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Molecular identification of yeasts from Turkish traditional cheeses: Extracellular enzyme activities and physiological properties important for dairy industry

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Abstract. *Gunay M, Genc TT. 2023. Molecular identification of yeasts from Turkish traditional cheeses: Extracellular enzyme activities and physiological properties important for dairy industry. Nusantara Bioscience 15: 1-11.* The determination of yeast microbiota in cheeses and the physiological properties of yeasts are very important for the dairy industry. In addition, the physiological features, proteolytic and lipolytic activities, and stress tolerance of yeasts have a significant role in the selection of starter yeast species for cheese ripening. This study aimed to determine industrially important yeasts isolated from cheese samples. Molecular techniques identified the isolated yeast strains. The yeast strains' extracellular enzyme activities, fermentation capacities, and thermotolerance and osmotolerance properties were also evaluated. A total of 81 yeast strains were isolated and characterized from three types of cheese samples. PCR-RFLP determined the isolated yeast strains and sequence analysis of ITS1-5.8S-ITS2 and 26S rDNA regions. A maximum parsimony tree was constructed by MEGA X software to evaluate the phylogenetic relationship of identified yeast strains. *Candida intermedia*, *Candida parapsilosis*, *Clavispora lusitaniae*, *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Pichia kudriavzevii*, and *Wickerhamomyces anomalus* yeast species were identified on cheese samples. The distribution of identified yeast species on cheese samples was determined as 48.1% for *W. anomalus*, 17.3% for *K. marxianus*, 14.8% for *C. parapsilosis*, 8.6% for *D. hansenii*, 4.9% for *Cl. lusitaniae*, 3.7% for *C. intermedia* and 2.5% for *P. kudriavzevii*. The *W. anomalus* yeast species was common in three cheese types. All strains of *W. anomalus* and *P. kudriavzevii* yeast species, three *C. parapsilosis*, and two *Cl. lusitaniae* yeast strains have important physiological properties for industrial applications. These yeast strains have the potential to be used in combination as starter cultures to improve cheese maturation in the future. This comprehensive study identifies yeast species by ITS1-5.8S-ITS2 and 26S rDNA regions and determines industrially important yeast species using multiple criteria (extracellular enzyme activity, stress tolerance, and fermentation capacity).

Keywords: Cheese-related yeast, fermentation, lipase, protease, stress tolerance

INTRODUCTION

Yeasts have been used in industrial and biotechnological applications. They also possess significant roles in various fields such as food, pharmacy, agriculture, and the fermentation process. *Saccharomyces cerevisiae* and other non-*Saccharomyces* yeast species are used in baking, wine making, dairy production, biomedical research, and drug discovery. They are also used as a biocontrol agent, as in *Metschnikowia pulcherrima* and *Aureobasidium pullulans* (Parafati et al. 2015; Settier-Ramírez et al. 2021). Conventional and non-conventional yeast strains are also frequently used in the production of bioethanol, heterologous proteins and industrial enzymes (Steensels and Verstrepen 2014; Settier-Ramírez et al. 2021; Vincent et al. 2021). Stress-resistant yeasts with high osmo-, thermo- and ethanol tolerance are preferred especially in industrial areas involving fermentation processes such as wine and bioethanol production (Balakumar and Arasaratnam 2012). Yeasts are used in the fermentation process for different purposes such as increasing the flavor of the product, controlling microbial spoilage, and adjusting the alcohol and nutrient levels (Steensels and Verstrepen 2014). The main producers of industrially important microbial enzymes are bacteria, filamentous fungi and a limited number of yeast species. *Candida boidinii*, *Candida pseudotropicalis*, *Candida rugosa*, *Cryptococcus laurentii*, *Geotrichum candidum*, *Kluyveromyces marxianus*, *Komagataella pastoris*, *Ogataea polymorpha*, *Pseudozyma antarctica*, *Rhodotorula* spp., *S. cerevisiae*, *Sporobolomyces salmonicolor*, *Trichosporon fermentum*, *Yarrowia lipolytica*, and *Zygosaccharomyces rouxii* yeast species are used for the production of industrial enzymes such as phenylalanine ammonia lyase (PAL), L-gutaminase, α-galactosidase, phytase, chymosin, lactase, inulinase, invertase, lipase and protease (Fonseca et al. 2008; Johnson and Echavarri-Erasun 2011; Johnson 2013a,b). Yeast proteases and lipases are important as they contribute to cheese flavor during the ripening process, even if they cause adverse taste, appearance and odor. However, the identification of new yeast strains with high protease and lipase activity is important for the dairy industry to increase product efficiency. It is known that the strains of *Clavispora*

lusitaniae and *Candida parapsilosis* yeast species have proteolytic activity while the strains of *K. marxianus* yeast species have both proteolytic and lipolytic activity (Binetti et al. 2013). The extracellular enzyme properties of yeast species may differ between strains of the same species. They can also be different depending on growth conditions and environmental factors (de Araújo et al. 2010; Molnárová et al. 2014; Delgado-Ospina et al. 2020).

The maturation and production of cheese is a very sophisticated process that involved different biochemical reactions. The cheese microbiota, which includes bacteria, molds and yeasts, contributes to the characteristic features of cheese and the ripening process, allowing different cheese varieties to be obtained (Montel et al. 2014; Banjara et al. 2015). Yeasts associated with cheese can have beneficial and non-beneficial effects on cheeses. Some yeasts may improve cheese quality by affecting its texture, ripening, and flavor, while others may cause spoilage, discoloration, and unpleasant odor or taste (Binetti et al. 2013; Hatoum et al. 2013). For example, *Debaryomyces hansenii* and *Candida krusei* yeast species promote the ripening of German Harzer and Quark cheeses, whereas *K. lactis* and *K. marxianus* cause spoilage of white-brined cheeses (Fröhlich-Wyder et al. 2019; Geronikou et al. 2020). Although the positive or negative contributions of yeasts vary at both species and strain levels, overgrowth of yeasts negatively affects cheese quality. Therefore, determining the microbial diversity of cheeses is important for the manufacturers in terms of increasing the quality and shelf life of cheese. In previous studies, different yeast strains have been isolated and identified from many commercial and local dairy products. The most commonly identified yeast species belong to *Candida*, *Cryptococcus*, *Debaryomyces*, *Geotrichum*, *Kluyveromyces*, *Trichosporon*, *Rhodotorula*, *Torulaspora*, *Saccharomyces*, and *Yarrowia* genera (Vasdinyei and Deak 2003). Yeast species isolated and identified from some local cheeses (white, tulum, Mihalic cheeses, etc.) belong to the genera of *Debaryomyces*, *Pichia*, *Geotrichum*, *Trichosporon*, *Kluyveromyces* and *Saccharomyces* (Hayaloğlu et al. 2002; Kavas et al. 2006; Çorbaci et al. 2012; Karasu-Yalcin et al. 2017). Generally, *D. hansenii*, *K. marxianus*, *K. lactis*, and *Y. lipolytica* are the predominant yeast species in the different cheese types (Togay et al. 2020; Çorbaci et al. 2012).

The diversity and density of yeasts vary according to temperature, ability to use lactose and other carbon sources, extracellular enzyme activities, stress tolerance, cheese-type, ripening process and also geographical locations (Merchan et al. 2021). Therefore, the main purpose of this study is to identify new yeast strains with potential for industrial applications. For this purpose, the isolated yeast strains from the traditional cheese samples (white, goat and cheddar) were identified by physiological (API-ID32C aux system) and molecular (PCR-RFLP and sequence analysis of both ITS1-5.8S-ITS2 and 26S rDNA regions) methods. Next, extracellular enzyme activities (for protease and lipase), stress tolerance and fermentation abilities of the identified yeast strains were determined to evaluate whether the yeast strains are industrially important. Seven yeast species were identified from a total of 81 yeast strains isolated from cheese samples. *Wickerhamomyces anomalus* and *Pichia kudriavzevii* were determined as potential yeast species for industrial applications. This is the first comparative study to identify yeast strains using PCR-RFLP and sequence analysis of both ITS1-5.8S-ITS2 and 26S rDNA regions at the same time. It is also a comprehensive study that determines yeast species of industrial importance by using more than one criterion (extracellular enzyme activity, stress tolerance and fermentation capacity).

