



# Microbiota profiling and screening of the lipase active halotolerant yeasts of the olive brine

Hasan Demirci<sup>1</sup> · Günseli Kurt-Gur<sup>1</sup> · Emel Ordu<sup>1</sup>

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## Abstract

Searching for novel enzymes that could be active in organic solvents has become an area of interest in recent years. Olive brine naturally provides a suitable environment for the survival of halophilic and acidophilic microorganisms and the resulting genome is thought to be a gene source for determining the halophilic and acidophilic proteins that are active in a non-aqueous organic solvent medium, and so it has been used in several biotechnological and industrial applications. In this study, microbial analysis of natural, cracked green olive brine from the southern region of Turkey has been made by next-generation sequencing of the brine metagenome for the first time in the literature. The number of reads assigned to fungal operational taxonomic units was the highest percentage (73.04%) with the dominant representation of Ascomycota phylum (99% of fungi). Bacterial OTU was 3.56% of the reads and Proteobacteria phylum was 65% of the reads. The lipase production capacity of the yeasts that were grown on the media containing elevated concentrations of NaCl (1–3 M) was determined on a Rhodamine B-including medium. Molecular identification of the selected yeasts was performed and 90% of sequenced yeasts had a high level of similarity with *Candida diddensiae*, whereas 10% showed similarity to *Candida boidinii*. The hydrolytic lipase activities using olive oil were analyzed and both yeasts showed cell-bound lipase activity at pH 3.0.

**Keywords** Acidophilics · Halophilics · Metagenomics · Microbial lipases · Olive brine

## Introduction

Metagenomic mining into different microbial environments allows for discovery of novel enzymes and has enormous scope for biotechnological development in addition to understanding fundamental microbiological correlations. Therefore, in this study, metagenomic analysis of salt-saturated olive brine from the southern region of Turkey was performed to understand the potential of halophilic enzymes which are thought to be ideal catalysts for a non-aqueous reaction medium due to the effect of salt on lowering water activity.

Although the activity of enzymes in organic solvents, such as supercritical fluids, fluoruous solvents and ionic liquids, is lower than in water, which is the conventional reaction medium, organic solvents enhance reaction rate

by increasing the solubility of nonpolar substrates, eliminating unfavorable side reactions, minimizing microbial contamination, enhancing thermostability, and allowing reactions to occur that are usually not favored in aqueous solution (Wang et al. 2016a, b; Priyanka et al. 2019). The common characteristic of these solvents is their non-aqueous (low or no water) organic solvent medium. Therefore, the isolation of novel enzymes that could be active and stable in non-aqueous organic solutions has become an area of interest in recent years (Bollinger et al. 2020; Wu et al. 2020; Al-Limoun et al. 2019; Zhang et al. 2018; de Borba et al. 2018; Sengupta et al. 2017). Enzymes derived from halophilic microorganisms are ideal for low-water or anhydrous solvent applications due to the effect of salt on lowering water activity (Wang et al. 2020; Noby et al. 2020; Huo et al. 2017; Xin and Hui-Ying 2013). The salt-saturated table olive fermentation medium (8–12% NaCl concentration, pH 3–4), which facilitates the survival of halophilic microorganisms, is a proper resource for the investigation of the halophilic microorganism and halophilic enzymes. Microorganisms, providing fermentation, enhance the nutritional characteristics and health benefits

✉ Emel Ordu  
bemel@yildiz.edu.tr

<sup>1</sup> Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Yıldız Technical University, Davutpasa Campus, 34220 Esenler, Istanbul, Turkey

of olives via the production of a variety of metabolic compounds (Grounta et al. 2016). The microbial composition of some table olives during fermentation was previously characterized and reviewed (Botta and Cocolin 2012; Heperkan 2013) which used classical culture-based methods or sequencing of the 16S rRNA gene clone library for profiling microbial diversity.

Reported studies show that the microbiota of processed olives and/or brines are composed of a complex association of bacteria, such as lactic acid bacteria, Enterobacteriaceae, Clostridium, Staphylococcus, yeasts and, occasionally, molds (Almeida 2014). Limited data are available in the literature on the detailed analysis of all microbial content of olive brine. Biochemical reactions that take place during the fermentation process of olives are quite complicated, and are determined by the enzymatic activity of the raw material and the metabolic activity of the indigenous microflora of the olives together with a variety of contaminating microorganisms. However, the fermentation process provides preservation against pathogenic microorganisms by lowering the pH of the brine. Spontaneous homemade table olive production increases the contamination risk by undesirable microorganisms, which could be coming from different sources such as soil, pipelines, pumps, water or poor hygiene conditions, and inadequate cleaning of the equipment and olive fruits (Lanza 2013; Botta et al. 2012; Panagou et al. 2013). Although pathogenic bacteria transmission through fermented olives has not been documented so far, several pathogens have been reported to survive in the fermentation process (Medina-Pradas and Arroyo-López 2015) with reported studies showing there is wide heterogeneity in the dominating microflora as a result of where and how fermentation was performed (Doulgeraki et al. 2012). Therefore, each investigated brine sample will enable the discovery of different microorganisms that can survive in a low pH and high salinity environment. While the identification of these microorganisms leads to the improvement of olive brine quality, their genomes will also provide a unique source for biotechnological research.

In this study, both culture-dependent and culture-independent approaches have been used. A detailed taxonomic analysis of the microorganisms that can be found in spontaneous natural cracked green olive brine has been made by next-generation sequencing of all microorganisms' genomes in the sample instead of a specific marker such as 16S rRNA, in order to investigate the gene sources and to isolate the novel enzymes.

Furthermore, culture-dependent isolation has also been performed by screening lipase producing halophilic yeasts on solid media containing elevated concentrations of NaCl followed by molecular identification with the sequencing of the internal transcribed spacer region.

## Materials and methods

### Olive brine sampling

Natural, cracked green olive (*Olea europaea* L.) brine, which has 8–12% NaCl concentration, was kindly provided by a local farm from the Toroslar Region of Turkey. The pH of the sample brine was measured as  $4 \pm 2$ . In this type of fermentation, the olives were washed with tap water and then cracked and fermented conventionally by indigenous bacteria and yeasts.

### DNA extraction and next-generation sequencing

DNA was extracted from the brine sample after 90 days of fermentation. The brine sample was transferred into sterile tubes and centrifuged at  $22,500 \times g$  for 30 min. 5 g of the pellet was obtained and the total DNA was extracted using the PowerSoil®DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and concentration of the DNA were determined using 1% agarose gel and analyzed through spectrophotometric measurements at 260 and 280 nm using NanoDrop® ND-1000 Spectrophotometer (ThermoFisher Scientific Inc., Milan, Italy). The total DNA concentration was adjusted to  $100 \text{ ng } \mu\text{l}^{-1}$  and sent for sequencing using Illumina HiSeq ( $2 \times 150 \text{ bp}$ ) platform (Eurofins GATC Biotech GmbH). Library preparation incorporated adaptor sequences and indexing compatible for Illumina sequencing technology, using proprietary methods of Eurofins GATC Biotech GmbH. The quality of the final library was assessed by determination of the size distribution and quantification. Sequencing was carried out on the Illumina HiSeq platform and raw data were obtained as a result of a primary analysis using Illumina's CASAVA software.

