
Safae Er Raouan,
Yassir Lekbach,
Hamza Ettadili,*

Microbial Biotechnology Laboratory, Faculty of Sciences and Technologies,
Sidi Mohamed Ben Abdellah University, Fez, Morocco

Saad Ibnsouda Koraichi,

Microbial Biotechnology Laboratory, Faculty of Sciences and Technologies,
Sidi Mohamed Ben Abdellah University, Fez, Morocco
Regional University Center of Interface, Sidi Mohamed Ben Abdellah
University, Fez, Morocco

Mohamed Ali Tahri Jouti,

Microbial Biotechnology Laboratory, Faculty of Sciences and Technologies,
Sidi Mohamed Ben Abdellah University, Fez, Morocco

Doi:10.19044/esj.2019.v15n30p464 [URL:http://dx.doi.org/10.19044/esj.2019.v15n30p464](http://dx.doi.org/10.19044/esj.2019.v15n30p464)

Abstract

In this study, three samples of olive oil wastes: Olive Rinse Water (OR), Olive Mill Wastewater (OM) and Olive Pomace (OP), which were collected from an olive oil mill located at Fez-Morocco, were analyzed for their microbiota. A total of 81 isolates were obtained and constituted a microbial bank formed of 35 bacteria, 41 yeasts and 5 fungi. The microbial communities have been compared in term of metabolic potential by testing hydrolytic enzymes activities of lipase, protease, amylase, cellulase, pectinase and tannase on agar plate media. The results reveal that among the examined microorganisms, 68 isolates were able to produce at least one of the screened enzymes. The pectinase activity was the predominant one (39.51%), followed by cellulase activity (34.57%) and by lipase activity (27.16%). However, the amylase activity was observed only for 11.11% of the studied isolates, followed by protease activity (9.88%) and by tannase activity 6.17%. Thereby, considering the isolates ability to produce enzymes, they can be considered as potential candidates for industrial and biotechnological applications. Lipases

are versatile enzymes that are used widely, it's why the mostly aim of this work was to isolate new bacterial strains producing high level of lipases with interesting biochemical criteria. Thus, two isolates OR34 and OM2 showed the maximum lipase production under the standard conditions, about 9 U/ml and 11 U/ml, respectively, using pH-stat technique. They were identified as *Proteus vulgaris* and *Serratia marcescens*, respectively, via partial 16S rDNA gene sequence analysis.

Keyword: Olive oil wastes, Microbiota, Enzymes, Biotechnological applications, Lipase

Introduction

Olive oil production is of vital importance for the economy and social life in most Mediterranean countries such as Spain, Italy, Greece, Turkey, Tunisia and Morocco. However, this activity is often associated with the generation of enormous quantities of liquid wastes (olive-mill wastewater OMW) and solid press-cake (olive-mill pomace), which represent a serious environmental problem; mainly for their high organic load and chemical composition that renders them resistant to degradation. (Cegarra et al., 2006; Morillo et al., 2009). The OMW is considered as the most critical waste, emitted by olive-mills, in terms of both quantity and quality. This black liquid wastewater is composed of the olive fruit vegetation water, washing and processing water, a portion of the olive pulp and small quantities of oil (Scioli and Vollaro, 1997). The dark brownish color of OMW has been attributed to the polymerization of tannins and polymeric phenolic compounds similar in structure to lignin (Pérez et al., 1998). In general, the composition and the quantity of OMW and consequently the environmental impact, depend on many factors, such as the olive variety, the cultivating techniques, the harvesting period and the extraction method (Tsiamis et al., 2011). The olive-mill pomace is composed of a mixture of olive pulp and olive stones (Morillo et al., 2009). Both OMW and olive-mill pomace have a phytotoxic and antimicrobial effects, due to the low pH that ranges generally between 4 and 5, the presence of organic acids and mostly the presence of phenols, negatively affecting aquatic and terrestrial ecosystems (Ben Sassi et al., 2006; Kistner et al., 2004; Rodriguez et al., 1988; Fiestas Ros de Ursinos et al., 1992).

As a consequence, several techniques and processes have been proposed for appropriate olive-mill wastes management such as composting, aerobic and anaerobic digestion and physico-chemical treatments (Morillo et al., 2009). Among the different options, biological methods are considered to be more environmentally-friendly and less expensive enabling at the same time some of their primary components to be recovered (Ouzounidou et al., 2010). In this way, OMW can be used as a fertilizer as it represents a valuable source

of minerals (potassium), organic matter and nutrients for plants (Altieri, and Esposito, 2008). It can be also used as substrate for the culture of certain microorganisms in order to obtain a potentially useful microbial biomass as an additive to animal feed and/or to induce a partial bioremediation of the residues (Laconi et al., 2007). In addition, the olive oil by-products contain useful compounds that can be used as substrates for the production of biofuels (Gelegenis et al., 2007; Zanichelli et al., 2007) and enzymes (Salgado et al., 2016; Leite et al., 2016; D'Annibale et al., 2005).

Regarding to biological methods, microbial olive mill wastes biodegradation was attempted through the use of indigenous microorganisms, or by inoculating arbitrarily chosen strains which are known for their ability to bioconvert wastes of similar nature, or by exploiting commercial consortia of microorganisms (Ouzounidou et al., 2010). Indigenous microorganisms, which have been adapted to the adverse conditions of olive mill wastes, are more likely to effectively colonize the effluent (Ntougias et al., 2013). Despite the importance of bioremediation as an alternative to olive-mill wastes detoxification and recycling, little is known about the indigenous microbiota and their potential for carrying out biodegradation (Millan et al., 2000; Giannoutsou et al., 2004, Federici et al., 2011). There are only few reports describing the microbial communities of olive-mill wastes (Tsiamis et al., 2011; Kavroulakis et al., 2011; Ntougias et al., 2013). They reported that OMW microbiota appears to have originated from soil and freshwater environments, while the cultivation and harvesting practice highly influenced the microbial community.

The fact that these microorganisms are well adapted to the extreme conditions of OMW makes them attractive for potential industrial applications. So, besides bioremediation, they can be used for others *in vitro* applications, thanks to their biotechnological properties such as production of enzymes. Some authors described yeast and fungi biodiversity from oleic ecosystems and screened their potential enzymatic activities for biotechnological use (Romo-Sánchez et al., 2010; Baffi et al., 2012). In fact, enzymes are biological catalysts that speed up biochemical reactions in living organisms, and which can be extracted from cells and then used to catalyze a wide range of commercially important processes (Robinson 2015). They have found application in many industries including pulp and paper, leather, detergents and textiles, pharmaceuticals, chemical, biofuels, food and animal feed, among others. In all areas of application, enzymes are used to effectively facilitate transformations or to replace traditional chemical processes making them cleaner with less impact on the environment (Adrio and Demain 2014; Lobedanz et al., 2016). Microbial enzymes are the most requested because of their ease of production and extraction, and owing to their special characteristics such as stability in large range of temperature and pH (Nigam

2013). Microorganisms, as enzymes source, are of much attention because they can be grown economically and are amenable to genetic modification. Researchers are looking for new microbial strains in order to produce different enzymes which fulfill the current industrial requirements (Sanchez and Demain, 2017).

The aim of this study was to isolate the microorganisms (bacteria, yeasts and fungi) present in olive oil wastes from Moroccan mill located at Fez, and to investigate their ability to produce enzymes for biotechnological applications. For this purpose, the activity of various enzymes (lipase, protease, amylase, cellulase, pectinase and tannase) was evaluated, with a special focus on bacterial lipases, because they tend to have alkaline pH optima, requested in detergent application.

I-Materials and methods:

1. Sampling :

Olive oil by-products samples: Olive Rinse Water (OR), Olive Pomace (OP) and Olive Mill Wastewater (OM) were collected from a triturating unity of olives located at Fez-Morocco and transferred to the laboratory in sterile and labeled glass vials. Then, they have been stored at 4°C until analyzed.

2. Microbiological analysis:

For isolation of microorganisms, aliquots of 1 g or 1 ml of each sample were suspended in 9 ml of sterile physiological water, homogenized for 5 min and then serially diluted. Volumes of 100 µl of each dilution were spread on three agar media. The Luria-Bertani agar (LB) containing (g/l): 10 tryptone, 5 yeast extract, 10 NaCl and 15 agar, with a final pH of 7 was used for isolation of bacteria. The Potato Dextrose agar (PDA) composed of (g/l): 20 dextrose, 4 potato extract and 15 agar, with a pH adjusted to 5.6 was used for isolation of yeasts. The Malt Extract agar (ME) containing (g/l): 30 malt extract, 5 peptone and 15 agar, with a final pH of 5.4 was used for isolation of fungi. Ampicilin (100 µg/ml) was added to the two later agar media in order to avoid bacterial growth. LB agar plates were incubated at 37 °C for 24 h, while PDA and ME agar plates were incubated at 30 °C for 2-5 days. Microbiological counts were performed in duplicate and the results are expressed in CFU (colony forming units). The colonies with different phenotypic aspects were purified by repeated streaking on the appropriate culture media plates. Finally, the purified isolates were designated according to the original biotope (OR, OM, OP) and preserved at -20 ° C in a liquid culture medium containing 30% glycerol.

3. Microscopic observation:

On the one hand, the microbial genus of each isolate (bacterium, yeast or fungus) was determined using optical microscope. On the other hand, the isolated bacteria were classified according to their morphologies (coccus or rod) and their cell wall composition (Gram negative or Gram positive) using Gram stain method, as described by Gram, 1884.

4. Screening for extracellular enzymes:

The different isolates are tested for their potential enzymatic activities, using the following revelation solid media:

4.1. Lipase activity :

The isolates were inoculated on one of the solid culture media (LB, PDA or ME) supplemented after autoclaving with 1% of olive oil and 1% of Rhodamine-B (w/v). After incubation at 37 ° C for bacteria and 30 ° C for yeasts and fungi, the lipase activity was detected by irradiating the plates with ultra violet light at 350 nm. The lipolytic activity is indicated by the appearance of orange fluorescence halos around the colonies (Geoffry and Achur, 2018; Kouker and Jaeger, 1986).