MATERIALS AND METHODS

Sampling and yeast isolation

Three different cheese samples (white, cheddar and goat cheeses) were collected from 6 different dairy producers in Bolu, Turkey. Five grams from each cheese sample were homogenized in a 2% sodium citrate solution and spread onto yeast extract glucose chloramphenicol agar medium (40 g/L, YGC) including 0.1% sodium propionate. The colony-forming units (CFU) were determined after 3 days of incubation at 30°C. Yeast strains having different colony morphology (colour, shape, size, or texture) were selected randomly and streaked on the YP medium (10 g/L yeast extract, 20 g/L bacto-peptone, 20 g/L agar) supplemented with 2% Dextrose. Yeast isolates were maintained at 4°C for the biochemical tests and molecular identification, and stored at -80°C in 20% glycerol for further studies.

Physiological characterization of isolated yeasts

The biochemical profile of isolated yeast strains was performed by the API-ID32C aux system (Bio-Mérieux, France) following the manufacturer's instructions. The strip includes 32 wells to perform 29 assimilation tests for carbohydrates, organic acids, and amino acids. Exponentially grown yeast cells (OD_{600}=0.8-1.0) were resuspended in API C medium and 135 µL of the suspension was dispensed into each cupule of the strip. The results were recorded by direct reading after 24 hr or 48 hr of incubation at 30°C.

Molecular identification of isolated yeasts using rDNA sequence

Genomic DNA isolation was conducted by using a previously improved procedure (Lõoke et al. 2011). The one yeast colony was suspended in 100 µL 200 mM LiOAc and 1% SDS solution. The cell suspension was incubated at 70°C for 5 min, and then 300 µL of absolute ethanol was added. The cell suspension was centrifuged for 3 min at 15000 rpm and the supernatant was discarded. The pellet was washed with 1 mL 70% ethanol and centrifuged for 3 min at 15000 rpm again. The pellet was dissolved in 100 µLTE buffer (pH 8) and stored at -20ºC.

PCR-RFLP analysis

ITS1-5.8S-ITS2 rDNA region was amplified by using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (White et al.

1990) for PCR-RFLP (Restriction Fragment Length Polymorphism) analysis. D1/D2 rDNA region was amplified by using NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') primers for PCR-RFLP analysis (Kurtzman and Robnett 1998). PCR amplification was studied with BIO-RAD thermal Cycler in 25 µL final volume, including 10X reaction buffer, 3mM $MgCl_2$, 10mM of dNTP, 10 pmol/µL of related primers, 1.25U Taq polymerase, and 50-100 ng DNA as a template. PCR conditions were determined as initial denaturation at 95°C for 5 min; 30 cycles of denaturing at 94°C for 3 min; annealing at 60°C (for ITS1-ITS4 primers) and 52°C (for NL1-NL4 primers) for 1 min, and extension at 72°C for 1 min; and a final extension step of 10 min at 72°C. PCR amplicons of ITS1-5.8S-ITS2 and D1/D2 rDNA region were digested with *Hae*III, *Hinf*I, *Hha*I (*Cfo*I), *Msp*I, and *Alu*I restriction endonucleases according to the supplier's instructions. PCR products and the restriction fragments were electrophoresed in 1.5% and 3% agarose gels, respectively, and photographed. The length of PCR amplicons and restriction fragments were calculated by using Gel-Pro Analyzer v4.0 software. The yeast strains were grouped according to restriction patterns.

Sequencing and phylogenetic analysis

Yeast strains having different restriction profiles were selected randomly and PCR products of ITS1-5.8S-ITS2 and D1/D2 rDNA were sequenced with the Applied Biotechnologies 3500xl Genetic Analyzer. The obtained sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) web server (Altschul et al. 1990). ITS1-5.8S-ITS2 and D1/D2 rDNA region sequences of the selected yeast strains were submitted to the GenBank database to get the accession number. Phylogenetic analysis of sequenced yeast strains was determined with MEGA-X (Molecular Evolutionary Genetics Analysis) software (Kumar et al. 2018). ITS1-5.8S-ITS2 and D1/D2 rDNA sequences of yeast strains were aligned with ClustalW (v1.6) parameters. The maximum parsimony tree of these regions was constructed by utilizing Subtree-Pruning-Regrafting (SPR) parameters and the bootstrap method. 1000 bootstrap replicates were used to determine branch support and bootstrap values below 50% were not shown.

Proteolytic activity

Extracellular protease activity of the isolated yeast strains was determined with the skimmed milk agar (SMA) plate test (Abdelmoteleb et al. 2017). Skimmed milk powder stock solution (10%) and agar solution were autoclaved separately and mixed where the final concentration of skimmed milk was 1%. Yeast strains were grown in YPD broth up to exponential phase and inoculated as 5 µL droplets into SMA plates. Plates were incubated for up to 10 days at 30°C. The clear zone around the yeast colonies was considered protease activity. All yeast strains were grouped according to the zone diameter as having high, middle, and low protease activity.

Lipolytic activity

Lipolytic activities of yeast strains were detected with Tween 20/80 precipitation test (Kumar et al. 2012). Tween 20 and Tween 80 contain esters of low fatty acid chains and oleic acid, respectively. Therefore, Tween 20 is used for the detection of esterase activity and Tween 80 is used for lipase activity. Tween plates (10 g/L peptone, 5 g/L $NaCl_2$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 20 g/L agar) supplemented with 10 mL Tween 20 or Tween 80 were prepared and poured into plates. Yeast cells growing exponentially in YPD broth were inoculated onto Tween plates as 5 µl droplets and incubated at 30°C for 3-7 days. The appearance of white visible precipitate around the boundary of the colonies because of the deposition of calcium crystal salts was an indicator of lipolytic activity. All yeast strains were grouped according to the zone diameter as having high, middle and low lipase activity.

Temperature and glucose tolerance test

The temperature tolerance tests of yeast strains were performed on a YPD medium. The plates were incubated at 25, 30, 37, and 45°C for 2-3 days. The glucose tolerance tests of yeast strains were performed on a YP medium supplemented with 50% dextrose. The plates were incubated at 30°C for 2-3 days. The growth of yeast strains was signed positive or negative according to the growth situations.

Fermentation test

The carbohydrate utilization test was performed using a YP medium supplemented with 1.6% bromothymol blue as pH indicator and fermentable carbon sources (2% each of dextrose, galactose, sucrose, lactose and maltose). The Durham tubes were also placed into the media to detect gas production (Karki et al. 2017). The yeast strains were inoculated to cultures and incubated at 30°C for up to 15 days. The color change in the growth medium from blue-green to yellow indicated acid production, and the presence of bubbles in the inverted Durham tube indicated gas production. The yeast strains showing both acid and gas production were recorded as fermentation positive. All experiments were carried out under anaerobic conditions.

Statistical analysis

All the biochemical tests were assayed in at least triplicate. The results were analyzed using the SPSS software (version 10.0) to obtain means and standard deviations (SD). P values of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Yeast identification and diversity

The white, cheddar and goat cheese samples were collected from six different dairy farms in Bolu, Turkey. A total of 81 yeast strains were isolated from cheese samples according to different colony morphology. The fungal load of cheese samples was determined as 5.34 log cfu/g for white cheese, 4.44 log cfu/g for cheddar cheese and 4.74

log cfu/g for goat cheese. Yeast counts in cheeses can vary between 2 and 9 log cfu/g depending on the ripening period and cheese types (Karasu-Yalcin et al. 2017; Bintsis 2021). In the present study, the yeast loads were found to be similar to the yeast counts in previous studies (Bintsis 2021). All isolated yeast strains were characterized using the API ID32C system and the results were analyzed with API-WEB v1.2.1 fungal database. The identified yeast strains were belonging to *Candida kefyr, C. parapsilosis, Candida pelliculosa, Candida famata, Candida intermedia, C. krusei* and *Candida lusitaniae* yeast species. It was observed that the assimilation profiles of the identified yeast strains were different. The yeast strains belonging to *C. kefyr* and *C. parapsilosis* showed seven different assimilation profiles (P1-P7). Similarly, six and three assimilation profiles were determined for *C. pelliculosa* and *C. famata* yeast strains, respectively. The other yeast strains belonging to *C. intermedia, C. krusei* and *C. lusitaniae* yeast species showed 2 assimilation profiles (Table 1).