### Data analysis

Abundance, measured by the percentage of OTU assigned reads from various taxonomic levels, was determined after paired-end alignments and quality filtering, removal of adapters and primers, and deletion of plant and human-derived host sequences using the Kraken database. The base quality of each sequence was inspected, and calls with low quality were removed from the 3' and 5' ends if the average Phred quality was below 15. Only mate pairs (forward and reverse read) were used for the next analysis step. The most abundant sequences in each Operational Taxonomic Unit (OTU) were selected as representative

sequences and used for the taxonomic assignment using the BLAST algorithm (Altschul et al. 1990).

Taxonomic profiling results produced by Kraken (Wood and Salzberg 2014) were used to generate interactive plots using Krona (Ondov et al. 2011) visualization tool to represent relative abundances within the complex hierarchy of metagenomic classification.

### Screening of the halophilic yeasts in the olive brine

In order to isolate yeast strains from the olive brine, the brine was centrifuged at  $5000 \times g$  for 10 min at room temperature, the sediment was serially diluted with 0.1% (w/v) peptone water, and aliquots of several dilutions were inoculated on solid YPD medium (yeast extract, 10 g/L; peptone, 20 g/L; dextrose, 20 g/L; agar, 20 g/L). Cells were incubated at 30 °C under aerobic conditions for 72 hours. Isolated colonies were streaked onto the YPD medium containing 1M, 2M, and 3M of NaCl and incubated at 30 °C for 72 h.

### Lipase activity screening

The YPD medium was autoclaved and then cooled down to about 65 °C. Then, 1% (v/v) filter-sterilized triacylglycerol and 0.01% (w/v) filter-sterilized Rhodamine B in H<sub>2</sub>O were added with vigorous shaking. After the medium was allowed to stand for 10 min at 60 °C to reduce foaming, 20 mL was poured into each plate. Cultures were inoculated as a small spot on the screening agar plate and incubated at 30 °C for yeasts. Lipase production was detected by irradiating plates with UV light at 350 nm after 72 h of incubation.

### Genomic DNA isolation and polymerase chain reaction

Selected yeast colonies were picked from the culture plates and suspended in 100 µL of 200 mM LiOAc, 1% SDS solution. After incubation at 70 °C for 15 minutes and the addition of 300 µL 96% ethanol, the samples were mixed by brief vortexing and then centrifuged for 3 min at  $16,000 \times g$ . The supernatant was removed and the pellet was resuspended in 100 µL TE. Cell debris was spun down by centrifugation for 1 min at  $16,000 \times g$ , and 1 µL of the supernatant was used for PCR.

PCR was performed using ITS1 and ITS4 primers to identify samples considered to be lipase-positive yeast grown in YPD (White and Lee 1990). For each sample, the total reaction volume was 20 µL consisting of 12.5 µL Dream Taq Buffer, 0.4 µM (each) of the ITS1 primer and ITS4 primer, and 5 ng of DNA template. PCR was carried out using the following conditions: initial denaturation at 95 °C for 3 min; 35 cycles of denaturation (95 °C for 1 min), annealing (51 °C for 1 min), and extension (72 °C for 30 s);

and a final extension step at 72 °C for 8 min. A negative control was performed with each run by replacing the template DNA with sterile water in the PCR mixture.

### Sequence analysis

Nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) of all the sequences obtained in this study was performed at the NCBI website to determine the pairwise level of similarity of these sequences with other sequences available in the NCBI database.

The bootstrap consensus tree was formed using MegaX (Kumar et al. 2018) by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura 1992). To construct the tree, the GenBank accessions having similarity between 77 and 100% (94–100% query coverage; e-value > e-100) were selected for YLP7, whereas the gene sequences showing 89.83–99.70% identity (93–100% query coverage; e-value > e-100) were used for YLP9.

### Growth conditions of the isolates

To determine cell-bound lipase activity, selected yeast isolates were grown overnight in 50 mL of YPD medium (yeast extract, 10 g/L; peptone, 20 g/L; dextrose, 20 g/L) at 28 °C. Then 300 µL of pre-culture was inoculated in 500 mL-shake-flasks containing 100 mL of production medium, pH 7.0 (0.1% (w/v) yeast extract, 1.6% (w/v) sodium chloride, 0.03% (w/v) magnesium sulphate heptahydrate, 0.2% (w/v) dipotassium hydrogen phosphate and 1% (w/v) ammonium sulfate) using 3% (v/v) olive oil as inducer (Wang et al. 2007) for 4 days at 25 °C under continuous shaking at 170 rpm. 1 mL of the samples was collected and the biomass concentration was determined by filtration (0.45 µm), and then dried to constant weight at 80 °C. The biomass was expressed as cell dry weight (CDW, mg/mL). The samples were then centrifuged ( $9000 \times g$ , 15 min) at 4 °C and the pellet and supernatant were used to determine lipase activity separately. All experiments were performed in triplicate.

### Hydrolytic activity assay

The natural olive oils (10%, w/v) were emulsified in buffers at different pHs containing 5% (w/v) Triton X-100. 0.1 M citrate buffer (pH 3.0–5.0) and 0.1 M potassium phosphate buffer (pH 6.0–8.0) were used as reaction buffers. The reaction was started by adding 1 mL of sample to 5 mL of this emulsion and then maintained for 30 min at 40°C with 300 rpm orbital agitation. The reaction was stopped by adding 5 mL of acetone: ethanol solution (1:1, v/v) to the mixture. The blank was set up by addition of relevant buffer to the emulsion, whereas the positive control was also prepared by adding the enzyme to the olive oil/buffer mixture

which was stopped with acetone: ethanol solution. Following the addition of 20  $\mu\text{l}$  of phytalein indicator, the fatty acids released were titrated to pH 11 with a 0.05 M NaOH solution. All assays were carried out in triplicate. One unit of enzyme activity was defined as the amount of enzyme that releases 1  $\mu\text{mol}$  of fatty acid per mL per min. The specific activity of the enzyme was expressed as  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  cell dry weight.