4.2. Protease activity:

The isolates were screened for their ability to hydrolyze casein in nutrient agar milk containing (g/l): 3 yeast extract, 5 casein peptone, 15 agar supplemented after autoclaving with 250 ml of sterile skimmed milk. After incubation of the plates at the appropriate temperature, colonies with a clear zone formed by the hydrolysis of casein were evaluated as protease producers (Ozturkoglu-Budak et al., 2016).

4.3. Amylase activity:

The isolates were inoculated on the appropriate agar plate (LB, PDA or ME), supplemented with 0.25% starch. After incubation, an iodine solution (1 g of I₂ and 2 g of KI in 300 ml of distilled water) was poured on the agar plates. The appearance of clear zones around the colonies indicates the presence of amylase activity (Madhav et al., 2011).

4.4. Cellulase activity:

Detection of cellulolytic activity was carried out on agar media previously mentioned (LB, PDA or ME) which were supplemented with 1% of carboxyl methyl cellulose (CMC). After isolates inoculation and incubation, the zone of clearance around the colonies was observed by adding Congo red solution (1%) for 15 min, as a detecting agent, then rinsing with NaCl solution (1M) (Hemati et al., 2018).

4.5. Pectinase activity:

Pectinolytic activity of recovered isolates was evaluated on the suitable agar plate (LB, PDA or ME) containing 1% of apple pectin. After incubation, the plates were flooded with 1% of cetyl trimethyl ammonium bromide (CTAB) solution. Strains with clear zones around their colonies were considered as pectinolytic enzymes producers (Patil and Chaudhari, 2010).

4.6. Tannase activity:

Tannase activity was detected using the above solid culture media (LB, PDA or ME) containing each one 0.5% of tannic acid. After incubation at the appropriate temperature, tannase producing strains were determined by visualizing the dark zone of hydrolysis formed around their colonies (Brahmbhatt and Modi 2015).

5. Production of bacterial lipases in liquid medium:

The mostly aim of this work was to isolate bacterial strains that can produce extracellular lipases for biotechnological interest. For this reason, the bacteria showing a lipolytic activity on olive oil agar plate were selected. Thus, to produce lipase in liquid medium, bacteria were firstly pre-cultured in 50 ml Erlenmeyer flasks, each containing 10 ml of nutrient broth, then shaken at 180 rpm and 37°C for 12 h. Secondly, the pre-cultures were used as inoculums and cultivated in 250 ml Erlenmeyer flasks containing 50 ml of a lipase production medium composed of (g /l): 15 casein peptone, 5 yeast extract, 5 NaCl, 2.5 glucose, 3 K₂HPO₄, 1 KH₂PO₄, with a final pH of 7.4. The initial absorbance (OD) measured at 600 nm was adjusted to an approximate 0.2 value. Finally, the inoculated flasks were incubated during 72 h on a rotary shaker at 37 °C and 180 rpm. The bacterial growth was followed by measuring the OD of the cultures at 600 nm (Horchani et al., 2009; Eddehech et al., 2018).

6. Crude extracellular lipase extraction:

After incubation, the cultures were harvested and centrifuged at 13,000 rpm for 20 min at 4 °C. The clear supernatants were collected and used for quantitative lipase assay.

7. Quantitative lipase activity assay:

The lipase activity was determined by potentiometric method using the pH-Stat technique (Metrohm type). It consists in measuring the lipolytic activities at constant pH using emulsified substrates. During the hydrolysis of a triacylglycerol emulsion by lipase, the fatty acids released are titrated by adding sodium hydroxide (0.1 N) to the reaction medium. This contains 10 ml of 10% olive oil emulsion in gum arabic (10% w/v), 20 ml of Tris-HCl buffer (2 mM Tris, 150 mM NaCl, pH 8), 3 mM CaCl₂ and 2 mM sodium

deoxycholate (NaDC). The enzymatic activity was expressed in International Units (1 IU corresponds to 1 μ mol of fatty acid released per min) (Laachari et al., 2013; Chahinian et al., 2002; Rathelot et al., 1976).

8. Molecular identification:

Molecular identification was performed based firstly on the extraction of the DNA followed by the amplification of the 16S rDNA gene through Polymerase Chain Reaction (PCR) in a thermocycler (Techgene^R), using universal primers fD1 (5 'AGAGTTTGATCCTGGCTCAG 3') and RS16 (5 'TACGGCTACCTTGTTACGACTT 3') (Weisburg et al., 1991). Genomic DNA of bacteria was extracted from freshly-grown isolates by organic solvents method, which based on alkaline lysis as described by Marmur 1961 with slight modifications. The reaction mixture of the PCR having a final volume of 50 μ l was composed of: Taq buffer (10X) with MgCl₂ (50 mM), dNTP (10 mM), Taq polymerase (5 U/ μ l), 10 μ M of each primer, 1 μ l of DNA template and pure water (Lorenz, 2012). The amplification program was run under the following conditions: initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 45 sec and extension at 72 °C for 1 min 30 sec. At the end of the cycles, a final extension was performed at 72 °C for 10 min in order to complete the synthesis of unfinished DNA strands (Hassi et al., 2007). Amplification efficiency was confirmed by electrophoresis on 1% agarose gel. The PCR products were then purified and sequenced according to the Sanger method using the same primers on an automatic sequencer (BIOSYSTEME 3130 xl) (Sanger et al., 1977). Finally, with a view to the taxonomic identification of the isolates, the sequences obtained were compared with a database (National Information Center for Biotechnology) thanks to the BLAST program (Basic Local Alignment Search Tool).

9. Statistical analysis:

The results are expressed on average and the experiments were conducted in triplicate. The data were analyzed for significant differences using the two-way analysis of variance and the Posthoc Tukey test by means of Graph Pad Prism software, version 8.0.2 (GraphPad Software, San Diego, California, USA).

2. Results and discussion:

1. Microbiological characterization:

A total of 81 isolates, with different macroscopic aspects, were isolated from the three samples, of which 36 are from the OR (44.44%), 22 from the OM (27.16%) and 23 from the OP (28.4%). In fact, a high microbial load was present in all three samples. Several studies showed that olive wastes contain

a significant microbial diversity which is greatly influenced by the olive fruit variety. Indeed, Tsiamis et al., 2011, reported that bacterial communities in olive mill wastes generated from various olive-fruit varieties had only 15% of the OTUs (Operational Taxonomic Unit) identified in common, indicating a cultivar-specific bacterial profile. Also, based on the data provided by Romo-Sanchez et al., 2010, yeast diversity in olive wastes appeared to be fruit variety dependent. Moreover, the microbial profile of olive wastes can also depend on the extraction process. According to Mouncif et al., 1993, a net difference between the traditional mill samples and the industrial samples was observed for their microbiota analysis. Furthermore, it has been shown that the majority of olive wastes microbiota has originated from soil and freshwater environments, while the cultivation and harvesting practice highly influenced the microbial community structure. Fecal bacteria have been also identified in olive mill wastes (Kavroulakis 2011; Tsiamis et al., 2011; Ntougias et al., 2013).

The results concerning the enumeration of colonies grown on LB, PDA and ME agar media, after incubation, are shown in Figure 1. It is noted that the OR contained the highest microbial load on the three culture media, followed by OM and OP. The difference between the microbial load of the three samples is statistically significant (P value <0.0001). Concerning the OR, this microbial richness was particularly marked on the LB medium with 39×10^5 CFU/ml. However, for OM and OP the microbial load was more accentuated on the PDA medium with 1.01×10^5 and 0.41×10^5 CFU/ml, respectively. The microbial load of the three samples was significantly different from culture medium to another (P value <0.0001). Some previous works have interested to analyze the types germs present in olive wastes, using different culture media. Indeed, Esmail et al., 2014, focused on the enumeration of the microbial burden of OMW, from three Morocco regions, to establish the link between the presence and absence of microorganisms and the effect of phenolic compounds. Taking the example of Fez-Boulman station sample (same sampling region of the present study), the total aerobic mesophilic flora (FMAT) counted on PCA (Plate Count Agar) medium, was 2.9×10^5 CFU/ml. The yeasts and molds, enumerated on Sabouraud chloramphenicol agar, presented 21.7×10^5 CFU/ml. The lactic acid bacteria counted on MRS (Man, Rogosa, Sharpe agar) medium, were 2.2×10^5 CFU/ml. These values are higher than those obtained here, in OM and OP samples, but lower than those obtained in OR sample. Moreover, Elharchli et al., 2015 estimated the biomass of yeasts and fungi in the OMW obtained from olive mill located at "Hamria" region in Fez-Morocco, which were respectively 10^6 CFU/ml and 6×10^6 CFU/ml. These values are higher than those of yeasts and fungi obtained here in the three samples. However, they showed that no bacteria could grow in LB medium, contrary to what is demonstrated here. In

addition, Fiestas Ros de Ursinos et al., 1992, reported that the concentration of microorganisms in OMW ranges from 10^5 to 10^6 CFU/ml, comprising several bacteria which the most common was *Pseudomonas*, yeasts of *Sacharomyces* type and the molds *Aspergillus* and *Penicillium* were also found. Another study focused on the assessment of the physicochemical and microbiological quality of OMW from different Tunisian regions, in order to determine their pollution levels, reported that the southern region samples revealed a high number of FMATs compared to the other regions (Zaier et al., 2017). This can be mainly related to the extraction process, the adopted operating conditions, the region, the storage conditions and the physicochemical characteristics which hinder the microorganism's growth.