Traditional identification methods are complicated processes and time-consuming and could give incorrect results (Pincus et al. 2007). Therefore, different commercial kit systems based on biochemical and assimilation tests, and DNA-based identification techniques (PCR-RFLP analysis, ITS1-5.8S-ITS2 and 26S rDNA sequences) have been used to identify yeast strains (Garnier et al. 2017; Karasu-Yalçın 2017; Benito et al. 2018; Moubasher et al. 2018; Haastrup et al. 2018; Genç and Günay 2020; Togay et al. 2020). Therefore, the isolated yeast strains were identified by PCR-RFLP and rDNA region sequence analysis. The genomic DNA extraction of all isolated yeast strains was carried out, and ITS1-5.8S-ITS2 and 26S rDNA regions were amplified. ITS1-5.8S-ITS2 and 26S rDNA regions of yeast strains were digested with five restriction endonucleases, and yeast strains were grouped for the restriction profiles (Table 2 and Table 3). Yeast strains showed 7 and 10 restriction profiles according to the PCR-RFLP results of ITS1-5.8S-ITS2 and 26S regions, respectively. It was determined that yeast strains grouped according to assimilation results showed a similar distribution according to ITS1-5.8S-ITS2 restriction profiles. However, there were differences between the groups of assimilations and the restriction profiles of the 26S region.

Table 1. Assimilation profile of yeast strains and identified species

Yeast species	Strain codes	Number of yeast strains
Candida kefyr	P1: W-13, W-14, W-18, W-19, W-20, W-22, W-24 P2: W-4, W-5 P3: W-9 P4: W-11 P5: C-28 P6: C-29 P7: C-20	14
Candida parapsilosis	P1: W-15, W-21 P2: W-6, W-7, W-12 P3: W-17 P4: W-3, W-8, W-25 P5: W-10 P6: W-16 P7: W-23	12
Candida pelliculosa	P1: W-2, C-2, C-3, C-7, C-8, C-10, C-11, C-16 P2: G-1, G-13, G-27 P3: C-1, C-5, C-6 P4: G-2, G-16 P5: C-4, C-9, C-12, C-13, C-14, C-15, C-21 P6: G-5, G-6, G-7, G-8, G-9, G-10, G-11, G-12, G-14, G-15, G-19, G-21, G-23, G-24, G-25, G-26	39
Candida famata	P1: C-18, C-22, C-23 P2: C-24, C-25, C-26 P3: C-19	7
Candida intermedia	P1: C-17, C-27 P2: W-1	3
Candida crusei	P1: G-3 P2: G-4	2
Candida lusitaniae	P1: G-17, G-18 P2: G-20, G-22	4

Note: "P" indicates assimilation profile; "W" indicates white cheese; "C" indicates cheddar cheese; "G" indicates goat cheese

Table 2. PCR-RFLP results of ITS-5.8S rDNA region

Strain code	*Hae*III	*Hinf*I	*Hha*I	*Alu*I	*Msp*I
W-4, W-5, W-9, W-11, W-13, W-14, W-18, W-19, W-20, W-22, W-24, C-20, C-28, C-29	650-90	280-190-110-90-90	290-175-150-90	375-175-170	-
W-2, C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, C-10, C-11, C-12, C-13, C-14, C-15, C-16, C-21, G-1, G-2, G-5, G-6, G-7, G-8, G-9, G-10, G-11, G-12, G-13, G-14, G-15, G-16, G-19, G-21, G-23, G-24, G-25, G-26, G-27	-	315-310	575-60	500-105-50	-
W-3, W-6, W-7, W-8, W-10, W-12, W-15, W-16, W-17, W-21, W-23, W-25	450-125	300-300	320-240	540-50	500-90
C-18, C-19, C-22, C-23, C-24, C-25, C-26	425-140-85	315-310	290-290-60	-	-
G-17, G-18, G-20, G-22	-	200-200	200-190-90	-	275-110
W-1, C-17, C-27	-	200-200	190-180	-	300-100
G-3, G-4	390-95	260-210	200-190-90	390-95	250-215

Table 3. PCR-RFLP results of 26S rDNA region

Strain code	*Hae*III	*Hinf*I	*Hha*I	*Alu*I	*Msp*I
W-4, W-5, W-9, W-11, W-13, W-14, W-18, W-19, W-20, W-22, W-24, C-20, C-28, C-29	475-150	400-200	-	240-150-120-90	-
W-2, C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, C-10, C-11, C-12, C-13, C-14, C-15, C-16, C-21, G-1, G-2, G-6, G-7, G-8, G-9, G-10, G-11, G-12, G-13, G-14, G-15, G-16, G-19, G-21, G-23, G-24, G-25, G-26, G-27	475-150	250-180-175	-	250-190-110-80	-
G-5	290-200-125	390-175-80	-	250-190-110-80	-
W-3, W-6, W-7, W-8, W-10, W-12, W-15, W-16, W-17, W-21, W-23, W-25	475-150	400-200	-	250-190-110-80	490-150
C-18, C-19, C-22, C-23, C-24, C-25, C-26	475-150	400-200	-	225-200-100-90	-
G-17, G-18, G-20, G-22	290-110-100-85	300-115-100-80	290-125-120-75	225-200-100-90	400-175-60
W-1, C-17	390-95-90	380-225	340-150-100	250-190-110-80	290-180-140
C-27	475-150	400-200	340-150-100	225-200-100-90	290-180-140
G-3	290-200-125	300-300	250-280-150	430-190	420-200
G-4	225-220-90-90	400-120-75	250-280-150	430-190	420-200

Note: *All the yeast strains have the same PCR length for 26S rDNA region of about 600-650bp. Thus, data not shown in the table

Table 4. Identification of selected yeasts isolated from Turkish traditional cheeses

Yeast Strains	Identified yeast strains (Ref. Acc. Number)	GenBank Acc. No	Identified yeast strains (Ref. Acc. Number)	GenBank Acc. No
W-1	*C. intermedia* (DQ657830.1)	MT321268	*C. intermedia* (KX981200.1)	MT334438
W-3	*C. parapsilosis* (MK998693.1)	MT321173	*C. parapsilosis* (MK940816.1)	MT334455
W-16	*C. parapsilosis* (MH445556.1)	MT321276	*C. parapsilosis* (FJ432673.1)	MT334446
W-22	*K. marxianus* (KY103821.1)	MT321174	*K. marxianus* (KJ641888.1)	MT334456
W-24	*K. marxianus* (MH595342.1)	MT321278	*K. marxianus* (KJ641888.1)	MT334448
C-4	*W. anomalus* (KY105866.1)	MT321170	*W. anomalus* (MG773348.1)	MT334452
C-17	*C. intermedia* (DQ657830.1)	MT321171	*C. intermedia* (KX981200.1)	MT334453
C-21	*W. anomalus* (KY105868.1)	MT321272	*W. anomalus* (MG773348.1)	MT334442
C-22	*D. hansenii* (KP835570.1)	MT321273	*D. hansenii* (KY107525.1)	MT334443
C-25	*D. hansenii* (KY103209.1)	MT321172	*D. hansenii (*KY107525.1)	MT334454
C-27	*C. intermedia* (DQ657830.1)	MT321275	*C. intermedia* (KX981200.1)	MT334445
G-3	*P. kudriavzevii* (MH545928.1)	MT321265	*P. kudriavzevii* (MK881743.1)	MT334435
G-4	*P. kudriavzevii* (MH545928.1)	MT321167	*P. kudriavzevii* (MF377363.1)	MT334449
G-5	*W. anomalus* (KY105880.1)	MT321168	*W. anomalus* (KF612003.1)	MT334450
G-7	*W. anomalus* (KY105894.1)	MT321266	*W. anomalus* (MG773348.1)	MT334436
G-18	*Cl. lusitaniae* (KY102565.1)	MT321169	*Cl. lusitaniae* (MH892862.1)	MT334451

When assimilation groups were compared with the restriction groups of the 26S region, one of 39 yeast strains (G-5) defined as *C. pelliculosa* was separated from the other 38 yeast strains with *Hae*III and *Hinf*I restrictions. Similarly, C-27 yeast strain was separated from other two *C. intermedia* strains (W-1 and C-17) with the *Hae*III restriction. In addition, the strains of *C. krusei* yeast species, G-3 and G-4, showed different profiles with the *Hae*III and *Alu*I restrictions. It was observed that *C. kefyr*, *C. famata*, *C. parapsilosis* and *C. lusitaniae* yeast strains distributed in similar groups according to the assimilation test and restriction profile of the 26S region.