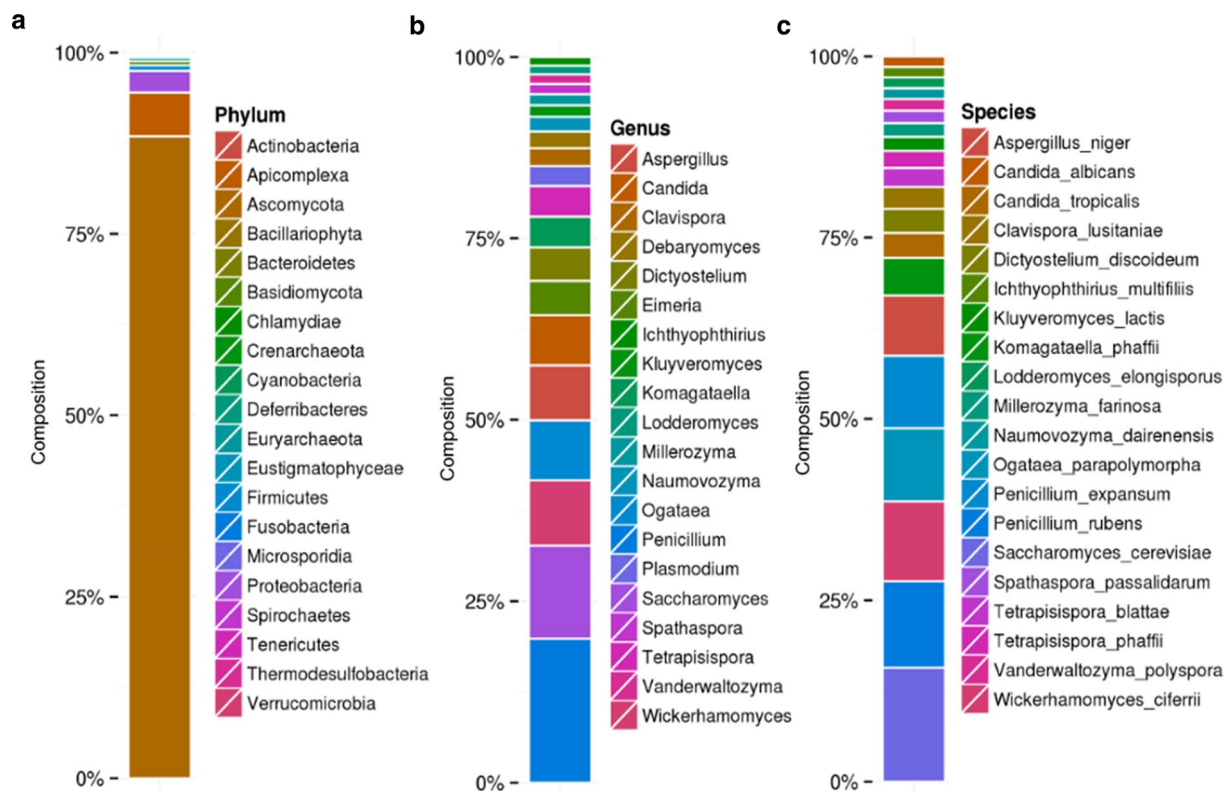
## Results

### Taxonomic profiling

A total of 47,737,960 high-quality reads were recovered and 7.27% (3,768,746) could be classified. After screening and removing host sequence reads coming from plants and humans, non-host reads were subjected to a taxonomic profiling algorithm. Taxonomic profiling was performed using Kraken (Wood and Salzberg 2014) and the MiniKraken reference database with default parameters. Taxonomic profiling results produced by Kraken were used to generate interactive plots using Krona (Ondov et al. 2011) for intuitive exploration of relative abundance and confidences

within the complex hierarchies of metagenomic classifications. The number of reads assigned to fungal OTUs was the highest percentage (73.04%); protozoa was 20% and bacteria was 3.56%. Archaea and viruses were both represented under 1%, while 3.20% of reads could not be assigned to any specific kingdom. Figure 1 shows the taxonomic distribution of microbial diversity detected from all domains. Based on the results from the metagenomic analysis, members of the phylum Ascomycota (82%) were dominant in the olive brine sample, followed by Apicomplexa (6%) and Proteobacteria (3%) (Fig. 1a). The most abundantly-detected genera were *Penicillium* with 15% average relative abundance (Fig. 1b). *Saccharomyces cerevisiae* was the most abundant species (11%) after *Penicillium rubens* (8%) and *Wickerhamomyces ciferrii* (8%) (Fig. 1c).

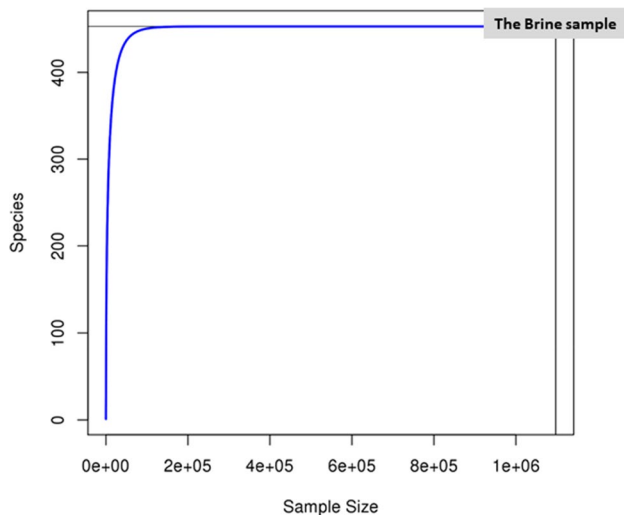
Species richness of the brine sample was also shown by the rarefaction curve (Fig. 2). This curve was calculated from the species abundance table and formed by plotting the total number of distinct species found as a function of the number of sequences sampled. This curve rose quickly and then became saturated when more than 400 species were found in the sample.



**Fig. 1** Bar plots showing the taxonomic abundance across the samples. **a** Taxa-level: Phylum; the most abundant phyla are Ascomycota. **b** Taxa-level: Genus; the most abundant genera are *Penicillium*. **c**

Taxa level: Species; the most abundant three species are *Saccharomyces cerevisiae*, *Penicillium rubens* and *Wickerhamomyces ciferrii*





**Fig. 2** The rarefaction curve of the olive brine

### Fungal diversity

Metagenomic profiling revealed that Ascomycota phylum (99% of fungi) was dominantly represented in the olive brine sample. The other fungi phyla Basidiomycota (0.7%), Microsporidia (0.1%) and Chytridiomycetes (0.005%) were also detected in a very-low frequency (Fig. 3a). Metagenomic profiling revealed that OTUs within the Ascomycota were largely identified as members of the classes Saccharomycetales (67%), Eurotiomycetes (28%) and Sordariomycetes (2%). OTU assigned to Dothideomycetes, Leotiomycetes, *Tuber melanosporum*, Orbillicaceae, Shizosaccharomyces and *Pneumocystis murina* classes was under 1%. The members of the two common classes Saccharomycetales and Eurotiomycetes are shown in Fig. 3b, c. Basidiomycota, the second common phylum, was represented mainly by members of the classes Agaricomycetes (40%), Pucciniales (17%), *Walleria* (13%), *Ustilaginomycetes* (13%) and *Tremellomycetes* (11%). Overall, the genera *Saccharomyces*, *Tetrapisispora*, *Naumovozyma*, *Candida*, *Debaryomyces*, *Millerozyma*, *Spathaspora*, *Wickerhamomyces*, and *Komagataella* were the most abundant fungi detected. The abundance of the most represented species is shown in Fig. 3. *Dictiostellium*, *Eimeria*, *Ichthyophthirius* and *Plasmodium* also exist as representatives of genera from other eukaryota.