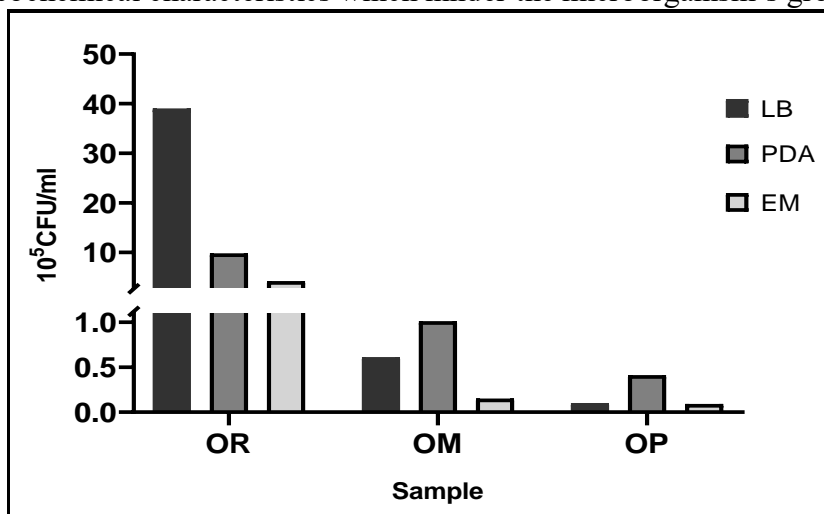


Figure 1: microbial load of each sample according to the culture medium

The different colonies isolated and purified constitute a microbial bank formed of 35 bacteria (43.21%), 41 yeasts (51.62%) and 5 fungi (6.17%). Figure 2 shows the inventory of purified isolates from each sample. It is noted that the isolates obtained from OR consisted mainly of bacteria (21) followed by yeasts (14), then fungi (1). Concerning OM and OP, yeasts were the most abundant (13 and 14, respectively), followed by bacteria (6 and 8, respectively) and fungi (3 and 1, respectively). The difference between the number of bacteria, yeasts and fungi in the same sample is statistically significant (P value <0.0001). The findings published previously, showed that yeasts and fungi represent the major flora of OMW (Zaier et al., 2017). These groups seem to be best suited to the high acidity and salinity of OMW and they resist more than bacteria to the toxicity of phenolic substances. Indeed, Mouncif et al., 1993 demonstrated that the yeasts, molds and lactic acid bacteria were the main microorganisms found in the OMW which are known by their activities on some inhibitors such as polyphenols and can survive in

the medium. Another study comparing wastewaters from three olive oil extraction processes (traditional, semi-modern and continuous) in the region of Marrakech-Morocco, revealed that yeast counts were close for all OMW samples and showing higher concentrations than fungi and bacteria (Ben Sassi et al., 2006). Nevertheless, Millan et al., 2000 showed that the number of viable cells and the proportion of the different microbial groups (molds, yeasts and bacteria) changed from one OMW pond to another.

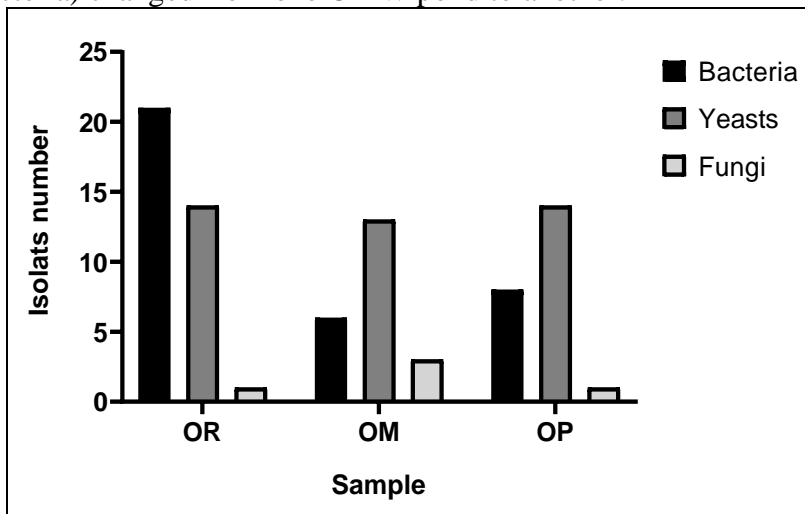


Figure 2: Inventory of purified isolates from each sample

2. Microscopic observation:

The 35 isolated bacteria were classified according to their Gram types (Figure 3-a). In fact, 17 bacteria were Gram-positive (48.57%) while 18 were Gram-negative (51.43%). For OR, there were more Gram-negative bacteria (15) than Gram-positive bacteria (6). In contrast, for OM and OP, there was an abundance of Gram-positive bacteria (4 and 7, respectively) compared to Gram-negative bacteria (2 and 1, respectively). The differences are statistically significant (P value <0.0001). Many bacteria isolated from olive oil wastes, known in the literature, can be either Gram-negative or Gram-positive bacteria. Indeed Ereqat et al., 2017 showed that six bacteria among seven, isolated from both liquid and solid olive oil wastes, collected from south of Palestine, were Gram-negative. However, according to Jones et al., 2000, all nine bacteria isolated from Spanish olive pomace were Gram-positive and two bacteria, among three, isolated from Greek olive pomace were also Gram-positive. In addition, a total of 17 bacteria were isolated from OMW originated in olive oil production plants located in Turkey, of which only two were Gram-negative (Ertuğrul et al., 2007).

Regarding the form, 16 isolates were coccus (45.71%), while 19 isolates were rod (54.29%) (Figure 3-b). It is noted that OR contained more

rod (15) than coccus (6), while OM and OP contained more coccus (4 and 6 respectively) than rod (2 for each one). The differences are statistically significant (P value <0.0001). Some studies determined the morphology of bacteria isolated from olive oil wastes. Indeed, Ereqat et al., 2017, reported that six bacteria among seven, isolated from Palestinian olive oil wastes, were rod. Moreover, all the bacteria isolated from the Spanish and Greek olive pomace were rod (Jones et al., 2000). In addition, Ertuğrul et al., 2007 isolated 17 bacteria from OMW, of which only two were coccus.

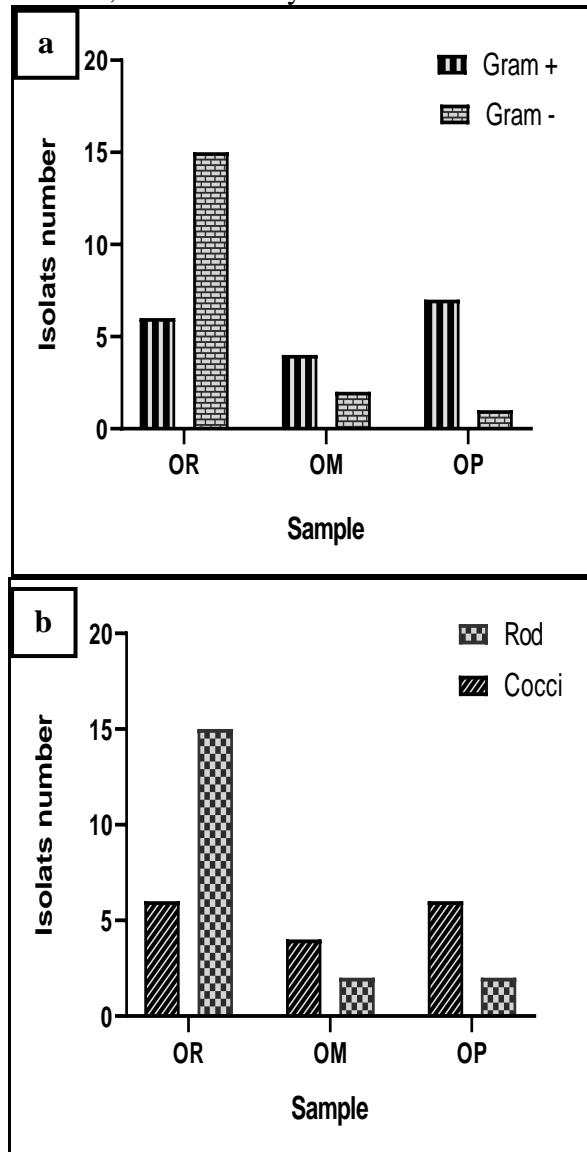


Figure 3: Gram types (a) and morphology (b) of bacteria isolated from each sample.

3. Screening of enzymatic activities:

All 81 purified isolates were tested for their enzymatic potentials; the six enzymatic activities studied for all isolates are grouped in Figure 4. The isolates showing hydrolase activities are classified in Table 1, indicating their microbial genus (bacteria, yeast or fungi), their type of Gram (positive or negative), their morphology (rod or coccus) and the enzymes that they are able to produce. The distribution of these enzymatic activities on the isolates from each sample is shown in Figure 5.