The different restriction profiles obtained from PCR-RFLP analysis can represent the different yeast species (Gibson et al. 2011). Although seven yeast species have been identified based on assimilation tests, ten different yeast species may have been isolated depending on the ten restriction profiles of the 26S region. Therefore, at least one yeast strain was randomly selected from each restriction profile of ITS-5.8S and 26S regions, and the amplification product of both ITS-5.8S and 26S regions was sequenced. The sequences of sixteen yeast strains (W-1, W-3, W-16, W-22, W-24, C-4, C-17, C-21, C-22, C-25, C-27, G-3, G-4, G-5, G-7, G-18) were analyzed by BLAST online tool from NCBI web server. The nucleotide sequences were submitted to the GenBank database and accession numbers were obtained for all sequences (Table 4). According to BLAST results, all yeast strains showed 97-99% similarity with reference strains for ITS-5.8S and 26S regions. G-3 and G-4 yeast strains were identified as *P. kudriavzevii*, G-5, G-7, C-4 and C-21 yeast strains identified as *W. anomalus*, G-18 yeast strain identified as *Cl. lusitaniae*, C-22 and C-25 yeast strains identified as *D. hansenii*, W-22 and W-24 yeast strains identified as *K. marxianus*, W-1, C-17 and C-27 yeast strains identified as *C. intermedia* and W-3 and W-16 yeast strains identified as *C. parapsilosis*. According to the APIWEB current database and previous studies, *C. krusei*, *C. pelliculosa*, *C. lusitaniae*, *C. famata*, and *C. kefyr* yeast species are synonymous with *P. kudriavzevii*, *W. anomalus*, *Cl.*

lusitaniae, *D. hansenii*, and *K. marxianus* yeast species, respectively (Kurtzman et al. 2011). Therefore, the yeast species identified by assimilation tests were identical to yeast species identified by sequence analysis. It has been previously reported that the percent confidence of API-ID32C results is less than that of molecular identification methods (Pincus et al. 2007). However, in recent studies, it has been reported that the reliability between the ID32C API System and 26S rDNA sequencing methods revealed a high correlation (Ceugniez et al. 2015). In this study, the results obtained with the API-ID32C identification kit system were found to be fully compatible with the results obtained by molecular identification methods. In addition, although the API-ID32C assimilation profile varies between strains of the same species, it was observed that the identification of yeasts was quite accurate. This may be because updates and revisions made in the API-ID32C kit and database allow for correct identification of yeasts.

The percent distribution of identified yeast species was determined as 48.1% for *W. anomalus*, 17.3% for *K. marxianus*, 14.8% for *C. parapsilosis*, 8.6% for *D. hansenii*, 4.9% for *Cl. lusitaniae*, 3.7% for *C. intermedia* and 2.5% for *P. kudriavzevii*. The diversity of yeast species on cheese types was given in Figure 1. The *W. anomalus* was abundant in goat cheese (21 of 27 strains) and cheddar cheese (17 of 29 strains). The *K. marxianus* and *C. parapsilosis* yeast species were distributed predominantly in white cheese samples. The other yeast species *Cl. lusitaniae* and *P. kudriavzevii* were distributed only in goat cheese samples, and *D. hansenii* was distributed only in cheddar cheese samples.

So far, *C. parapsilosis*, *C. albicans*, *C. diddensiae*, *C. haemulonii*, *C. membranifaciens*, *C. sake*, *C. tropicalis*, *C. versatilis*, *C. zeylanoides*, *Cl. lusitaniae*, *D. hansenii*, *Galactomyces geotrichum*, *K. lactis*, *K. marxianus*, *Meyerozyma guilliermondii* (formerly *P. guilliermondii*), *P. anomala*, *Rhodotorula mucilaginosa*, *S. cerevisiae*, *Torulaspora delbrueckii*, *Williopsis californica* and *Y. lipolytica* yeast species have been identified from different traditional Turkish cheeses (tulum, Kashkaval, Mihalic, örgü, white, sepet, and goat) (Yalçın and Uçar 2009; Çorbacı et al. 2012; Togay et al. 2020; Esen and Çetin 2021).

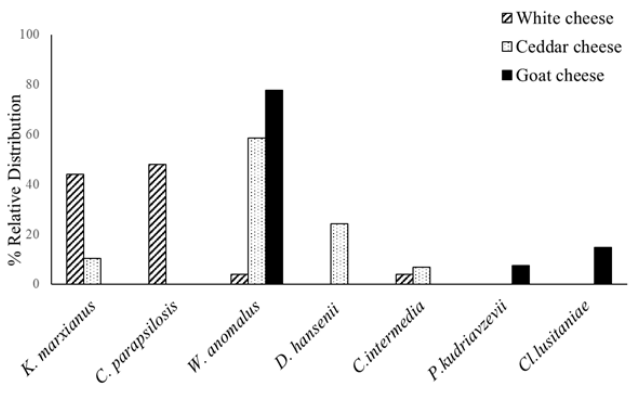

Figure 1. The relative distribution of identified yeast species

In addition to these species, many yeast species have been isolated and identified from local cheeses of different countries in the last decade. The *C. boidinii*, *C. butyric*, *C. mogii*, *C. sphaerica*, *Cr. albidus*, *K. blattae*, *K. thermotolerance*, *P. farinose*, *P. membranifaciens*, *R. glutinis* and *Z. rouxii* yeast species were recorded from Cyprus, Denmark, Macedonia, France and South Africa (Bintsis 2021). The *C. inconspicua*, *C. xylopsoci*, *G. candidus* and *P. kudriavzevii* yeast species were identified from Bryndza cheese of Slovakia (Pangallo et al. 2014). Similarly, *C. catenulate*, *C. etchellsii*, *C. glaebosa*, *G. candidum*, *Kazachstania unispora*, *Kodomaea ohmeri*, *Saturnispora mendoncae* and *T. ovoides* yeast species were identified from local cheeses of Brazil, England, France, Italy, Mexico and Spain (Gkatzionis et al. 2014; Tofalo et al. 2014; Padilla et al. 2014; Cardoso et al. 2015; Ceugniez et al. 2015; Chombo-Morales et al. 2016; Dugat-Bony et al. 2016). These studies showed that *D. hansenii*, *K. lactis*, *K. marxianus* and *Y. lipolytica* are the most common yeast species in many cheese types. In this study, *K. marxianus* and *W. anomalus* yeast species were found at high prevalence in cheese samples. Also, *P. kudriavzevii* yeast strain was reported for the first time in this study as a yeast species isolated from Turkish cheeses.