### Bacterial communities

Analysis of the metagenomic sequences revealed that a total of 4% reads belong to the bacteria, and the two bacterial phyla Proteobacteria and Firmicutes have the greatest number of reads (65% and 22% of bacteria, respectively) among them. The dominant class of bacteria was Gammaproteobacteria (65%) followed by Bacilli (19%), while

Enterobacteriaceae (27%) and Pseudomonadales (26%) were the dominant bacterial families of the sample (Fig. 4). The phylum with the second major number of sequences was Firmicutes with the order Lactobacillales (6%). The genera of Lactic Acid Bacteria, which were sequenced in our olive brine sample, were *Aerococcus* (3%), *Streptococcaceae* (2%), *Lactobacillus* (0.6%), *Leuconostocaceae* (0.2%) and *Enterococcaceae* (0.2%).

### Screening the halophilic populations having lipase activity

In this study, yeasts on the YPD agar plate were inoculated to other YPD agar plates containing elevated concentrations of NaCl (1 M-3 M) to screen the halophilic yeasts. In total, 300 yeast colonies survived, which were 130, 102, and 68 in 1 M, 1.5 M, and 3 M of NaCl including media, respectively.

Based on their extremely halophilic characteristics, yeasts that survived in 3 M NaCl including media were chosen for further lipase activity screening assay. The lipolytic activity of 68 colonies was evaluated in agar plates via the detection of enzymatic activity formed by Rhodamine B as described by Kouker and Jaeger (1987). The results of the preliminary plate screening showed that 13 of the 68 colonies were found to have lipase activity, while a bright orange halo occurred under UV light (350 nm) indicating positive lipase activity (Fig. 5).

### Molecular identification of halophilic yeasts by ITS sequencing

In this study, 10 halophilic yeasts with the highest lipolytic activity were chosen from selective media, and species determination was performed by ITS-PCR analysis. Following genomic DNA isolation from selected yeasts, the ITS region, including the 5.8S gene, was amplified by the universal primer pair ITS 1 and ITS 4, and the amplified regions were sequenced by Sanger sequencing. BLASTn was used to perform species identification employing the generated sequences as query against standard databases using default settings. In total, 90% of the sequenced yeasts showed significant similarity to *Candida diddensiae*, whereas 10% of the yeasts were closely related to *Candida boidinii*.

The bootstrap consensus tree was formed using MegaX (Kumar et al. 2018) with the Maximum Likelihood method and Tamura 3-parameter model (Tamura 1992) based on the partial sequence of the small subunit ribosomal RNA gene, the complete sequence of internal transcribed spacer 1, 5.8S ribosomal RNA gene, the complete sequence of internal transcribed sequence 2, and partial sequence of the large subunit ribosomal RNA gene. The evolutionary analyses of two yeasts designated as YLP7 and YLP9 were conducted by bootstrap test (500 replicates) and all positions with less than

**Fig. 3** Interactive plots generated by Krona. Species diversity of the brine sample is represented for fungi (a), Saccharomycetales (b) and Eurotiomycetes (c). Two common eukaryotic classes and their species are given in the plots

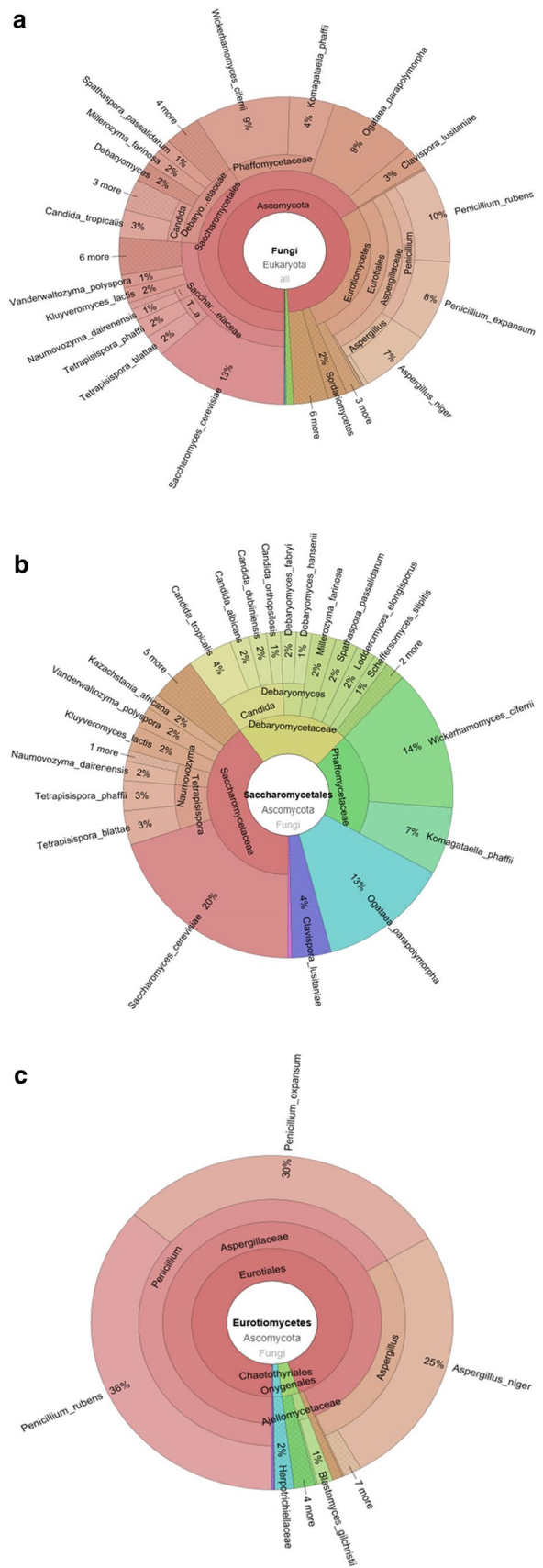
95% site coverage were eliminated by partial deletion option (Felsenstein 1985). *Pichia norvegensis* was used as an outgroup for analysis of YLP7, while *Pichia sp.* SG6L04 strain was used as an outgroup for phylogenetic analysis of YLP9. The phylogenetic tree revealed that the closest relative (with 100% identity) of YLP7 isolate was *Candida boidinii* strain CBS:3092 (GenBank Accession number: KY101986.1), which is from Saccharomycetales order, *Pichiaceae* family, *Ogataea* genus, Ogataea/Candida clade, whereas YLP9 isolate was closely related (with 99.70% similarity) to *Candida diddensiae* CBS:2214 strain (GenBank Accession Number: MK394116.1) from Saccharomycetales order, *Debaromyetaceae* family, *Yamadazyma* genus, Yamadazyma/Candida clade (Fig. 6).

