GIDA
THE JOURNAL OF FOOD
E-ISSN 1309-6273, ISSN 1300-3070

Research/ Araştırma
GIDA (2021) 46 (5) 1081-1091
doi: 10.15237/gida.GD21088

ENZYMATIC CHARACTERIZATION OF YEAST ISOLATED FROM NATURALLY FERMENTED HERBS*

Erkan Güneş[1], Furkan Aydın[2], İbrahim Çakır[3]**

[1] Amasya University Rectorate, Internal Audit Unit, Amasya, Turkey,
[2] Aksaray University, Faculty of Engineering, Food Engineering Department, Aksaray, Turkey.
[3] Bolu Abant İzzet Baysal University, Faculty of Engineering, Food Engineering Department, Bolu, Turkey.

Received/ Geliş: 17.05.2021; *Accepted*/ Kabul: 06.07.2021; *Published online*/ Online baskı: 02.08.2021

Güneş, E., Aydın, F., Çakır, İ. (2021). Enzymatic characterization of yeast isolated from naturally fermented herbs. GIDA (2021) 46 (5) 1081-1091 doi: 10.15237/gida.GD21088

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ABSTRACT

This study aims to identify yeasts from spontaneously fermented herbs used for Van herby cheese and to evaluate their enzymatic activities. Sequencing of partial 26S rRNA gene revealed the species of *Pichia membranifaciens* (n = 8), *Kazachstania servazzii* (n = 6), *Debaryomyces hansenii* (n = 2), *Kluyveromyces marxianus* (n = 2), and *Pichia fermentans* (n = 2). Enzyme profiles were determined using API-ZYM strips. The isolates had diverse enzyme activities, including alkaline and acid phosphatase, esterase, esterase lipase, aminopeptidases, and proteases mostly at different levels, which may have crucial roles during ripening of the cheese. *K. marxianus* M8.1, *D. hansenii* M6.1, and M6.3, *P. membraniciens* M13.1, M13.2, and M14.1 had superior and diverse enzymatic characteristics. Such enzymatic activities could be of great technological importance for the determination of adjunct culture along with starter lactic acid bacteria for the production of Van herby cheese.

Keywords: Brine solution, herb, yeast, enzyme activity.

FERMENTE SALAMURA OTLARDAN İZOLE EDİLEN MAYALARIN ENZİMATİK KARAKTERİZASYONU

ÖZ

Bu çalışmada, Van otlu peyniri üretiminde kullanılan fermente salamura otlarından mayaların izolasyonu, identifikasyonu ve enzimatik aktivitelerinin belirlenmesi amaçlanmıştır. Yirmi adet maya izolatı izole edilerek moleküler olarak tanımlanmıştır. Tanımlamada 26S rRNA gen bölgesinin D1/D2 alt bölgesi çoğaltılarak sekanslanmıştır. Sekanslama sonucunda *Pichia membranifaciens* (n = 8), *Kazachstania servazzii* (n = 6), *Debaryomyces hansenii* (n = 2), *Kluyveromyces marxianus* (n = 2) ve *Pichia*

* *This paper includes data from MSc thesis of Erkan Güneş. A part of this study was presented as a poster at the 2nd International Congress on Food Technology, November 05-07, 2014, Kuşadası, Aydın Turkey and published as an abstract in Congress Abstract Book*. / Bu çalışma Erkan Güneş'in yüksek lisans tez çalışmasından veriler içermektedir. Bu çalışmanın bir kısmı 2. Uluslararası Gıda Teknolojisi Kongresi, 05-07 Kasım 2014, Kuşadası, Aydın, Türkiye'de poster olarak sunulmuş ve kongre kitabında özeti bildiri olarak basılmıştır.

** *Corresponding author* / Yazışmalardan sorumlu yazar:
✉: ibrahimcakir@ibu.edu.tr ✆ (+90) 374 254 1000/ 4827 🖷: (+90) 374 253 4558

Erkan Güneş; ORCID no: 0000-0001-8666-9124
Furkan Aydın; ORCID no: 0000-0003-2924-5434
İbrahim Çakır; ORCID no: 0000-0001-7775-1871

fermentans (*n* = 2) türleri tanımlanmıştır. İzolatların enzim profilleri API-ZYM test kitleri kullanılarak belirlenmiştir. İzolatların, peynirin olgunlaşması sırasında önemli rollere sahip olabilecek alkali ve asit fosfataz, esteraz, esteraz lipaz, aminopeptidazlar ve proteazlar dahil olmak üzere çeşitli enzim aktivitelerine sahip olduğu belirlenmiştir. İzolatlar arasından, *K. marxianus* M8.1, *D. hansenii* M6.1 ile M6.1, *P. membraniciens* M13.1, M13.2 ile M14.1 suşlarının üstün enzimatik özellikler taşıdığı görülmüştür. Bu tür enzimatik faaliyetlerin, Van otlu peyniri üretimi için starter laktik asit bakterileri ile birlikte ek kültürün belirlenmesi için teknolojik bir öneme sahip olabileceği düşünülmektedir.
Anahtar kelimeler: Salamura çözeltisi, ot, maya, enzim aktivitesi.