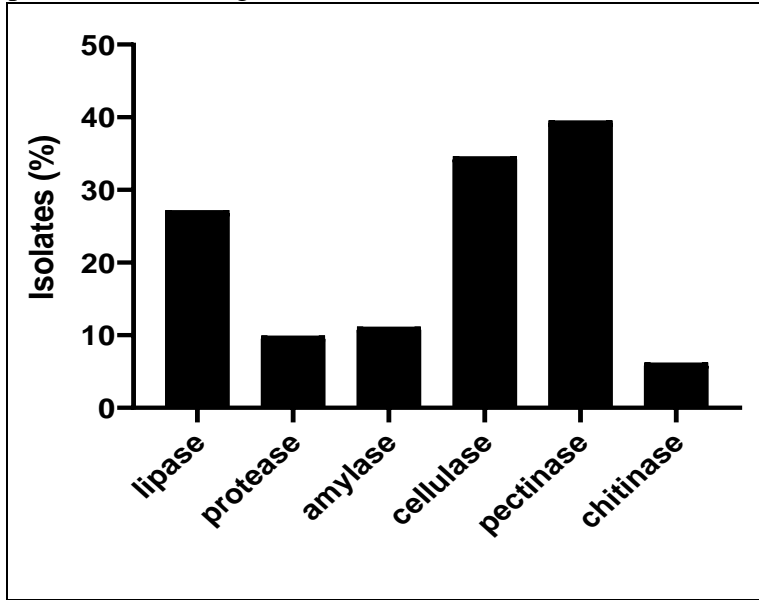


Figure 4: Percentage of enzymatic activities of purified isolates

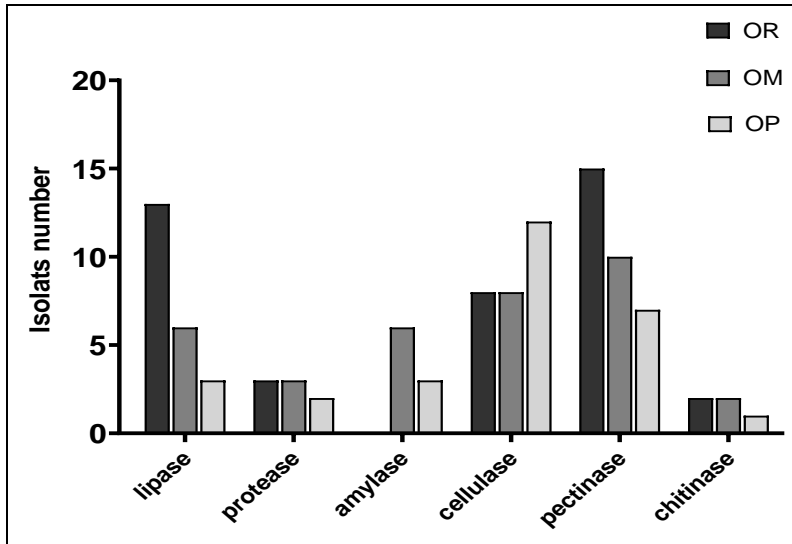


Figure 5: Distribution of enzymatic activities on the three samples

3.1. Lipase activity:

The lipolytic activity was observed for 27.16% of the purified isolates. After irradiation with UV light of plates, 22 isolates showed fluorescent halos around their colonies reflecting their ability to produce extracellular lipase. In fact, olive wastes with their lipid contents can be a good biotope for microorganisms producing lipase. Some studies focused on the isolation of lipolytic microorganisms from olive oil wastes (Mouncif et al., 1993). Indeed, Ertuğrul et al., 2007, demonstrated that *Bacillus* sp. isolated from OMW exhibited a high lipase activity. Also, several olive oil yeast species, belonging especially to the genus *Candida* (Ciafardini and Zullo, 2018), *Saccharomyces cerevisiae* and *Williopsis californica* (Ciafardini et al., 2006), were able to produce lipase, but it can be inhibited by high concentration of the phenols present in olive oil. Moreover, *Debaryomyces hansenii* yeast strain, producing lipase, was isolated from dry-salted olives of Thassos variety (Papagora et al., 2013).

Lipolytic activity was abundant, especially in OR isolates (13), followed by those of OM (6) and those of OP (3). Lipase activity of isolates from each sample was significantly different (P value <0.0001). Among the all lipase producers isolates, 12 were yeasts, 8 were bacteria and 2 were fungi. In fact, several studies showed that OMW can be used as culture medium for different lipolytic yeast strains, like *Yarrowia lipolytica* (Lanciotti et al., 2005) and *Candida cylindracea* (Brozzoli et al., 2009). It can be also considered as valuable liquid growth medium for the production of lipase by fungi (D'Annibale et al., 2005), such as *Aspergillus ibericus* (Oliveira et al., 2011; Abrunhosa et al., 2012) and *Penicillium citrinum* (D'Annibale et al., 2006). Another study showed that *A. ibericus* achieved the highest level of lipase production, during the bioremediation of undiluted OMW, probably due to its higher lipids content which act as inducer for the production of lipases (Salgado et al., 2016). In addition, *Geotricum candidum* was able to grow in OMW and produced lipase in order to control decolourisation and biodegradation of OMW (Asses et al., 2009). Olive pomace has been also successfully utilized for lipase production using thermostable fungal cultures of *Rhizomucor pusillus* and *Rhizopus rhizopodiformis* (Cordova et al., 1998).

3.2. Protease activity:

Among 81 analyzed isolates, only 8 (9.88%) displayed proteolytic activity, which 3 were from OR, 3 from OM and 2 from OP. They showed degradation halos of casein on solid medium, reflecting their ability to produce extracellular proteases. There is no difference between the protease activity of isolates from OR and isolates from OM (P value > 0.9999). To our knowledge, no protease-producing microorganism has been isolated from olive oil wastes before. In contrast, Salgado et al., 2016 demonstrated that three strains of

Aspergillus can produce proteases during bioremediation of OMW and the highest level was achieved by *A.uvarum*.

3.3. Amylase activity:

The amylase activity was present in 11.11% of isolates examined. So, 9 isolates showed transparent halos indicating their ability to degrade starch. No isolate from OR showed amylase activity. Statistically a significant difference was found in amylase activity between isolates from OM (6 isolates) and OP (3 isolates) (P value < 0.0001). In fact, Karam et al., 2017 isolated an amylase producing fungus, from an Egyptian olive oil cake, identified as *A. awamori*. This fungus amylase was constitutively produced using various agro-industrial wastes and the highest enzyme production was obtained by using olive oil cake. Moreover, Ramachandran et al., 2004, reported the α -amylase production by *A. oryzae* using different oil cakes, and indicated that groundnut oil cake (GOC) was the best one.

3.4. Cellulase activity:

The results show that 34.57% of the tested isolates are endowed with cellulase activity. A number of 28 isolates were able to degrade CMC and showed yellow halos under their colonies, of which 8 were from OR, 8 from OM and 12 from OP. The cellulase activity of the OP isolates was significantly different from that of OR and OM isolates (P value <0.0001). In fact, some of the yeast have been isolated from paste, pomace and olive fruits, and exhibited cellulase activity (Romo-Sánchez et al., 2010). Moreover, a total of 53 fungi strains were isolated from olive phyllosphere in Castilla La Mancha region, among them 43 presented cellulase activity (Baffi et al., 2012). Furthermore, Leite et al., 2016, reported the use of crude olive pomace (COP) and exhausted olive pomace (EOP) as solid substrate for cellulase and xylanase production. The cellulase production was carried out using *A. ibericus*, *A. niger* and *A. uvarum* and was highest in EOP using *A. niger* strain.

3.5. Pectinase activity:

The findings revealed that the highest percentage of the examined isolates (39.51%) exhibited pectinase activity. So that 32 isolates of them showed clear zones indicating their ability to degrade pectin. OR sample was the richest in pectinase-producing microorganisms (15), followed by OM (10) and then OP (7). The differences observed between the pectinase activity of the isolates from OR, OM and OP were statistically significant (P value <0.0001). In fact, olive oil sediments are rich in many nutrients originating from fruit mesocarp, including pectin. Consequently, pectinolytic yeasts can utilize this pectin for growth and have been previously identified as the causative agent of the spoilage defects in table olives (Ciafardini and zullo,

2018; Golomb et al., 2013). In addition, Federici et al., 1988, reported that OMW contained simple sugars, polysaccharides and pectin, which can be good substrates for suitable microorganisms. They indicated also, that *Cryptococcus albidus* var. *albidus* was capable to grow in OMW pretreated, with the release of significant levels of pectinase activity (Crognale et al., 2006).

3.6. Tannase activity:

The results indicated that the lowest percentage of the studied isolates 6.17% endowed tannase activity. So that 5 isolates showed dark halos of tannic acid degradation. They are distributed as 2 from OR, 2 from OM and only one from OP. There is statistically no difference between tannase activity of isolates from OR and isolates from OM. In fact, Pepi et al., 2013 indicated that four bacterial strains isolated from OMW were capable of growing in the presence of tannic acid as sole carbon and energy source. The most efficient one was identified as *Klebsiella* sp. and showed 60% of tannic acid utilization. In addition, Pepi et al., 2010 reported that three bacterial strains isolated from OMW were able to degrade tannic acid. Two among them belong to the genus of *Serratia* and the third one was identified as *Pantoea* sp. which showed the highest tannase activity. Also, 22 tannase-positive bacterial isolates were obtained from the thermophilic and the mesophilic phase of OMW composting (Federici et al., 2011). Indeed, microbial tannases catalyze the degradation of tannins that are among the most abundant polyphenolic compounds found in olive plants (Bhat et al., 1998).

Table 1: Genus, morphology, Gram type and enzymatic activities of purified isolates

Isolate	Genus	Morphology	Gram	Lipase	Protease	Amylase	Cellulase	Pectinase	Tannase
OR1	yeast			+					
OR2	yeast			+			+		
OR3	yeast			+				+	
OR4	yeast			+				+	
OR5	bacterium	Coccus	Positive	+					
OR6	bacterium			+					
OR8	bacterium	Coccus	Negative				+		
OR9	bacterium	Rod	Positive				+		
OR10	bacterium	Rod	Positive	+			+		
OR13	fungus			+					
OR14	yeast			+					
OR15	bacterium	Coccus	Positive					+	
OR17	bacterium	Rod	Negative					+	
OR18	yeast							+	
OR19	bacterium	Coccus	Negative		+			+	
OR20	bacterium	Coccus	Negative		+		+		
OR21	bacterium	Rod	Negative					+	+
OR24	bacterium	Coccus	Positive					+	
OR27	yeast							+	
OR28	yeast							+	
OR33	yeast							+	
OR34	bacterium	Rod	Negative	+			+	+	+
OR35	bacterium	Rod	Negative	+	+		+	+	
OR36	bacterium	Rod	Negative	+				+	
OR37	bacterium	Rod	Negative	+			+	+	
OM1	bacterium				+				
OM2	bacterium	Coccus	Negative	+				+	
OM3	bacterium	Rod	Positive			+	+	+	
OM5	yeast			+			+	+	
OM6	yeast			+					
OM7	bacterium	Coccus	Positive		+				
OM8	fungus					+		+	
OM9	yeast				+	+		+	
OM10	bacterium	Coccus	Positive				+		
OM11	bacterium	Coccus	Positive			+			
OM12	yeast						+		
OM13	yeast						+		
OM14	yeast			+					
OM15	yeast							+	+
OM16	yeast							+	
OM17	yeast							+	
OM18	yeast					+	+		
OM19	yeast					+		+	
OM20	yeast							+	+
OM21	bacterium	Rod	Negative	+			+		