Phylogenetic analysis of identified yeast species was carried out by MEGA-X software. The obtained sequence of ITS-5.8S and 26S regions were aligned using ClustalW v1.6 tool, and the maximum parsimony tree was generated by utilizing MEGA-X (Kumar et al. 2018). *S. cerevisiae* was selected as an outgroup. Yeast species were divided into three main clades according to the maximum parsimony tree result of the ITS-5.8S region (Figure 2). The first clade including 6 yeast species was separated into 2 sub-clades. The *C. intermedia* and *Cl. lusitaniae* species are localized in the same sub-clade while the other sub-clade includes *P. kudriavzevii* yeast species. The second clade consists of eight yeast species and is divided into 3 sub-clades, the first sub-clade contains four *W. anomalus* yeast species, the second sub-clade includes *D. hansenii*, and the third sub-clade includes *C. parapsilosis* yeast species. The last clade includes only one yeast species, *K. marxianus*. According to the maximum parsimony tree of the 26S rDNA region, yeast strains were divided into 2 main clades (Figure 3). The first clade consists of 3 sub-clades including *C. intermedia*, *C. parapsilosis*, *Cl. lusitaniae*, *D. hansenii*, *P. kudriavzevii* and W. *anomalus* yeast species and the second clade contains only one yeast, *K. marxianus*.

Screening of protease and lipase activities

Extracellular proteases and lipases have been used in various industrial areas, including the pharmaceutical, detergent, leather, waste management, drug designs, cosmetic, biodiesel production, food and dairy industry (Escribano et al. 2017; Liu et al. 2020; Naveed et al. 2021). Several studies revealed that anti-inflammatory drugs, pesticides, anti-Alzheimer drugs, and analgesics could be produced by using yeast lipases. In addition, the importance of biodiesel production is increasing rapidly due to the worldwide depletion of fossil fuels (Adlercreutz

2013; Sharma and Kanvar 2014; Gupta et al. 2015). So, the isolation and identification of non-*Saccharomyces* yeast species with high protease and lipase activity are important for industrial applications. Therefore, protease and lipase activities of all isolated yeast strains were determined (Table 5). All strains of *P. kudriavzevii* and *W. anomalus* yeast species showed a high protease activity. In addition, all strains of *C. intermedia*, three yeast strains of *D. hansenii* (C-24, C-25, C-26) and three yeast strains of *K. marxianus* (W-4, W-9, W-11) yeast species displayed a protease activity in a moderate level. The strains of *C. parapsilosis* and *Cl. lusitaniae* yeast species did not have protease activity. Interestingly, lipase activity was not detected in all isolated yeast strains except for three strains of *C. parapsilosis* (W-7, W-17, W-21), which exhibited low lipase activity.

When the protease and lipase activities of 81 yeast strains were evaluated, it was determined that 61.7% of the yeast strains showed protease activity, while only 2.5% showed lipase activity. In previous studies, it was determined that *C. parapsilosis*, *Cl. lusitaniae*, *K. marxianus*, *M. pulcherrima* and *W. anomalus* yeast species have industrially important protease activity (Binetti et al. 2013; Escribano et al. 2017). And, *R. mucilaginosa*, *C. parapsilosis*, *K. marxianus* and *W. anomalus* yeast species have industrially important lipase activity (Binetti et al. 2013; Yalçın et al. 2014; Gupta et al. 2015).

Table 5. Extracellular enzyme profiles of cheese-related yeast strains

Yeast species	Yeast strains	Protease	Lipase
K. marxianus	W-4, W-9, W-11	++	-
	W-5, W-13, W-14, W-18, W-19, W-20, W-22, W-24, C-20, C-28, C-29	-	-
W. anomalus	W-2, C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, C-10, C-11, C-12, C-13, C-14, C-15, C-16, C-21, G-1, G-2, G-5, G-6, G-7, G-8, G-9, G-10, G-11, G-12, G-13, G-14, G-15, G-16, G-19, G-21, G-23, G-24, G-25, G-26, G-27	++++	-
C. parapsilosis	W-3, W-6, W-8, W-10, W-12, W-15, W-16, W-23, W-25	-	-
	W-7, W-17, W-21	-	++
D. hansenii	C-18, C-19, C-22, C-23	-	-
	C-24, C-25, C-26	++	-
Cl. lusitaniae	G-17, G-18, G-20, G-22	-	-
C. intermedia	W-1, C-17, C-27	++	-
P. kudriavzevii	G-3, G-4	++++	-

Note: Negative sign indicates the absence of a clear zone around the colony; Zone diameter (mm): ++++ (>15); +++ (6–15); ++ (3–6); + (1–3)

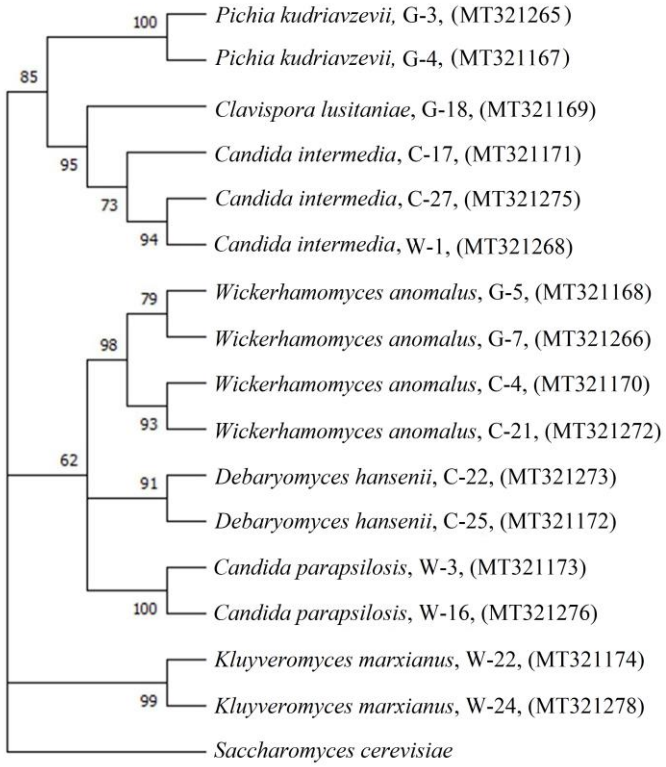

Figure 2. Phylogenetic placement of yeast species based on the sequence of ITS1-5.8S-ITS2 rDNA region. Reference sequences retrieved from the GenBank database are included. The tree was constructed with the maximum parsimony method and the Subtree-Pruning Regrafting algorithm. Numbers on branches represent the bootstrap values (>50%) from 1000 random replicates. The consistency index is (0.689320), the retention index is (0.777003), and the composite index is 0.545709 (0.535604) for all sites and parsimony-informative sites (in parentheses)

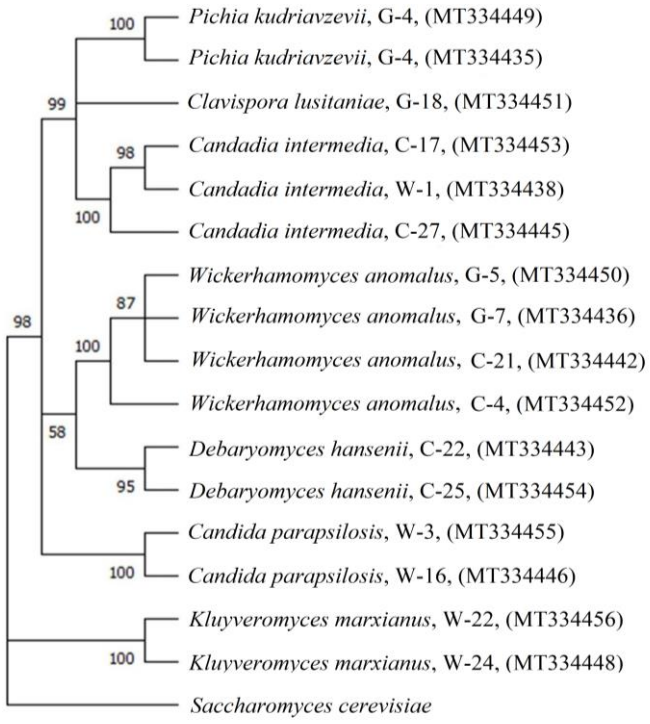

Figure 3. Phylogenetic placement of yeast species based on the sequence of D1/D2 rDNA region. Reference sequences retrieved from the GenBank database are included. The tree was constructed with the maximum parsimony method and using the Subtree-Pruning Regrafting algorithm. Numbers on branches represent the bootstrap values (>50%) from 1000 random replicates. The consistency index is (0,695906), the retention index is (0,828383), and the composite index is 0,610828 (0,576477) for all sites and parsimony-informative sites (in parentheses)