**Olive oil hydrolysis activities of halophilic yeasts**

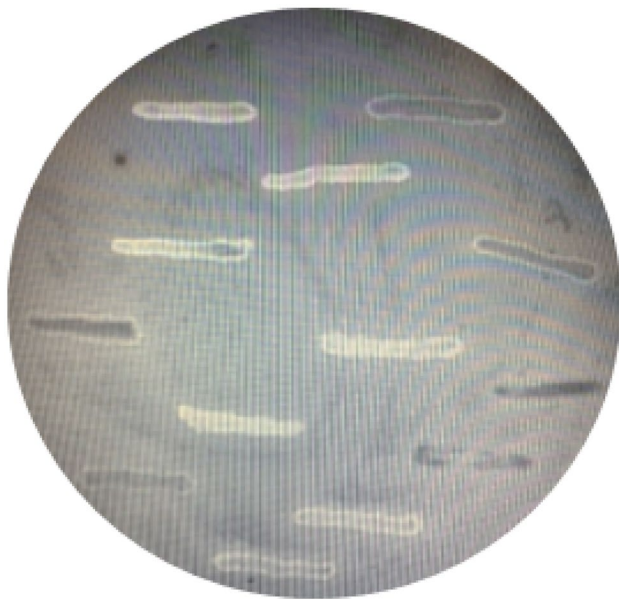
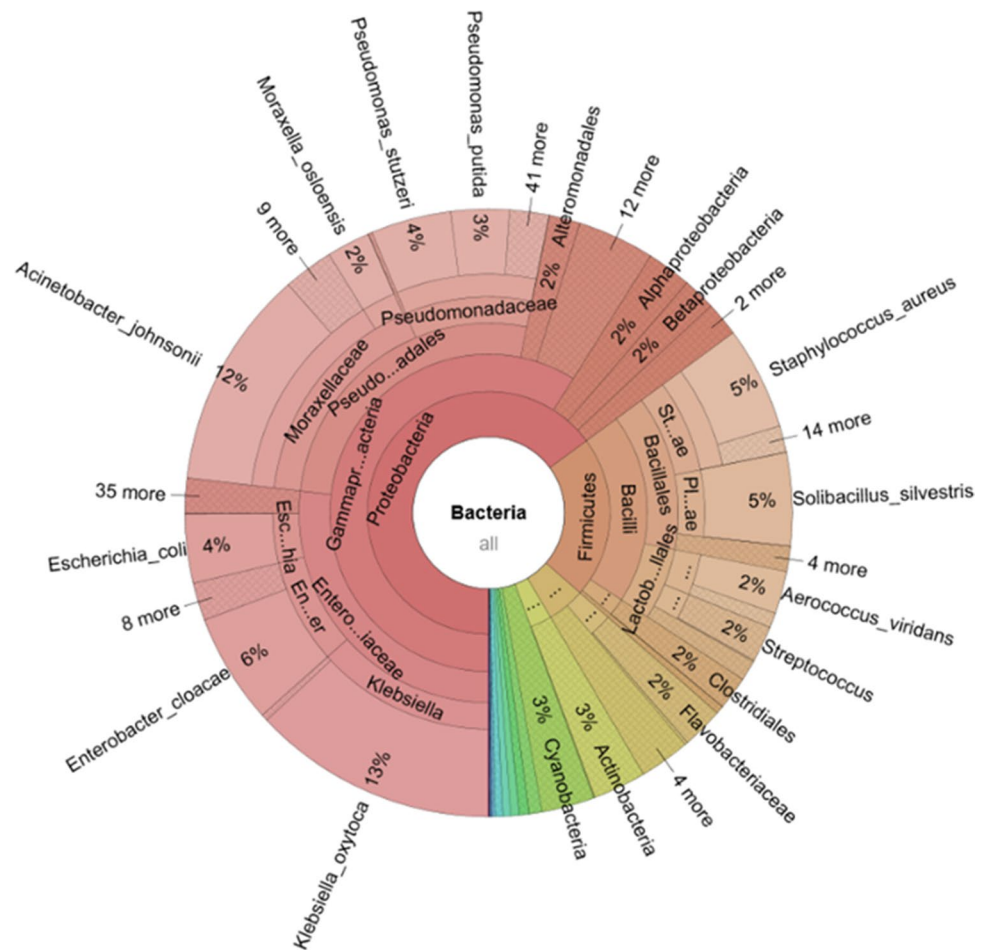
To determine the crude lipase activity of the previously selected isolates YLP7 and YLP9, the cells were incubated in the growth medium, including olive oil as lipase inducer. On the fourth day of cultivation, the cells were precipitated by centrifugation and the pellet fraction was used for analysis. The cell-bound lipase activity was determined in the reaction medium using buffers at different pHs (pH 3.0–7.0) and olive oil as substrate. The results showed that maximum hydrolytic activity was recorded at pH 3.0 for YLP7 (*C. boidinii*) and YLP9 (*C. diddensiae*), 222.2  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  cell dry weight and 162.7  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  cell dry weight, respectively. The activity of both yeasts at pH 4.0 was 200.04  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  CDW and 100.29  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  CDW, respectively and no activity was measured at increasing pH (Table 1).

**Discussion**

Isolation of novel industrially-important enzymes, such as hydrolases that could be active and stable in non-aqueous organic reaction environments has become an area of interest in recent years. Enzymes of halophilic microorganisms are ideal for low-water or anhydrous solvent applications since the salt in the environment has an effect on lowering the water (Wang et al. 2020; Noby et al. 2020; Huo et al. 2017; Xin and Hui-Ying 2013). The salt-saturated olive brine (8–12% NaCl concentration, pH 3–4) is a suitable source for the investigation of halophilic microorganisms producing halophilic enzymes that are stable in organic solvents.