OM22	fungus			+					
OM23	fungus						+		
OP1	yeast			+					
OP2	yeast			+					
OP3	yeast			+					
OP4	yeast						+		
OP5	yeast				+		+		
OP6	yeast						+		
OP9	bacterium	Coccus	Positive			+			
OP10	bacterium	Coccus	Positive			+			
OP11	bacterium	Coccus	Positive			+	+		
OP13	bacterium	Coccus	Positive				+		
OP14	bacterium	Rod	Negative				+	+	
OP15	yeast							+	
OP16	yeast						+	+	
OP17	yeast							+	
OP18	yeast						+		
OP19	bacterium	Coccus	Positive					+	
OP21	yeast						+		
OP22	yeast				+		+		
OP23	bacterium	Coccus	Positive					+	
OP24	bacterium	Rod	Positive				+	+	
OP25	fungus						+		+
total = 68				22	8	9	28	32	5

4. Bacterial lipase production in liquid medium:

As the main objective of this study was to isolate lipase-producing bacteria for biotechnological interest, the 8 bacteria that showed fluorescent halos on Rhodamine-B agar plates supplemented by olive oil (Figure 6) were selected for further work. Among them, 6 bacteria were isolated from OR and only 2 from OM, and none from OP. In fact, microbial lipases are generally more stable than animal or plant lipases. Indeed, they constitute an important group of biotechnologically valuable enzymes, mainly because of the versatility of their applied properties and ease of mass production (Hasan et al., 2006). Microbial lipases have a wide range of biochemical properties depending on their sources with respect to substrate specificity, regio-and/or stereoselectivity, thermostability, pH optimum, etc. Due to these characteristics, the application of these enzymes extends to various sectors, such as detergent, cosmetics, flavor and food, bioremediation, biofuels, biocatalysis and production of pharmaceutical drugs, among others (Verma et al., 2012; Lai et al., 2018; Jaeger and Eggert, 2002). So, for each desired application, the suitable lipase can be found. Moreover, bacterial strains are generally more used as they offer higher activities compared to yeasts and tend to have neutral or alkaline pH optima and are often thermostable (Hasan et al., 2006).

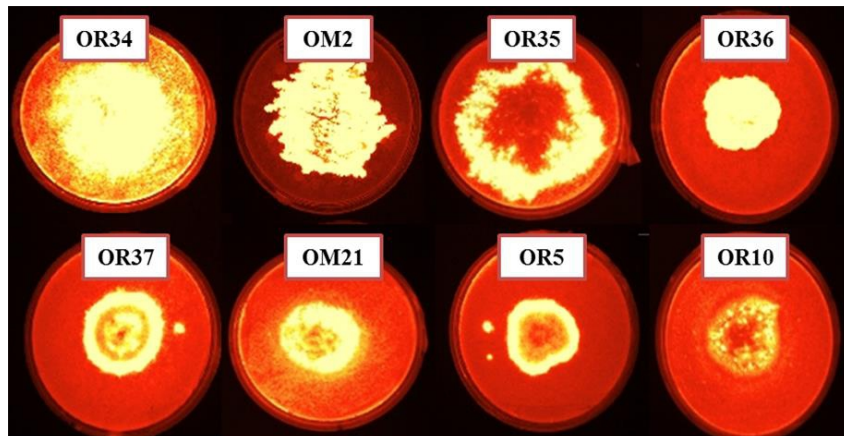


Figure 6: fluorescent halos of lipase-producing bacteria on Rhodamine-B agar medium

Growth and lipase production profiles of the 8 selected bacteria are presented in Figure 7. The results showed that the 8 bacteria grew well on this liquid culture medium, with different growth rates, ranging from 4.75 to 8.98 at 600 nm. In fact, OM 2 and OM 21 bacteria represented the highest growth rates, which were 8.98 (after 30 h of incubation) and 8.5 (after 24 h of incubation), respectively. Concerning lipase activity, the levels were ranging from 0.83 to 11 U/ml and the highest one was obtained by OR 34 (11 U/ml) after 30 h of incubation at 37 °C, followed by OM 2 (9U/ml) after 24 h of incubation at 37 °C. The profiles also show that the lipase production increases with the increase of the cell growth to reach its maximum during the beginning of the stationary phase and started to decrease afterwards.

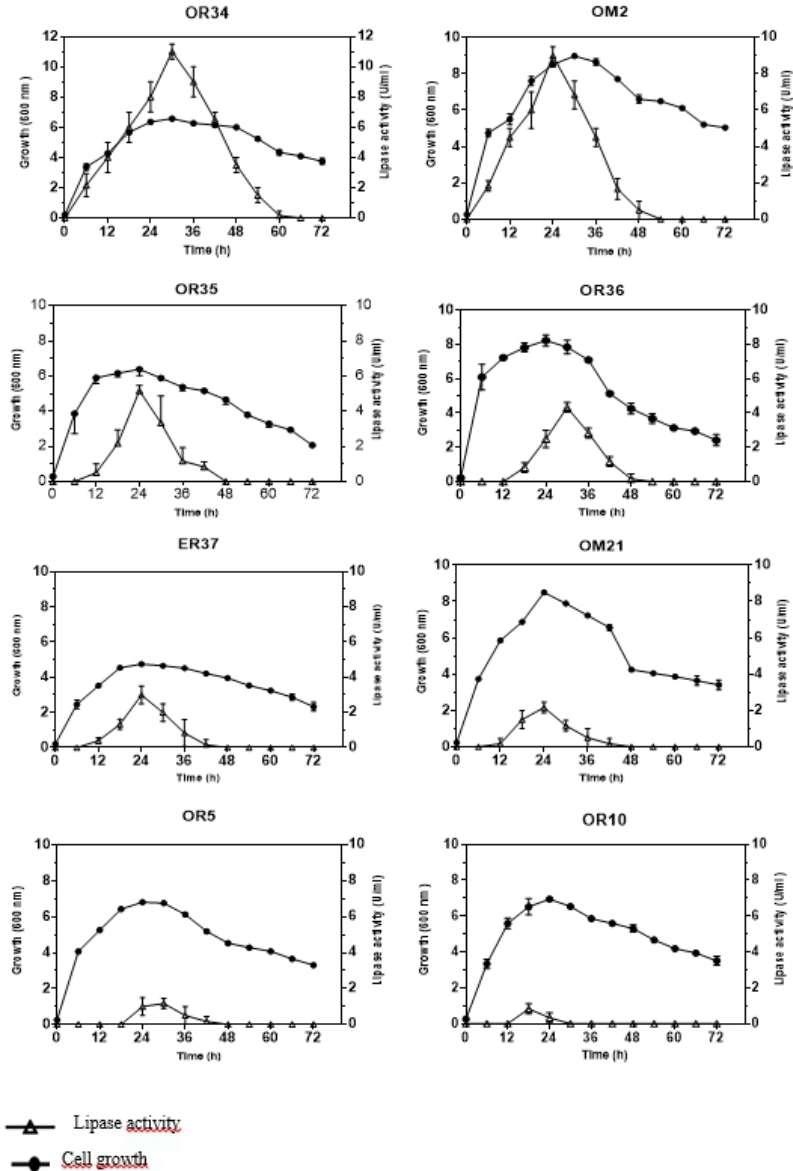


Figure 7: Time course of growth and lipase production kinetic of selected bacteria in liquid production medium under the standard conditions

As demonstrated here, it seems that, on the one hand, the rates of enzymatic production and the cell growth differed from one strain to another and according to the incubation period. On the other hand, the lipase production was dependent to the cell growth density. In fact, Sharma et al., 2014, showed that the lipase production by *Bacillus* sp. KS4 increased rapidly during log phase of growth curve and reached the maximum (0.612 U/ml) after 28 h of incubation with final cell density of 1.633, after which the lipase

activity decreased gradually. While an incubation period of 16 h was sufficient for *Acinetobacter* sp. AU07 to produce the maximum of lipase activity that was 14.5 U/ml during the stationary phase (OD 600 = 1.3) and then gradually decreased (Gururaj et al., 2016). Also, both bacterial cell growth and lipase production of *Halobacillus trueperi* RSK CAS9 reached the maximum (1410.17 U/ml) at 42 h and started to decrease gradually after 48 h (Sathishkumar et al., 2015). Furthermore, the halotolerant strain *Staphylococcus* sp. CJ3 showed the highest extracellular lipase production amounting 5 U/ml which was achieved after 24 h of cultivation and corresponded to the beginning of stationary phase (OD 600 = 2.5). After that period, the lipase activity decreased slightly and a residual activity could still be measured at 96 h (Daoud et al., 2013).

The profiles obtained in this study, may vary if the medium and culture conditions are varied. In fact, various factors influence enzyme production and bacterial growth, including the type and concentration of nutrients, the pH of the medium, the incubation temperature, inoculum's size, the presence or absence of metallic ions and inductors (Abbas et al., 2017; Shyamala Devi et al., 2012; Sharma et al., 2014). For this reason, it is necessary to optimize the culture parameters for each strain, in order to increase the cell growth and the level of lipase production.

Based on the current results, the two bacteria presenting the highest lipase production (OR 34 and OM2) under the standard conditions were selected and subjected to molecular identification.

5. Molecular identification:

Sequencing of the 16S rDNA with the primers fD1 and RS16 provided sequences larger than 500 bp. In the literature, identifications were performed using 500 bp sequences (Hall et al., 2003, Patel et al., 2000), others were based on the sequencing of about 800 bp (Bosshard et al., 2003) and even less than 200 bp (Wilck et al., 2001). Table 2 shows the bacterial species whose 16S rDNA gene showed a strong identity with that of the two selected bacteria. Thus, the OR 34 strain was identified as *Proteus vulgaris* and the OM 2 strain was identified as *Serratia marcescens*. These data are corroborated with the literature which shows that these species are well known by their lipase production.