However, in this research, the strains of *C. parapsilosis*, *Cl. lusitaniae* and *K. marxianus* (except three strains) yeast species did not show proteolytic activity. Similarly, all isolated yeast strains of *C. parapsilosis* (except three strains), *K. marxianus* and *W. anomalus* yeast species did not show lipolytic activity. The extracellular enzyme activities of yeast species can show variations between the strains of the same species. For example, the production of proteases is affected by growth conditions (such as components of medium, inoculum size and dissolved oxygen) and environmental factors (such as pH, temperature and incubation time) (de Araújo et al. 2010; Molnárová et al. 2014). In this study, it was observed that the proteolytic and lipolytic activities were strain-specific as previously stated. In addition, all strains of *P. kudriavzevii* and *W. anomalus* yeast species have great potential as candidate yeasts for the dairy industry due to their high proteolytic activity.

Screening thermotolerant and osmotolerant properties

Since cheese is a fermentation product, the composition of cheese is indirectly regulated by microbial biota. The presence of thermotolerant and osmotolerant yeasts in this microbial biota is important. Thermotolerant and osmotolerant yeasts are capable of surviving and growing at high temperatures and osmotic environments (such as high sugar or salt concentrations), respectively. So, thermotolerance and osmotolerance properties of yeast strains were also investigated in this work. Thermotolerance properties of yeast strains were determined by incubation in a rich medium at 25, 30, 37 and 45°C (Table 6). All yeast strains showed a well growth at 25 and 30°C (data not shown). *D. hansenii* and *C. intermedia* yeast strains did not grow at both 37 and 45°C. The strains of *W. anomalus* yeast species grew at 37°C but not at 45°C. *K. marxianus*, *C. parapsilosis*, *Cl. lusitaniae* and *P. kudriavzevii* yeast strains showed a well growth at both 37 and 45°C. The osmotolerant yeast strains were determined by incubating yeast strains in the rich medium supplemented with 50% glucose at 30°C (Table 6). The results showed that all the yeast strains (except *K. marxianus*) showed growth in an osmotic environment. It has been previously reported that some strains of *C. famata, C. parapsilosis, D. hansenii, P. kudriavzevii* and *W. anomalus* possess osmophilic properties (Breuer and Harms 2006). Furthermore, *D. hansenii*, *K. marxianus* and *P. kudriavzevii* yeast species have both thermophilic and osmophilic properties (Breuer and Harms 2006; Yamamoto et al. 2015; Choi et al. 2017). In this research, *C. parapsilosis*, *Cl. lusitaniae* and *P. kudriavzevii* yeast strains were determined as both thermotolerant and osmotolerant yeast species. In addition, no variation was observed between the thermotolerant and osmotolerant profiles of yeast strains belonging to the same species.

Table 7. Fermentation capacity of yeast strains in different carbon sources

Yeast species	Yeast strains	Dex	Gal	Suc	Lac	Mal
K. marxianus	W-4, W-11, W-13, W-14, W-20, W-5, W-9, W-19, W-22, W-24, C-20, C-29	+	+	+	+	+
	W-18, C-28	+	+	+	+	-
W. anomalus	W-2, C-2, C-4, C-5, C-6, C-7, C-9, C-10, C-11, C-13, C-14, C-16, G-5, G-6, G-7, G-9, G-10, G-11, G-12, G-13, G-14, G-15, G-21, G-23, G-26, G-27	+	+	+	+	+
	C-1, C-3, C-8, C-12, C-15, C-21, G-1, G-2, G-8, G-16, G-19, G-24, G-25	+	+	+	-	+
C. parapsilosis	W-3, W-6, W-7, W-12, W-17, W-23, W-8, W-15, W-16	+	+	+	-	+
	W-10, W-21, W-25	+	+	+	+	+
D. hansenii	C-23, C-24, C-25, C-26	+	+	+	+	+
	C-18, C-19, C-22	+	+	+	+	-
Cl. lusitaniae	G-17, G-18	+	+	+	+	+
	G-20, G-22	+	+	+	-	+
C. intermedia	W-1, C-17, C-27	+	+	+	+	+
P. kudriavzevii	G-3	+	+	+	-	+
	G-4	+	+	-	-	-

Note: Negative sign indicates the absence of growth; Positive sign indicates the presence of growth. Dex: Dextrose, Gal: Galactose, Suc: Sucrose, Lac: Lactose, Mal: Maltose

Table 6. Thermotolerant and osmotolerant yeast strains

Yeast species	37°C	45°C	50% Dex
K. marxianus	+	+	-
W. anomalus	+	-	+
C. parapsilosis	+	+	+
D. hansenii	-	-	+
Cl. lusitaniae	+	+	+
C. intermedia	-	-	+
P. kudriavzevii	+	+	+

Note: Negative sign indicates the absence of growth; Positive sign indicates the presence of growth

Screening fermentation ability

The fermentation ability of the yeast strains was analyzed by using five different fermentable carbon sources: dextrose, galactose, sucrose, lactose, and maltose. Fermentation tests of all yeast strains yielded positive results on dextrose, galactose, and sucrose carbon sources, except G-4 yeast strain of *P. kudriavzevii* which gave a negative result on sucrose (Table 7). The strains of *C. intermedia*, *D. hansenii* and *K. marxianus* yeast species showed positive results on lactose. However, thirteen yeast strains of *W. anomalus*, nine yeast strains of *C. parapsilosis*, two yeast strains of *Cl. lusitaniae* and all strains of *P. kudriavzevii* yeast species gave negative results for the lactose. *C. intermedia*, *C. parapsilosis*, *Cl. lusitaniae* and *W. anomalus* yeast strains showed positive results in the maltose fermentation test, while two yeast strains of *K. marxianus*, three yeast strains of *D. hansenii* and one strain of *P. kudriavzevii* showed negative results in the maltose test (Table 7). As a result, 61.8% of isolated yeast strains gave positive results in all fermentable carbon sources used.

Due to their high fermentation capacity in different carbon sources and thermotolerant/osmotolerant properties, twenty-six strains of *W. anomalus*, three strains of *C. parapsilosis* (W-10, W-21 and W-25) and two strains of *Cl. lusitaniae* (G-17 and G-18) have been identified as yeast species with industrial potential. On the other hand, fermentative yeasts are often responsible for the deterioration of cheeses. Although *P. kudriavzevii* yeast strain (G-4) can grow in sucrose, lactose and maltose carbon sources, it cannot ferment them. For this reason, it may not cause deterioration even if it is used as a starter culture in cheese production. In this respect, G-4 strain of *P. kudriavzevii* is industrially important.

In conclusion, yeasts are an important part of the cheese microbiota. While yeasts have positive effects on improving the sensory properties of cheeses, they sometimes cause the cheese to deteriorate. Therefore, the determination of yeast diversity in cheese biota is important for the development of new protective precautions and new starter cultures. Biochemical and molecular identification methods are of great importance for the identification of yeast samples in cheese biota. In this study, seven different yeast species were identified from traditional cheese samples, and *C. parapsilosis*, *Cl. lusitaniae*, *P. kudriavzevii* and *W. anomalus* yeast species were determined to be industrially important. The strains of these yeast species have the potential for industrial applications according to their extracellular enzyme activities, fermentation capacity and thermotolerant-osmotolerant properties.

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REFERENCES

Abdelmoteleb A, Troncoso-Rojas R, Gonzalez-Soto T, González-Mendoza D. 2017. Antifungical activity of autochthonous *Bacillus subtilis* isolated from *Prosopis juliflora* against phytopathogenic fungi. Mycobiol 45: 385-391. DOI: 10.5941/MYCO.2017.45.4.385.