**Fig. 4** Species diversity of Bacteria in the olive brine sample

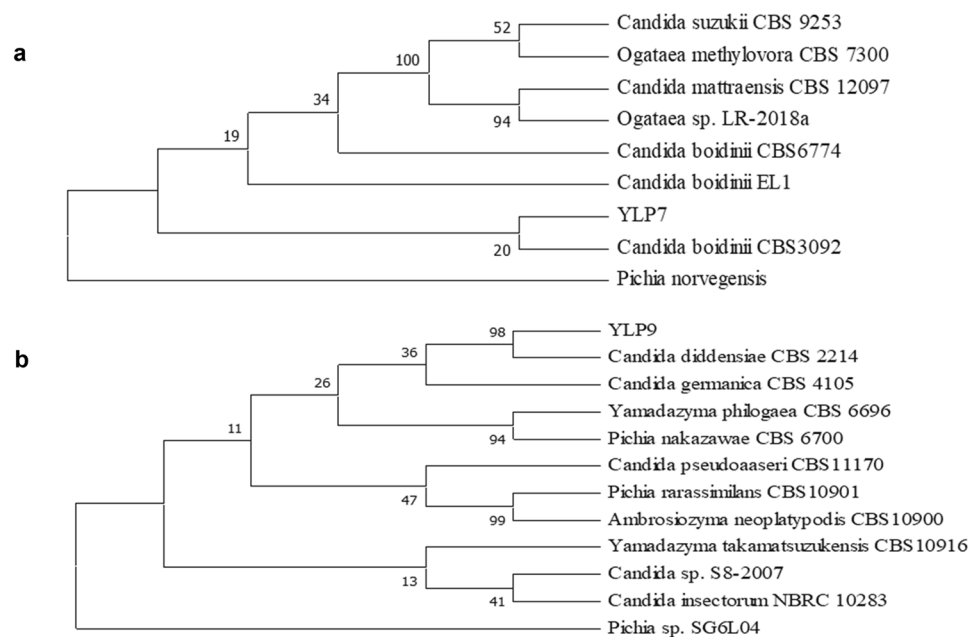


**Fig. 5** Screening of lipolytic activity on Rhodamine B agar plate with substrate hydrolysis leading to formation of an orange fluorescent halo upon exposure to UV irradiation

Table olives are an indispensable fermented food for Mediterranean people and fermentation of the olive fruit constitutes a large economy (Perpetuini et al. 2020). The microbial composition during fermentation was previously characterized and it was reported that the microbial content of olive brine was mainly composed of bacteria, yeasts and occasionally molds. The dominating microflora vary depending on where and how fermentation is performed (Heperkan 2013). Therefore, each investigated brine sample will enable the discovery of different microorganisms that can survive in a low pH and high salinity environment. In contrast to previous studies, our study represents detailed taxonomic profiling of fungal and bacterial diversity of olive brine through taxonomic marker-independent sequencing. Next-generation sequencing of all metagenomes extracted from the olive brine microbial community allowed for determination of which microorganisms are involved in these complex and variable microbiota and enabled more reliable quantification of microorganisms through assessment of all genomic information, rather than of common markers such as 16S rRNA only. Thus, the olive brine microflora from the southern region of Turkey, which naturally provides a suitable environment for the survival of halophilic and



**Fig. 6** The bootstrap consensus trees of two representative yeasts isolated in this study, based on 5.8S rRNA including sequences of some accessions from GenBank resources. The evolutionary analyses were conducted in MEGA X by bootstrap test (500 replicates). The replicate trees' percentages, where the associated taxa clustered together in the bootstrap test, are shown on the branches: **a** Tree containing YLP7 isolate and 8 GenBank accessions, *Pichia norvegensis* was used as an outgroup. **b** Tree containing YLP9 isolate and 11 GenBank accessions, *Pichia sp.*SG6L04 strain was used as an outgroup



**Table 1** The cell-bound hydrolytic activities of YLP7 and YLP9 yeasts at different pHs using olive oil as substrate

	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0
Cell-bound hydrolytic activity ( $\mu\text{mol min}^{-1} \text{mg}^{-1} \text{CDW}$ )					
YLP7 ( <i>C. boidinii</i> )	222.2 $\pm$ 19.4	200.04 $\pm$ 33.3	—	—	—
YLP9 ( <i>C. diddensiae</i> )	162.7 $\pm$ 0	100.29 $\pm$ 24.88	—	—	—

acidophilic microorganisms, may serve as a gene source for studies searching for novel enzymes. Taxonomic profiling of the olive brine metagenome was carried out by next-generation sequencing. Fungal OTUs constituted the highest percentage of the reads followed by protozoa and bacteria. Metagenomic profiling revealed that Ascomycota phylum (99% of fungi) with the two common classes Saccharomycetales and Eurotiomycetes was dominantly represented in the olive brine sample and Basidiomycota, Microsporidia and Chytridiomycetes phyla were also detected in a very-low frequency. The genera *Saccharomyces*, *Tetrapisispora*, *Naumovozyma*, *Candida*, *Debaryomyces*, *Millerozyma*, *Spathaspora*, *Wickerhamomyces*, and *Komagataella* were the most abundant fungi detected. Similarly, studies on the fungal content of different olive cultivars from Portugal, Italy, Spain, Greece and Turkey reported *Saccharomyces* (*S. cerevisiae*, *S. rosinii*), *Candida* (*C. apicola*, *C. boidinii*, *C. diddensiae*, *C. oleophila*, *C. olivae*, *C. parapsilosis*, *C. quercitrusa*, *C. sorbosa*, *C. tropicalis*, *C. aaseri*, *C. atlantica*), *Citeromyces matritensis*, *Debaromyces* (*D. etchelsii*, *D. hanseii*), *Issatchenkia occidentalis*, *Pichia* (*P. caribbica*, *P.*

*mississippiensis*, *P. galeiformis*, *P. guilermundii*, *P. kluyveri*, *P. membranifaciens*, *Pichia kudriavzevii*), *Rhodotorula mucilaginosa*, *Zygorulasporea mrakii*, *Wickerhamomyces anomalus*, *Aurebasidium pullulans*, *Geotrichum candidum*, *Lachancea thermotolerans*, *Torulasporea delbrueckii*, *Zygosaccharomyces* (*Z. florentinus*, *Z. fermentati*) and *Penicillium* (*P. citrinum*, *P. roqueforti*, *P. brevicompactum*) yeasts (Heperkan, 2013; Leventdurur et al. 2016).

Although studies for determination of the microbial content of the naturally-fermented black olives in Turkey are available (Leventdurur et al. 2016; Müjdecı et al. 2018; Kumral et al. 2013; Ozsoy et al. 2017), species diversity of natural, cracked green olive brine has not been predicted with next-generation sequencing method before. In three different towns of the southern region of Turkey, the microbiota of the naturally fermented black olives of *Olea europaea* cv. Gemlik were identified by PCR-RFLP analysis of 5.8S ITS rRNA region and also sequencing of the D1/D2 domains of 26S rRNA gene. *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Candida boidinii*, *Candida diddensiae*, *Pichia galeiformis*, *Pichia membranifaciens* and *Kluyveromyces lactis* were found as commonly isolated species (Leventdurur et al. 2016). In the Tarsus and Bahçe districts, the most frequently identified species was *C. boidinii*, whereas *Saccharomyces sp* was the most representative of the Serinyol district, as determined in our study. It has been concluded that species diversity changes depending on the cultivation regions of the olives and also on the type of olive processing technique (Leventdurur et al. 2016).