Table 2: Molecular identification of the two lipase producing bacteria

Isolate	Primer	size of sequenced fragment	Identity (%)	Bacterial species showing strong homology with the isolate
OR 34	FD1	637 pb	98.04%	<i>Proteus vulgaris</i> strain CM_AC3
	RS 16	641 pb	98.39%	<i>Proteus vulgaris</i> strain L1-10-9
OM 2	RS 16	574 pb	99.63%	<i>Serratia marcescens</i> strain MTR13
	FD1	501 pb	98.56%	<i>Serratia</i> sp. PCSAS2-4

Indeed, Kim et al., 1996 isolated an extracellular alkaline lipase from the soil collected near a sewage disposal plant in Korea and identified to be a strain of *P.vulgaris* K 80. This enzyme was used as catalyst during the interesterification and transesterification reactions (Jo and Kim, 2016; Natalia et al., 2017). Another strain of *P.vulgaris* T6 was isolated from soil samples, which has potential applications in detergents and leather processing, because of its alkalophilic property (Fang et al., 2009). In addition, a lipase-producing bacterium K107 was isolated from soil samples of China and identified to be a strain of *Proteus* sp. and was applied in biodiesel production (Gao et al., 2009). It was used also into the resolution of 1-phenylethanol, the chiral derivatives of which were important building blocks for drug substrates and agricultural products (Gao et al., 2011).

Concerning lipases from *Serratia* genus, some authors showed that the high tolerance against organic solvent, along with a broad substrate spectrum, made the *S. marcescens* ECU1010 lipase a very attractive enzyme for potential industrial applications, particularly in the field of biocatalytic resolution (Li et al., 2011; Zhao et al., 2008; Long et al., 2007). Also, a psychrotrophic *S.marcescens* was isolated from raw milk and demonstrated as extracellular lipase producer. Moreover, Eddehech et al. 2018, reported the production, purification and biochemical characterization of a thermoactive alkaline lipase, produced by *Serratia* sp. W3 isolated from palm leaves Tunisian cultivar. It was considered as a potential candidate for industrial and biotechnological applications, such as synthesis of biodiesel and detergent industry.

Similarly, the lipases produced by the two bacteria, identified in this paper, must be biochemically characterized in order to test their performance in detergent industry and others, which will make the goal of the next work.

Conclusion

The findings presented in this study, explored on the one hand the microbial richness of olive oil wastes. The samples collected from the Moroccan olive mill had a heterogeneous microbial composition, consisting mainly of yeasts but also of bacteria and some fungi. On the other hand, this paper indicates that the microbial communities of olive oil wastes constitute a

potent source of useful enzymes for potential industrial applications. Indeed, among 81 examined isolates, 68 were able to produce at least one of the screened enzymes (lipase, protease, amylase, cellulase, pectinase and tannase). In addition, among the 8 lipolytic bacteria isolated, two were identified as *Proteus vulgaris* and *Serratia marcescens* and showed the highest lipase production in liquid medium. These lipases will be biochemically characterized in order to test their performance in various biotechnological applications.

References:

1. Abbas, N., Javed, J., Abbas, Z., Choudry, S., Ali, S. (2017). Lipase Production from *Bacillus subtilis* using various Agricultural wastes. *International Journal of Advanced Engineering, Management and Science (IJAEMS)*. 3: 405-409.
2. Abrunhosa, L., Oliveira, F., Dantas, D., Gonçalves, C. and Belo, I. (2012). Lipase production by *Aspergillus ibericus* using olive mill wastewater. *Bioprocess and Biosystems Engineering*. 36:285–291.
3. Adrio, J.L. and Demain, A.L. (2014). Microbial Enzymes: Tools for Biotechnological Processes. *Biomolecules*. 4 : 117-139. doi:10.3390/biom4010117
4. Altieri, R. and Esposito, A. (2008). Olive orchard amended with two experimental olive mill wastes mixtures: Effects on soil organic carbon, plant growth and yield. *Bioresource Technology*. 99(17): 8390–8393.
5. Asses, N., Ayed, L., Bouallagui, H., Ben Rejeb, I., Gargouri, M., Hamdi, M. (2009). Use of *Geotrichum candidum* for olive mill wastewater treatment in submerged and static culture. *Bioresource Technology* 100: 2182–2188.
6. Baffi, M.A., Romo-Sánchez, S., Ubeda-Iranzo, J. and Briones-Pérez, A.I. (2012). Fungi isolated from olive ecosystems and screening of their potential biotechnological use. *New Biotechnology*. 29:451-456.
7. Ben Sassi, A., Boularbah, A., Jaouad, A., Walker, G., Boussaid, A. (2006). A comparison of Olive oil Mill Wastewaters (OMW) from three different processes in Morocco. *Process Biochemistry*. 41 :74–78.
8. Bhat, T.K., Singh, B., Sharma, O.P. (1998). Microbial degradation of tannins-A current perspective. *Biodegradation*. 9: 343–357.
9. Bosshard, P.P., Abels, S., Zbinden, R., Bottger, E.C., Altwegg, M. (2003). Ribosomal DNA Sequencing for Identification of Aerobic Gram-Positive Rods in the Clinical Laboratory (an 18-Month Evaluation). *Journal of Clinical Microbiology*. 41 (9) : 4134–4140. DOI: 10.1128/JCM.41.9.4134–4140.2003

10. Brahmabhatt, D. and Modi H. A. (2015). Comparative Studies on Methods of Tannase Assay. *International Journal for Research in Applied Science & Engineering Technology (IJRASET)*. 3: 715-720.
11. Brozzoli, V., Crognale, S., Sampedro, I., Federici, F., D'Annibale, A., Petruccioli, M. (2009). Assessment of olive-mill wastewater as a growth medium for lipase production by *Candida cylindracea* in bench-top reactor. *Bioresource Technology*. 100:3395–3402.
12. Cegarra, J., Alburquerque, J.A., Gonzalez, J., Tortosa, G., Chaw, D. (2006). Effects of the forced ventilation on composting of a solid olive-mill by-product (“alperujo”) managed by mechanical turning. *Waste Management*. 26 :1377–1383.
13. Chahinian, H., Nini, L., Boitard, E., Dubès, J.-P., Comeau, L.-C., & Sarda, L. (2002). Distinction between esterases and lipases: A kinetic study with vinyl esters and TAG. *Lipids*. 37(7): 653–662. doi:10.1007/s11745-002-0946-7
14. Ciafardini, G., Zullo, B.A. (2018). Virgin olive oil yeasts: A review. *Food Microbiology*. 70:245-253.
15. Ciafardini, G., Zullo, B.A., Iride, A. (2006). Lipase production by yeasts from extra virgin olive oil. *Food Microbiology*. 23:60–67
16. Cordova, J., Nemmaoui, M., Ismaili-Alaoui, M., Morin, A., Roussos, S., Raimbault, M., Benjilali, B. (1998). Lipase production by solid state fermentation of olive cake and sugar cane bagasse. *Journal of Molecular Catalysis B: Enzymatic*. 5:75–78.
17. Crognale, S., D'Annibale, A., Federici, F., Fenice, M., Quaratino, D. and Petruccioli, M. (2006). Olive oil mill wastewater valorisation by fungi. *Journal of Chemical Technology and Biotechnology*. 81:1547–1555.
18. D'Annibale, A., Brozzoli, V., Crognale, S., Gallo, A.M., Federici, F. and Petruccioli, M. (2006). Optimisation by response surface methodology of fungal lipase production on olive mill wastewater. *Journal of Chemical Technology and Biotechnology*. 81:1586–1593.
19. D'Annibale, A., Sermanni, G.G., Federici, F., Petruccioli, M. (2005). Olive-mill wastewaters: a promising substrate for microbial lipase production. *Bioresource Technology*. 97 :1828–1833.
20. Daoud, L., Kamoun, J., Bou Ali, M., Jallouli, R., Bradai, R., Mechichi, T., Gargouri, Y., Ben Ali, Y., Aloulou, A. (2013). Purification and biochemical characterization of a halotolerant *Staphylococcus* sp. extracellular lipase. *International Journal of Biological Macromolecules*. 57: 232 237.
21. Eddehech, A., Zarai, Z., Aloui, F., Smichi, N., Noiriél, A., Abousalham, A., Gargouri, Y. (2018). Production, purification and biochemical characterization of a thermoactive, alkaline lipase from a