Adlercreutz P. 2013. Immobilisation and application of lipases in organic media. Chem Soc Rev 42: 6406-6436. DOI: 10.1039/C3CS35446F.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215: 403-410. DOI: 10.1016/S0022-2836(05)80360-2.

Balakumar S, Arasaratnam V. 2012. Osmo-, thermo- and ethanol-tolerances of *Saccharomyces cerevisiae* S1. Braz J Microbiol 43: 157-166. DOI: 10.1590/S1517-838220120001000017.

Banjara N, Suhr MJ, Hallen-Adams HE. 2015. Diversity of yeast and mold species from a variety of cheese types. Curr Microbiol 70: 792-800. DOI: 10.1007/s00284-015-0790-1.

Benito MJ, Gonçalves Dos Santosa MTP, Córdoba MG, Alvarenga N, Herrera SRMS. 2018. Yeast community in traditional Portuguese serpa cheese by culture-dependent and -independent DNA approaches. Intl J Food Microbiol 262: 63-70. DOI: 10.1016/j.ijfoodmicro.2017.09.013.

Binetti A, Carrasco M, Reinheimer J, Suarez V. 2013. Yeasts from autochthonal cheese starters: Technological and functional properties. J App Microbiol 115: 434-444. DOI: 10.1111/jam.12228.

Bintsis T. 2021. Yeasts in different types of cheese. AIMS Microbiol 7: 447-470. DOI: 10.3934/microbiol.2021027.

Breuer U, Harms H. 2006. *Debaryomyces hansenii* -an extremophilic yeast with biotechnological potential. Yeast 23: 415-437. DOI: 10.1002/yea.1374.

Cardoso VM, Borelli BM, Lara CA, Soares MA, Pataro C, Bovedan EC, Rosa CA. 2015. The influence of seasons and ripening time on yeast communities of a traditional Brazilian cheese. Food Res Intl 69: 331-340. DOI: 10.1016/j.foodres.2014.12.040.

Ceugniez A, Drider D, Jacques P, Coucheney F. 2015. Yeast diversity in a traditional French cheese "Tomme d'orchies" reveals infrequent and frequent species with associated benefits. Food Microbiol 52: 177-184. DOI: 10.1016/j.fm.2015.08.001.

Choi DH, Eun-Hee Park EH, Kim MD. 2017. Isolation of thermotolerant yeast *Pichia kudriavzevii* from nuruk. Food Sci Biotechnol 26: 1357-1362. DOI: 10.1007/s10068-017-0155-6.

Chombo-Morales P, Kirchmayr M, Gschaedler A, Lugo-Cervantes E, Villanueva-Rodríguez S. 2016. Effects of controlling ripening conditions on the dynamics of the native microbial population of Mexican artisanal Cotija cheese assessed by PCR-DGGE. Food Sci Technol 65: 1153-1161. DOI: 10.1016/j.lwt.2015.09.044.

Çorbaci C, Uçar FB, Yalçin HT. 2012. Isolation and characterization of yeasts associated with Turkish-style homemade dairy products and their potential as starter cultures. Afr J Microbiol Res 6: 534-542. DOI: 10.5897/AJMR11.895.

de Araújo VD, de Albuquerque LC, Neves RP, Mota CS, Moreira KA, de Lima-Filho JL, Cavalcanti MTH, Converti A, Porto ALF. 2010. Production stability of protease from *Candida buinensis*. App Biochem Biotechnol 162: 830-842. DOI: 10.1007/s12010-009-8779-5.

Delgado-Ospina J, Triboletti S, Alessandria V, Serio A, Sergi M, Paparella A, Rantsiou K, Chaves-López C. 2020. Functional biodiversity of yeasts isolated from Colombian fermented and dry cocoa beans. Microorganisms 8: 1086. DOI: 10.3390/microorganisms8071086.

Dugat-Bony E, Garnier L, Denonfoux J, Ferreira S, Sarthou AS Bonnarme P, Irlinger F. 2016. Highlighting the microbial diversity of 12 French cheese varieties. Intl J Food Microbiol 238: 265-273. DOI: 10.1016/j.ijfoodmicro.2016.09.026.

Escribano R, Lucia Gonzalez-Arenzana L, Garijo P, Berlanas C, Lopez-Alfaro I, Lopez R, Gutierrez AR, Santamaria P. 2017. Screening of enzymatic activities within different enological non-*Saccharomyces* yeasts. J Food Sci Technol 54: 1555-1564. DOI: 10.1007/s13197-017-2587-7.

Esen Y, Çetin B. 2021. Bacterial and yeast microbial diversity of the ripened traditional middle east surk cheese, Intl Dairy J 117: 105004. DOI: 10.1016/j.idairyj.2021.105004.

Fonseca GG, Heinzle E, Wittmann C, Gombert AK. 2008. The yeast *Kluyveromyces marxianus* and its biotechnological potential. Appl Microbiol Biotechnol 79: 339-354. DOI: 10.1007/s00253-008-1458-6.

Fröhlich-Wyder MT, Arias-Roth E, Jakob E. 2019. Cheese yeasts. Yeast 36 (3): 129-141. DOI: 10.1002/yea.3368.

Garnier L, Valence F, Pawtowski A, Auhustsinava-Galerne L, Frotté N, Baroncelli R, Deniel F, Coton E, Mounie J. 2017. Diversity of spoilage fungi associated with various French dairy products. Intl J Food Microbiol 241: 191-197. DOI: 10.1016/j.ijfoodmicro.2016.10.026.

Genç T, Günay M. 2020. Internal transcribed spacer (ITS) sequence-based identification of yeast biota on pomegranate surface and determination of extracellular enzyme profile. Nus Biosci 12: 59-67. DOI: 10.13057/nusbiosci/n120111.

Geronikou A, Srimahaeak T, Rantsiou K, Triantafillidis G, Larsen N, Jespersen L. 2020. Occurrence of yeasts in white-brined cheeses: methodologies for identification, spoilage potential and good manufacturing practices. Front Microbiol 11: 582778. DOI: 10.3389/fmicb.2020.582778.

Gibson BR, Pham T, Wimalasena T, Box WG, Koivuranta K, Storgards E, Smart KA. 2011. Evaluation of ITS PCR and RFLP for differentiation and identification of brewing yeast and brewery 'wild' yeast contaminants. J Inst Brew 117: 556-568. DOI: 10.1002/j.2050-0416.2011.tb00504.x.

Gkatzionis K, Yunita D, Linforth RS, Dickinson M, Dodd CER. 2014. Diversity and activities of yeasts from different parts of a Stilton cheese. Int J Food Microbiol 177: 109-116. DOI: 10.1016/j.ijfoodmicro.2014.02.016.

Gupta R, Kumari A, Syal P. Singh Y. 2015. Molecular and functional diversity of yeast and fungal lipases: Their role in biotechnology and cellular physiology. Prog Lipid Res 57: 40-54. DOI: 10.1016/j.plipres.2014.12.001.

Haastrup MK, Johansen P, Malskær AH, Castro-Mejía JL, Kot W, Krych L, Arneborg N, Jespersen L. 2018. Cheese brines from Danish dairies reveal a complex microbiota comprising several halotolerant bacteria and yeasts. Intl J Food Microbiol 285: 173-187. DOI: 10.1016/j.ijfoodmicro.2018.08.015.

Hatoum R, Labrie S, Fliss O. 2013. Identification and partial characterization of antilisterial compounds produced by dairy yeasts. Probiotics Antimicrob Proteins 5: 8-17. DOI: 10.1007/s12602-012-9109-8.

Hayaloğlu AA, Güven M, Fox PF. 2002. Microbiological, biochemical and technological properties of Turkish white cheese 'Beyaz Peynir'. Intl Dairy J 12: 635-648. DOI: 10.1016/S0958-6946(02)00055-9.

Johnson EA, Echavarri-Erasun C. 2011. Yeast biotechnology. In: Kurtzman CP, Fell JW, Boekhout T (eds). The Yeasts, A Taxonomic Study, Elsevier, Amsterdam. DOI: 10.1016/B978-0-444-52149-1.00003-3.