Yeasts in the olive fermentation medium have some biotechnological properties. They can produce ethanol, glycerol, higher alcohols, esters, flavor-generating volatile



compounds and vitamin B-complex during the fermentation process. Furthermore, yeasts are able to uptake and degrade oleuropein, which causes the bitter taste of the fermented olive product by beta glucosidase activity and also increases the free fatty acid content in olives by esterase and lipase activity (Heperkan 2013; Bevilacqua et al. 2012). In addition, the antibacterial activity of some yeasts on some food-borne pathogens has been observed (Silva et al. 2011). For example, *Debaromyces hansenii* and *S. cerevisiae* are known as halotolerant killer yeasts since they produce toxic proteins or glycoproteins (killer-toxins) in the presence of NaCl (Arroyo-López et al. 2016). Another yeast species of olive fermentation medium having biotechnological potential is *Candida tropicalis*, which can produce both ethanol fuel and xylitol by hydrolyzing olive prunings with low-cost agricultural residue. Additionally, this organism can metabolize phenolic compounds by preventing the inhibition of the fermentation process by any lignin degradation products (Martín et al. 2010).

The presence of the molds *Penicillium* and *Aspergillus* in the fermentation medium is not desirable due to the possibility of mycotoxin production, and also softening of the fruit by cellulase and xylanase activity, although the exact function of those two genera in the olive brine environment has not been elucidated yet. On the other hand, their mold enzymes such as beta-glucosidase, cellulase, xylanase, pectinase, lignin modifying, lipolytic and proteolytic enzymes can be of interest for several industrial applications (Bavaro et al. 2017; Arroyo-López et al. 2016). Based on the report of AMFEP (Association of Manufacturers and Formulators of Enzyme Products) in 2015, commercialized enzymes were categorized by their taxonomic origin and the majority of the commercial enzymes originated from fungi (Ascomycota, Basidiomycota and Mucorales phyla) and almost 25% of all enzymes came from *Aspergillus* genus. Fungal enzymes, encompassing glycoside hydrolases, proteases, lipases and phosphatases are used for large-scale applications in the textile, paper and pulp industries, biomass treatment and also manufacture of food products. Fungal microorganisms are also the main producers of primary metabolites (e.g. organic acids) and biologically active secondary metabolites, such as antibiotics and immune-suppressors (Marmeisse et al. 2017). Considering these properties of fungal enzymes, the occurrence of *Penicillium* and *Aspergillus* fungi and the activity of their industrially important enzymes in brine should be meticulously evaluated for the stability of these enzymes in the halophilic environment of olive brine.

Analysis of the metagenomic sequences revealed that a total of 4% reads belong to the bacteria and among them two bacterial phyla Proteobacteria and Firmicutes have the greatest number of reads, which are known to be common inhabitants of olive brine. This result also supports a recent study carried out by BenítezCabello et al. (2020). According

to metataxonomic analysis results obtained from 72 table olive samples which were collected around the World, it was revealed that the bacteria domains consisted of Proteobacteria 47.27%, Firmicutes 18.33%, Bacteroidetes 13.03%, and Actinobacteria 10.76% (Rodríguez-Gómez et al. 2017; BenítezCabello et al. 2020). In this study, the dominant class among the bacteria was Gammaproteobacteria followed by Bacilli, Enterobacteriaceae and Pseudomonadales. The phylum with the second major number of sequences was Firmicutes, with the order Lactobacillales. The genera of Lactic Acid Bacteria which were sequenced in our olive brine sample and which are among the identified species *Lactobacillus curvatus* and *Lactobacillus kefiranoferiens* were present in high numbers, which have not been reported as participating in the olive fermentation process. Previous studies on the olive fermentation environment have shown that bacteria of the Enterobacteriaceae family, different species of the genera *Enterococcus*, *Clostridium*, *Pseudomonas*, *Staphylococcus*, *Leuconostoc* (*Ln. citreum*, *Ln. mesenterodites*) and *Lactobacillus* (*L. brevis*, *L. coryniformis*, *L. paracesei*, *L. paracollinoides*, *L. paraplatarum*, *L. plantarum*) (Heperkan 2013) were identified in the fermentation medium. *L. pentosus*, *L. casei*, *L. curvatus*, *L. fermentum*, *L. cellobiosus*, *L. coryniformis*, *L. rhamnosus*, *L. vaccinoferens*, *L. lactis*, *Hafnia alvei* and *Propionibacterium acidipropionici* were also identified (De Angelis et al. 2015). However, yeasts and lactic acid bacteria (LAB) exist together in a brine medium. Yeasts synthesize the enzyme cofactors and thiamine, nicotinic acid, pyridoxine and pantothenic acid which are essential for lactic acid bacteria growth, whereas LAB allow lowering of the pH of the medium by lactic acid secretion and promote yeast growth (Arroyo-López et al. 2008). In this study, yeasts dominate the brine medium as also observed in another natural brine environment of *Olea europaea* cv. Gemlik (Kumral et al. 2013), whereas 0.03% of total reads belong to *Lactobacillus* genus. It is suggested that this is related not only to the existence of the phenolic compounds, hydroxytyrosol and oleuropein, but also to the temperature and salt concentration of the olive brine (Arroyo-López et al. 2016; Kumral et al. 2013).

Considering the potential of olive brine as a gene source for studies searching for novel enzymes, it is possible to find protein entries that are reported in the Protein Data Bank (PDB), Reference Sequence collection (RefSeq) and UniProtKB/Swiss-Prot databases. Currently, 221 lipase entries from 26 fungi, where 127 of the entries are of 16 *Ascomycetes* species, and 478 entries from 81 bacteria, where 154 of the entries are of 32 *Proteobacteria* species are reported in the Protein Data Bank (<https://www.ncbi.nlm.nih.gov/protein>; 15.07.2020). Furthermore, thousands of entries from industrial enzymes, such as cellulases, esterases, proteases, xylanases, catalases and pectinases from those taxonomic groups are reported in the databases. The reported sequences

could be used in heterologous expression of the relevant proteins in several hosts and may also provide for investigation of the biotechnological usage potential of those enzymes by characterizing their activity in different conditions.