- newly isolated *Serratia* sp. W3 Tunisian strain. *International Journal of Biological Macromolecules*. doi:10.1016/j.ijbiomac.2018.11.050
22. Elharchli, H., Ghomari, O., Chokrafi, F-Z., Chraibi, M., Khalil, F. (2015). Study of Physico-chemical and Microbiological Combined Treatment of Olive Oil Mill Wastewater. *International Journal of Scientific & Engineering Research*. 6 : 909-917.
 23. Ereqat, S.I., Abdelkader, A.A., Nasereddin, A.F., Al-Jawabreh, A.O., Zaid, T.M., Letnik, I. and Abdeen, Z.A. (2017). Isolation and characterization of phenol degrading bacterium strain *Bacillus thuringiensis* J20 from olive waste in Palestine. *Journal of Environmental Science and Health, Part A*. 0:1–7. DOI:10.1080/10934529.2017.1368300.
 24. Ertuğrul, S., Dönmez, G. and Takaç, S. (2007). Isolation of lipase producing *Bacillus* sp. from olive mill wastewater and improving its enzyme activity. *Journal of Hazardous Materials*. 149:720–724.
 25. Esmail, A., Abed, H., Firdaus, M., Chahboun, N., Mennane, Z., Berny, E.H., Ouhssine, M. (2014). Physico-chemical and microbiological study of oil mill wastewater (OMW) from three different regions of Morocco (Ouazzane, Fes Boulman and Béni Mellal). *Journal of Materials and Environmental Science*. 5:121-126.
 26. Fang, Y., Lu, Y., Lv, F., Bie, X., Zhao, H., Wang, Y., Lu, Z. (2009). Improvement of alkaline lipase from *Proteus vulgaris* T6 by directed evolution. *Enzyme and Microbial Technology*. 44 (2): 84-88. DOI:10.1016/j.enzmictec.2008.10.018
 27. Federici, E., Pepi, M., Esposito, A., Scargetta, S., Fidati, L., Gasperini, S., Cenci, G., Altieri, R. (2011). Two-phase olive mill waste composting: Community dynamics and functional role of the resident microbiota. *Bioresource Technology*. 102:10965–10972.
 28. Federici, F., Montedoro, G., Servili, M., Petruccioli, M. (1988). Pectic Enzyme Production by *Cryptococcus albidus* var. *albidus* on Olive Vegetation Waters Enriched with Sunflower Calathide Meal. *Biological Wastes*. 25:291-301.
 29. Fiestas Ros de Ursinos, J.A. and Borja Padilla, R. (1992). Use and treatment of olive mill wastewater: Current situation and prospects in Spain. *Grasas y Aceites*. 43: 101-106.
 30. from two-phase olive oil extraction waste products. *World Journal of Microbiology & Biotechnology*. 16: 555-561.
 31. Gao, B., Su, E., Lin, J., Jiang, Z., Ma, Y., Wei, D. (2009). Development of recombinant *Escherichia coli* whole-cell biocatalyst expressing a novel alkaline lipase-coding gene from *Proteus* sp. for biodiesel production. *Journal of Biotechnology*. 139 (2): 169–175. doi:10.1016/j.jbiotec.2008.10.004

32. Gao, B., Xu, T., Lin, J., Zhang, L., Su, E., Jiang, Z., Wei, D. (2011). Improving the catalytic activity of lipase LipK107 from *Proteus* sp. by site-directed mutagenesis in the lid domain based on computer simulation. *Journal of Molecular Catalysis B: Enzymatic*. 68: 286–291. doi:10.1016/j.molcatb.2010.12.001
33. Gelegenis, J., Georgakakis, D., Angelidaki, I., Christopoulou, N., Goumenaki, M. (2007). Optimization of biogas production from olive-oil mill wastewater, by codigesting with diluted poultry-manure. *Applied Energy*. 84(6): 646–663. doi:10.1016/j.apenergy.2006.12.001
34. Geoffry, K. and Achur R.N. (2018). Screening and production of lipase from fungal organisms. *Biocatalysis and Agricultural Biotechnology*. 14: 241-253. DOI:10.1016/j.bcab.2018.03.009.
35. Giannoutsou, E. P., Meintanis, C. and Karagouni, A. D. (2004). Identification of yeast strains isolated from a two-phase decanter system olive oil waste and investigation of their ability for its fermentation. *Bioresource Technology*. 93(3): 301–306. DOI:10.1016/j.biortech.2003.10.023.
36. Golomb, B.L., Morales, V., Jung, A., Yau, B., Boundy-Mills, K.L., Marco, M.L. (2013). Effects of pectinolytic yeast on the microbial composition and spoilage of olive fermentations. *Food Microbiology* 33:97-106.
37. Gram, H. C. (1884). Über die isolierte Färbung der Schizomyceten in Schnitt-und Trockenpräparaten. *Fortschr. Med.* 2:185-189.
38. Gururaj, P., Ramalingam, S., Nandhini Devi, G., Gautam, P. (2016). Process optimization for production and purification of a thermostable, organic solvent tolerant lipase from *Acinetobacter* sp. AU07. *Brazilian Journal of Microbiology*. 47: 647–657. DOI:10.1016/j.bjm.2015.04.002.
39. Hall, L., Doerr, K.A., Wohlfiel, S.L. and Roberts, G.D. (2003). Evaluation of the MicroSeq System for Identification of Mycobacteria by 16S Ribosomal DNA Sequencing and Its Integration into a Routine Clinical Mycobacteriology Laboratory. *Journal of Clinical Microbiology*. 41: 1447–1453.
40. Hasan, F., Shah, A.A., Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial Technology*. 39:235–251. DOI:10.1016/j.enzmictec.2005.10.016.
41. Hassi, M., Haggoud, A., El Mzibri, M., Ibnsouda, S., Houari, A., Iraqui, M. (2007). Isolation and identification of a staphylococcal strain with an antimycobacterial activity and study of its mode of action. *Annals of Microbiology*. 57 (4) : 651-656.

42. Hemati, A., Aliasghar zad, N., Khakvar, R. (2018). In vitro evaluation of lignocellulolytic activity of thermophilic bacteria isolated from different composts and soils of Iran. *Biocatalysis and Agricultural Biotechnology*. 14:424–430. DOI:10.1016/j.bcab.2018.04.010.
43. Horchani, H., Mosbah, H., Salem, N. B., Gargouri, Y., & Sayari, A. (2009). Biochemical and molecular characterisation of a thermoactive, alkaline and detergent-stable lipase from a newly isolated *Staphylococcus aureus* strain. *Journal of Molecular Catalysis B: Enzymatic*/ 56 (4): 237–245. doi:10.1016/j.molcatb.2008.05.011
44. Jaeger, K-E. and Eggert, T. (2002). Lipases for biotechnology. *Current Opinion in Biotechnology*.13:390–397.
45. Jo, J.C. and Kim, H.K. (2016). Production of cinnamoyl lipids using immobilized *Proteus vulgaris* K80 lipase and an evaluation of their antioxidant activity. *Journal of Molecular Catalysis B: Enzymatic*. 129: 54–60. DOI:10.1016/j.molcatb.2016.04.008
46. Jones, C.E., Murphy, P.J., Russell, N.J. (2000). Diversity and osmoregulatory responses of bacteria isolated
47. Karam, E.A., Abdel Wahab, W.A., Saleh, S.A.A., Hassan, M.E., Kansoh, A.L., Esawy, M.A. (2017). Production, Immobilization and Thermodynamic Studies of Free and Immobilized *Aspergillus awamori* Amylase. *International Journal of Biological Macromolecules*. 102:694-703. doi:10.1016/j.ijbiomac.2017.04.033.
48. Kavroulakis, N. and Ntougias, S. (2011). Bacterial and b-proteobacterial diversity in *Olea europaea* var. mastoidis- and *O. europaea* var. koroneiki-generated olive mill wastewaters: influence of cultivation and harvesting practice on bacterial community structure. *World Journal of Microbiology and Biotechnology*. 27:57–66. DOI 10.1007/s11274-010-0426-3.
49. Kim, H-K., Lee, J-K., Kim, H., Oh, T-K. (1996). Characterization of an alkaline lipase from *Proteus vulgaris* K80 and the DNA sequence of the encoding gene. *FEMS Microbiology Letters*. 135(1): 117–121.
50. Kistner, T., Nitz, G., Schnitzler, W.H. (2004). Phytotoxic effects of some compounds of olive mill wastewater (OMW). *Fresenius Environmental Bulletin*. 13: 1360-1361.
51. Kouker, G. and Jaeger, K.E. (1986). Specific and Sensitive Plate Assay for Bacterial Lipases. *Applied and Environmental Microbiology*. 53 (1): 211-213.
52. Laachari, F., Elabed, S., Sayari, A., Iraqui M., Harchali, H., Boubendir, A., Saad Ibsouda, K.S. (2013). Biochemical characterization of a thermoactive and thermostable lipase from a newly isolated *Trichosporon coremiiforme* strain. *African Journal of Biotechnology*. 12 (28): 4503-4511.

53. Laconi, S., Molle, G., Cabiddu, A., Pompei, R. (2007). Bioremediation of olive oil mill wastewater and production of microbial biomass. *Biodegradation*. 18(5) : 559–566. doi:10.1007/s10532-006-9087-1
54. Lai, O-M., Lee, Y-Y, Phuah, E-T. and Akoh, C.C. (2018). *Lipase/Esterase: Properties and Industrial Applications*. Reference Module in Food Science. DOI:10.1016/b978-0-08-100596-5.21640-5.
55. Lanciotti, R., Gianotti, A., Baldi, D., Angrisani, R., Suzzi, G., Mastrocola, D., Guerzoni, M.E. (2005). Use of *Yarrowia lipolytica* strains for the treatment of olive mill wastewater. *Bioresource Technology*. 96 :317–322.
56. Leite, P., Salgado, J.M., Venâncio, A., Domínguez, J.M., Belo, I. (2016). Ultrasounds pretreatment of olive pomace to improve xylanase and cellulase production by solid-state fermentation. *Bioresource Technology*. 214: 737–746. doi:10.1016/j.biortech.2016.05.028
57. Li, S., Pang, H., Lin, K., Xu, J-H., Zhao, J., Fan, L. (2011). Refolding, purification and characterization of an organic solvent-tolerant lipase from *Serratia marcescens* ECU1010. *Journal of Molecular Catalysis B: Enzymatic*. 71(3-4), 171–176. doi:10.1016/j.molcatb.2011.04.016
58. Lobedanz, S., Damhus, T., Borchert, T. V., Hansen, T. T., Lund, H., Lai, W., Lin, M., Leclerc, M., Kirk, O. (2016). *Enzymes in Industrial Biotechnology*. Kirk-Othmer Encyclopedia of Chemical Technology. 1-73. doi:10.1002/0471238961.091404211409
59. Long, Z., Xu, J., Pan, J. (2007). Immobilization of *Serratia marcescens* Lipase and Catalytic Resolution of Trans-3-(4'-methoxyphenyl)glycidic Acid Methyl Ester. *Chinese Journal of Catalysis*. 28(2) :175-179.
60. Lorenz, T.C. (2012). Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies. *Journal of Visualized Experiments*. (63), e3998. DOI: 10.3791/3998.
61. Madhav,K., Verma, S., Tanta, R. (2011). Isolation of amylase producing *Bacillus* species, from soil sample of different regions in Dehradun and to check the effect of pH and Temperature on their amylase activity. *Journal of Pharmaceutical and Biomedical Sciences*. 12 (03).
62. Marmur, J. (1961). A Procedure for the Isolation of Deoxyribonucleic Acid from Micro-organisms. *Journal of Molecular Biology*. 3:208-218. DOI: 10.1016/s0022-2836(61)80047-8.
63. Millan, B., Lucas, R., Robles, A., Garcia, T., Alvarez de Cienfuegos, G., Galvez, A. (2000). A study on the microbiota from olive-mill wastewater (OMW) disposal lagoons, with emphasis on filamentous