Johnson EA. 2013a. Biotechnology of non-*Saccharomyces* yeasts--the ascomycetes. Appl Microbiol Biotechnol 97: 503-517. DOI: 10.1007/s00253-012-4497-y.

Johnson EA. 2013b. Biotechnology of non-*Saccharomyces* yeasts--the basidiomycetes. Appl Microbiol Biotechnol 97: 7563-7577. DOI: 10.1007/s00253-013-5046-z.

Karasu-Yalcin S, Senses-Ergul S, Ozbas ZY. 2017. Enzymatic characterization of yeast strains originated from traditional Mihalic cheese. J Microbiol Biotechnol Food Sci 6: 1152-1156. DOI: 10.15414/jmbfs.2017.6.5.1152-1156.

Karki TB, Timilsina PM, Yadav A, Pandey GR, Joshi Y, Bhujel S, Adhikari R, Neupane K. 2017. Selection and characterization of potential baker's yeast from indigenous resources of Nepal. Biotechnol Res Intl 2017: 1925820. DOI: 10.1155/2017/1925820.

Kavas G, Kinik D, Uysal S, Kilic S, Celikel N, Akbulut N. 2006. Characterisation of yeasts isolated from artisanal Turkish dairy products. Intl J Dairy Sci 1: 44-50. DOI: 10.3923/ijds.2006.44.50.

Kumar D, Kumar L, Nagar S, Raina C, Parshad R, Gupta VK. 2012. Screening, isolation and production of lipase/esterase producing *Bacillus* sp. Strain DVL2 and its potential evaluation in esterification and resolution reactions. Arch App Sci Res 4: 1763-1770.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35: 1547-1549. DOI: 10.1093/molbev/msy096.

Kurtzman CP, Fell JW, Boekhout T. 2011. The Yeasts: A taxonomic Study. Elsevier B.V, Amsterdam.

Kurtzman CP, Robnett CJ. 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Anton Leeuw 73: 331-371. DOI: 10.1023/A:1001761008817.

Liu E, Li M, Abdella A, Wilkins MR. 2020. Development of a cost-effective medium for submerged production of fungal aryl alcohol oxidase using a genetically modified *Aspergillus nidulans* strain. Bioresour Technol 305: 123038. DOI: 10.1016/j.biortech.2020.123038.

Lõoke M, Kristjuhan K, Kristjuhan A. 2011. Extraction of genomic DNA from yeasts for PCR-based applications. Biotechniques 50: 325-328. DOI: 10.2144/000113672.

Merchan AV, Ruiz-Moyano S, Hernández MV, Benito MJ, Aranda E, Rodríguez A, Martín A. 2021. Characterization of autochthonal yeasts isolated from Spanish soft raw ewe milk protected designation of origin cheeses for technological application. J Dairy Sci 105: 2931-2947. DOI: 10.3168/jds.2021-21368.

Molnárová J, Vadkertiová R, Stratilová E. 2014. Extracellular enzymatic activities and physiological profiles of yeasts colonizing fruit trees. J Basic Microbiol 54: 74-84. DOI: 10.1002/jobm.201300072.

Montel MC, Buchin S, Mallet A, Delbes-Paus C, Vuitton DA, Desmasures N, Berthier F. 2014. Traditional cheeses: Rich and diverse microbiota with associated benefits. Intl J Food Microbiol 177: 136-154. DOI: 10.1016/j.ijfoodmicro.2014.02.019.

Moubasher AAH, Abdel-Sater MA, Soliman ZSM. 2018. Yeasts and filamentous fungi associated with some dairy products in Egypt. J Mycol Med 28: 76-86. DOI: 10.1016/j.mycmed.2017.12.003.

Naveed M, Nadeem F, Mehmood T, Bilal M, Anwar Z, Amjad F. 2021. Protease-A versatile and ecofriendly biocatalyst with multi-industrial applications: An updated review. Catal Lett 151: 307-323. DOI: 10.1007/s10562-020-03316-7.

Padilla B, Manzanares P, Belloch C. 2014. Yeast species and genetic heterogeneity within *Debaryomyces hansenii* along the ripening process of traditional ewes' and goats' cheeses. Food Microbiol 38: 160-166. DOI: 10.1016/j.fm.2013.09.002.

Pangallo D, Sakova N, Korenova J, Puškárová A, Kraková L, Valík L, Kuchta T. 2014. Microbial diversity and dynamics during the production of may bryndza cheese. Intl J Food Microbiol 170: 38-43. DOI: 10.1016/j.ijfoodmicro.2013.10.015.

Parafati L, Vitale A, Restuccia C, Cirvilleri G. 2015. Biocontrol ability and action mechanism of food-isolated yeast strains against *Botrytis cinerea* causing post-harvest bunch rot of table grape. Food microbiol 47: 85-92. DOI: 10.1016/j.fm.2014.11.013

Pincus DH, Orenga S, Chatellier S. 2007. Yeast identification--past, present, and future methods. Med Mycol 45: 97-121. DOI: 10.1080/13693780601059936.

Settier-Ramírez L, López-Carballo G, Hernández-Muñoz P, Fontana A, Strub C, Schorr-Galindo S. 2021. New isolated *Metschnikowia pulcherrima* strains from apples for postharvest biocontrol of *Penicillium expansum* and patulin accumulation. Toxins 13: 397. DOI: 10.3390/toxins13060397.

Sharma S, Kanwar SS. 2014. Organic solvent tolerant lipases and applications. Sci World J 2014: 625258. DOI: 10.1155/2014/625258.

Steensels J, Verstrepen KJ. 2014. Taming wild yeast: Potential of conventional and nonconventional yeasts in industrial fermentations. Ann Rev Microbiol 68: 61-80. DOI: 10.1146/annurev-micro-091213-113025.

Tofalo R, Fasoli G, Schirone M, Perpetuini P, Pepe K, Corsetti A, Suzzi G. 2014. The predominance, biodiversity and biotechnological properties of *Kluyveromyces marxianus* in the production of Pecorino di Farindola cheese. Intl J Food Microbiol 187: 41-49. DOI: 10.1016/j.ijfoodmicro.2014.06.029.

Togay SO, Capece A, Siesto G, Aksu H, Sandıkçı Altunalmaz S, Yılmaz Aksu F, Romano P, Karagül-Yüceer Y. 2020. Molecular characterization of yeasts isolated from traditional Turkish cheeses. Food Sci Technol 40: 871-876. DOI: 10.1590/fst.24319.

Vasdinyei R, Deak T. 2003. Characterization of yeast isolates originating from Hungarian dairy products using traditional and molecular identification techniques. Intl J Food Microbiol 86: 123-130. DOI: 10.1016/S0168-1605(03)00251-4.

Vincent M, Johnny Q, Adeni DSA, Suhaili N. 2021. Potential of *Candida glabrata* from ragias a bioethanol producer using selected carbohydrate substrate Nus Biosci 13: 1-10. DOI: 10.13057/nusbiosci/n130101.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds). PCR Protocols: A Guide to Methods and Applications. Academic Press, California.

Yalçın HT, Çorbacı C, Uçar FB. 2014. Molecular characterization and lipase profiling of the yeasts isolated from environments contaminated with petroleum. J Basic Microbiol 54: 85-92. DOI: 10.1002/jobm.201300029.

Yalçın HT, Uçar FB. 2009. Isolation and characterization of cheese spoiler yeast isolated from Turkish white cheeses. Ann Microbiol 59: 477-483. DOI: 10.1007/BF03175134.

Yamamoto H, Shima T, Yamaguchi M, Mochizuki Y, Hoshida H, Kakuta S, Kondo-Kakuta C, Noda NN, Inaqaki F, Itoh T, Akada R, Ohsumi Y. 2015. The thermotolerant yeast *Kluyveromyces marxianus* is a useful organism for structural and biochemical studies of autophagy. J Biol Chem 115: 6833-6842. DOI: 10.1074/jbc.M115.684233.