The biotechnological potential of microbial populations grown in the halophilic environment of olive brine has also been investigated. Olive brine is an acidic and halophilic environment, allowing only organisms that can survive under the conditions of high salinity and acidity. Halophiles have been known as potential sources of industrially important enzymes due to their exceptional stability. The present study has screened halophilic yeasts that can survive on solid YPD media supplemented with increased concentrations of NaCl (1, 1.5, and 3 M) and it has identified the hydrolytic enzymes occurring in the saline habitats of table olive brine. Former studies show that *Candida sp.* are tolerant to salt concentrations of 15–16%, however, in this study, the isolation has been carried out from concentrations of 17–18% which is even higher than previous reports (Llorente et al. 1997; Butinar et al. 2005). This provides the opportunity to investigate extreme halophilic industrial enzymes such as lipases.

Yeast colonies that survived in the media including 3 M NaCl were chosen for further lipase activity screening assay on agar plates containing Rhodamine B. The halophilic yeasts with highest lipolytic activity were identified by amplification of the ITS region of fungal rDNA including the 5.8S gene which is usually amplified by the universal primer pair ITS 1 and ITS 4 designed by White et al. (1990). Amplified regions were sequenced by Sanger sequencing. BLASTn was used to perform species identification employing the generated sequences as query against standard databases using default settings. As a result of the BLAST analysis of the sequences, it was determined that the selected microorganisms belong to two different species. Many organisms have similarity to YLP7 and YLP9 isolates and to infer the phylogenetic relationship, the bootstrap consensus tree was constructed using the Maximum Likelihood method and Tamura 3-parameter model in MegaX (Tamura 1992; Kumar et al. 2018). The gene sequences from GenBank were used to construct trees based on their similarity to YLP7 and YLP9 sequences. Evolutionary analyses revealed that the halophilic yeast isolate YLP7 was closely related to *Candida boidinii* CBS:3092 strain, while YLP9 isolate showed close relationship with *Candida diddensiae* CBS:2214 strain. Both yeasts were previously found in olive habitats. Leventdurur et al. (2016) reported that *Candida boidinii* was the predominant yeast during fermentation of some table olives from the southern region of Turkey in the presence of 8% NaCl. The presence of *Candida diddensiae* in olive oil was also reported (Zullo and Ciafardini 2020).

Since the activity of lipase, esterase, and beta glucosidases are among the industrially important enzymes that can

be found in olive-related environments, the lipase activity screening of the yeasts isolated from the olive brine was performed. Lipases (triacylglycerol acyl hydrolase, EC 3.1.1.3) catalyze the hydrolysis of the triglyceride ester bonds into diglycerides, monoglycerides, fatty acids and glycerol by acting as an interface between aqueous and non-aqueous phases (Wang et al. 2016a, b; Ciafardini and Zullo 2015; Glogauer et al. 2011). Although lipases are found in animals, plants, and microorganisms, microbial lipases constitute the most important group with their broad spectrum of usage in the fine chemistry, detergent, food, and textile industries (Druteika et al. 2020; Rani and Jagtap 2019; Yan et al. 2018; Cai et al. 2017). Here, the cell-bound lipase activity of isolated yeasts was analyzed since the cell-bound type of lipase has been reported from several *Candida* species before (Ciafardini and Zullo 2015). Besides, it is known that cell-bound lipases have an advantage as they show good operational stability and reduce the process costs by naturally immobilizing onto the cell wall (Fraga et al. 2018).

Previously selected isolates YLP7 and YLP9 were incubated in the growth medium containing olive oil as lipase inducer and the precipitate of cells was analyzed at different pHs (pH 3.0–7.0) using olive oil as substrate. The results showed that the highest hydrolytic activity was measured at pH 3.0 for both YLP7 (*C. boidinii*) and YLP9 (*C. diddensiae*) yeasts proving that both yeasts have acidic cell-bound lipases. Although both yeasts have activity at pH 4.0, hydrolysis of the olive oil was not detected at elevated pH levels. Previous studies revealed that *Candida sp.* are abundantly found in olive oil containing habitats, which are also known to have intracellular, cell-bound and extracellular lipase activity (Zullo and Ciafardini 2020; Arroyo-López et al. 2008). *Candida diddensiae* is a non-pathogenic dimorphic yeast and it has been found in extra virgin olive oil without deteriorating sensory attributes after four months of storage (Zullo and Ciafardini 2020). Its usage as a starter yeast has also been reported in 12% NaCl containing brines (Ciafardini and Zullo 2019). Although different cultivation and activity assays have been performed compared to this study, the cell-bound olive oil hydrolysis of different oil born *C. diddensiae* strains was between 3465 and 10,198  $\mu\text{g}$  oleic acid  $\text{g}^{-1}$  biomass  $\text{h}^{-1}$  (Ciafardini and Zullo 2015) and this shows that the cell-bound lipases for YLP7 and YLP9 isolates are consistent with the literature. Another study reported the presence of *Candida boidinii* in oily environments and its isolated extracellular lipase from spent olive Chemlal showed the maximal activity at pH 7.0 (Bataiche et al. 2014). Acidic lipases have potential in the food industry to hydrolyse and/or modify triacylglycerols to improve nutritional characteristics (de Almeida et al. 2013) and can also be used in flavour industries by formation of aroma esters at acidic conditions (Knob et al. 2020). It is

also possible to use acidic lipases as feed enzymes since the digestive environment of the stomach and intestine is acidic (Knob et al. 2020; Yan et al. 2018). The potential of the acidic lipases of YLP7 and YLP9 yeasts should be evaluated in terms of feasibility in these sectors. The acidic lipases analyzed in this study were determined to be true lipases, thanks to their ability to hydrolyze olive oil, which is a long-chain fatty acid containing triacylglycerol, as reported in another study isolating a true lipase from a fat-contaminated soil by metagenomic approach (Glogauer et al. 2011). Since the true lipases have broad substrate specificity, organic solvent stability and also regio- and stereo-selectivity as biocatalysts, the detected novel acidic lipases are candidates to be used in several biotechnological applications.

The catalytic properties, optimum pH, optimum temperature, thermal stability, and also stability in the presence of different organic solvents will be evaluated in further studies. This will illuminate the potential of the biotechnological and industrial usage of these novel acidic lipases of isolated halophilic yeasts.

## Conclusions

Olive fermentation brine is a valuable and promising biotechnological environment when searching for acidophilic and halophilic enzymes. The microbial flora of the olive brine from the Toroslar region of Turkey has been determined by NGS for the first time in the literature. Genome sequence knowledge will provide bioinformatics data, and mine new genes encoding industrially important proteins in future studies. Furthermore, two yeast colonies showing high percentages of similarity to *Candida diddensiae* and *Candida boidinii* were isolated by screening the yeasts of the olive brine at increasing concentrations of NaCl (up to 3M) and both yeasts gave significant cell-bound hydrolytic activity in acidic conditions (pH 3.0). Following heterologous expression in different hosts and detailed biochemical characterization of these acidic lipases, their industrial usage potential will also be evaluated in future studies.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no conflict of interest.

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