- fungi and their biodegradative potential. *Microbiological Research*. 155 :143-147.
64. Morillo, J. A., Antizar-Ladislao, B., Monteoliva-Sánchez, M., Ramos-Cormenzana, A., Russell, N. J. (2009). Bioremediation and biovalorisation of olive-mill wastes. *Applied Microbiology and Biotechnology*. 82(1): 25–39. doi:10.1007/s00253-008-1801-y
 65. Mouncif, M., Tamoh, S., Faid M. and Achkari-Begdouri, A. (1993). A study of chemical and microbiological characteristics of olive mill waste water in Morocco. *International journal of fats and oils*. 44: 335-338.
 66. Natalia, A., Kim, S-J., Kim, H.K. (2017). Antioxidant and antibacterial activity of fatty acid vanillyl ester produced by *Proteus vulgaris* K80 lipase-mediated transesterification, *Journal of Molecular Catalysis B: Enzymatic*.
 67. Nigam, P.S. (2013). Microbial Enzymes with Special Characteristics for Biotechnological Applications. *Biomolecules*. 3 : 597-611. doi:10.3390/biom3030597
 68. Ntougias, S., Bourtzis, K., Tsiamis, G. (2013). Review Article: The Microbiology of Olive Mill Wastes. *BioMed Research International*. (2013) :1–16. doi:10.1155/2013/784591
 69. Oliveira, F., Abrunhosa, L., Dantas, D., Gonçalves, C., Venâncio, A. and Belo, I. (2011). Lipase production from olive mill wastewaters by *Aspergillus ibericus*. Book of Abstracts of the 11th International Chemical and Biological Engineering Conference, Lisbon, Portugal, 5-7 September 2011.
 70. Ouzounidou, G., Zervakis, G.I., Graitis, F. (2010). Raw and Microbiologically Detoxified Olive Mill Waste and Their impact on plant Growth. *Terrestrial and aquatic environmental Toxicology*. 4: 21-38.
 71. Ozturkoglu-Budak, S., Wiebenga, A., Bron, P. A., de Vries, R. P. (2016). Protease and lipase activities of fungal and bacterial strains derived from an artisanal raw ewe’s milk cheese. *International Journal of Food Microbiology*. 237:17–27. DOI:10.1016/j.ijfoodmicro.2016.08.007.
 72. Papagora, C., Roukas, T., Kotzekidou, P. (2013). Optimization of extracellular lipase production by *Debaryomyces hansenii* isolates from dry-salted olives using response surface methodology. *Food and Bioproducts Processing*. 91 (4): 413-420
 73. Patel, J.B., Leonard, D.G.B., Pan, X., Musser, J.M., Berman, R.E. and Nachamkin, I. (2000). Sequence-Based Identification of Mycobacterium Species Using the MicroSeq 500 16S rDNA Bacterial Identification System. *Journal of Clinical Microbiology*. 38: 246–251.

74. Patil, N.P. and Chaudhari, B.L. (2010). Production and purification of pectinase by soil isolate penicillium sp. and search for better agro-residue for its SSF. *Recent Research in Science and Technology*. 2(7): 36-42.
75. Pepi, M., Cappelli, S., Hachicho, N., Perra, G., Renzi, M., Tarabelli, A., Altieri, R., Esposito, A., Focardi, S.E., Heipieper, H.J., (2013). *Klebsiella* sp. strain C2A isolated from olive oil mill waste is able to tolerate and degrade tannic acid in very high concentrations. *FEMS Microbiology Letters* .343:105–112.
76. Pepi, M., Lampariello, L.R., Altieri, R., Esposito, A., Perra, G., Renzi, M., Lobianco, A., Feola, A., Gasperini, S., Focardi, S.E. (2010). Tannic acid degradation by bacterial strains *Serratia* spp. and *Pantoea* sp. isolated from olive mill waste mixtures. *International Biodeterioration & Biodegradation*. 64:73-80.
77. Pérez, J., de la Rubia, T., Ben Hamman, O. and Martínez, J. (1998). *Phanerochaete flavido-alba* Laccase Induction and Modification of Manganese Peroxidase Isoenzyme Pattern in Decolorized Olive Oil Mill Wastewaters. *Applied and Environmental Microbiology*. 64 (7): 2726–2729.
78. Ramachandran, S., Patel, A.K, Nampoothiri, K.M., Chandran, S., Szakacs, G., Soccol, C.R. and Pandey, A. (2004). Alpha Amylase from a Fungal Culture Grown on Oil Cakes and Its Properties. *Brazilian Archives of Biology and Technology*. 47:309-317.
79. Rathelot, J., Julien, R., Canioni, P., Coeroli, C., Sarda, L. (1976). Studies on the effect of bile salt and colipase on enzymatic lipolysis. Improved method for the determination of pancreatic lipase and colipase. *Biochimie*. 57(10): 1117–1122. doi:10.1016/s0300-9084(76)80572-x
80. Robinson, P.K. (2015). Enzymes: principles and biotechnological applications. *Essays in Biochemistry*. 59:1-41. doi: 10.1042/BSE0590001
81. Rodriguez, M. M., Perez, J., Ramos-Cormenzana, A. and Martinez, J. (1988). Effect of extracts obtained from olive oil mill waste waters on *Bacillus megaterium* ATCC 33085. *Journal of Applied Bacteriology*. 64 (3): 219–226. DOI:10.1111/j.1365-2672.1988.tb03378.x.
82. Romo-Sánchez, S., Alves-Baffi, M., Arévalo-Villena, M., Úbeda-Iranzo, J., Briones-Pérez, A. (2010). Yeast biodiversity from oleic ecosystems: study of their biotechnological properties. *Food Microbiology*. 27:487–492.
83. Salgado, J.M., Abrunhosa, L., Venancio, A., Domínguez, J.M., Belo, I. (2016). Combined bioremediation and enzyme production by

- Aspergillus* sp. in olive mill and winery wastewaters. *International Biodeterioration & Biodegradation* 110:16-23.
84. Sanchez, S. and Demain, A. L. (2017). Useful Microbial Enzymes - An Introduction. *Biotechnology of Microbial Enzymes*. 1–11. doi:10.1016/b978-0-12-803725-6.00001-7
 85. Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors *Proceedings of the National Academy of Sciences of USA*. 74: 5463-5467.
 86. Sathishkumar, R., Ananthan, G., Iyappan, K., Stalin, C. (2015). A statistical approach for optimization of alkaline lipase production by ascidian associated-Halobacillus trueperi RSK CAS9. *Biotechnology Reports*. 8: 64–71. DOI:10.1016/j.btre.2015.09.002
 87. Scioli, C. and Vollaro, L. (1997). The use of *Yarrowia lipolytica* to reduce pollution in olive mill wastewaters. *Water Research*. 31(10): 2520–2524. doi:10.1016/s0043-1354(97)00083-3
 88. Sharma, D., Kumbhar, B. K., Verma, A. K., Tewari, L. (2014). Optimization of critical growth parameters for enhancing extracellular lipase production by alkalophilic *Bacillus* sp. *Biocatalysis and Agricultural Biotechnology*. 3: 205–211. DOI:10.1016/j.bcab.2014.04.004.
 89. Shyamala Devi, A., Chitra Devi, K., Rajendiran, R. (2012). Optimization of Lipase Production Using *Bacillus subtilis* by Response Surface Methodology. *International Journal of Biotechnology and Bioengineering*. 6: 840-845.
 90. Tsiamis, G., Tzagkaraki, G., Chamalaki, A., Xypteras, N., Andersen, G., Vayenas, D., Bourtzis, K. (2011). Olive-Mill Wastewater Bacterial Communities Display a Cultivar Specific Profile. *Current Microbiology*. DOI:10.1007/s00284-011-0049-4.
 91. Verma, N., Thakur, S. and Bhatt, A.K. (2012). Microbial Lipases: Industrial Applications and Properties (A Review). *International Research Journal of Biological Sciences*. 1: 88-92.
 92. Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. (1991). 16S Ribosomal DNA Amplification for Phylogenetic Study. *Journal of Bacteriology*. 173: 697-703
 93. Wilck, M.B., Wu, Y., Howe, J.G., Crouch, J.Y. and Edberg, S.C. (2001). Endocarditis Caused by Culture-Negative Organisms Visible by Brown and Brenn Staining: Utility of PCR and DNA Sequencing for Diagnosis. *Journal of Clinical Microbiology*. 39: 2025–2027.
 94. Zaier, H., Chmingui, W., Rajhi, H., Bouzidi, D., Roussos, S., Rhouma, A. (2017). Physico-chemical and microbiological characterization of olive mill wastewater (OMW) of different regions of Tunisia (North,

- Sahel, South). *Journal of new Sciences, Agriculture and Biotechnology*. 48:2897-2906.
95. Zanichelli, D., Carloni, F., Hasanaj, E., D'Andrea, N., Filippini, A., Setti, L. (2007). Production of Ethanol by an Integrated Valorization of Olive Oil Byproducts. The Role of Phenolic Inhibition. *Environmental Science and Pollution Research – International*. 14(1): 5–6. doi:10.1065/espr2006.06.316
96. Zhao, L-L., Xu, J-H., Zhao, J., Pan, J., Wang, Z-L. (2008). Biochemical properties and potential applications of an organic solvent-tolerant lipase isolated from *Serratia marcescens* ECU1010. *Process Biochemistry*. 43(6): 626–633. doi:10.1016/j.procbio.2008.01.023