INTRODUCTION

Yeast constitute the microflora of many artisanal cheeses (Frohlich-Wyder et al., 2019). Sources of yeast in cheese are quite complex due to their ability to tolerate extreme conditions such as low pH and reduced water activity with enhancing brine concentrations. They are widely dispersed in a cheese-making environment, raw milk, and on the surfaces of cheese-making tools (Banjara et al., 2015). The herbs and spontaneously fermented herbs are also great contamination sources of different yeast species as well as lactic acid bacteria (LAB) (Cakir, 2010). They play crucial roles in the production of nearly all types of artisanal cheeses (Haastrup et al., 2018). The initially abundant yeast species in cheese are reported to be salt-tolerant. They have the ability to metabolize lactate produced by LAB in the early stage of ripening as well as producing NH_3 from amino acids, thus increasing the pH of the cheese. The deacidification process gives rise to bacteria, mainly non-starter LAB, to grow and pursues the ripening (Frohlich-Wyder et al., 2019). Moreover, thanks to their lipolytic, proteolytic, and extensive extracellular enzymatic activity, they have substantial effects on the development of cheese flavor and texture (McSweeney, 2004). The contribution of yeast to cheese technology has enabled them to be included in adjunct culture studies for the recent years (Atanassova et al., 2016; Binetti et al., 2013; De Freitas et al., 2008; Kesenkas and Akbulut, 2008).

Herby (Otlu) cheese is a semi-hard and herb-flavored artisanal cheese manufactured mainly from raw sheep's milk in the eastern part of Turkey for over 200 years (Tarakci et al., 2004). Approximately 25 different herbs are used alone or as mixtures of a few prepared in brine, among which *Allium*, *Thymus*, *Anthriscus*, and *Ferula* are the most commonly used at levels of 0.5-2.0 kg per 100 kg milk (Coskun, 1998; Hayaloglu and Farkye, 2011). It still continues to gain popularity among consumers demanding robust flavors due to its unique garlic and thyme aroma distinguishing Van herby cheese from other artisanal cheeses in aroma perspective. Although the chemistry and biochemistry of Van herby cheese have been very-well studied, the microbiological aspect regarding the isolation and characterization of yeast remains unclear.

There has been a growing interest in studying yeast isolated from traditional cheeses in recent years, which mainly focus on genetic characterization, determining their roles in ripening and evaluating their technological and probiotic characteristics more in particular (Aydin et al., 2020; Binetti et al., 2013). Four significant yeast species, which are *Yarrowia lipolytica, Kluyveromyces marxianus*, *K. lactis*, and *Debaryomyces hansenii*, were reported to be the most isolated species from artisanal cheeses and their brine solutions and are described in more detail (Atanassova et al., 2016; Banjara et al., 2015; Ceugniez et al., 2015). On the other hand, *Pichia membranifaciens*, *P. fermentans*, *P. kudriavzevii, Galatactomyces candidum*, *Saccharomyces cerevisiae*, and some species belonging to *Candida* genus were isolated in lesser amounts (Aponte et al., 2010; Zheng et al., 2018). Taking into account that yeast plays essential roles during ripening as well as LAB, there is still a need for determining endogenous yeast microflora of artisanal cheeses and their technological properties extensively. Some studies regarding technological characteristics of yeast isolated from different artisanal cheeses, including Erzincan Tulum cheese, Mihalic cheese, Fossa cheese, and Serpa cheese, were carried out (Biagiotti et al., 2018;

Dos Santos et al., 2017; Karasu-Yalcin et al., 2012: 2017). Yet, to the best of our knowledge, yeast from Herby cheese or spontaneously fermented herb solutions has not been studied in detail. This study aims to identify yeast from naturally fermented herb brines used for the production of Herby cheese and evaluate their enzymatic characteristics.

MATERIALS AND METHODS

Fermented brine solutions

Fermented herb brine solutions containing *Allium schoenoprasum, Anthriscus nemorosa, Prangos ferulacea*, and *Chaerophyllum macropodum* alone and as a mixture of a few were provided by Professor Yusuf Tuncturk from Food Engineering Department of Van Yuzuncu Yil University (Van, Turkey). The detailed information regarding the isolation materials is given in Table 1 within the result and discussion section.

Isolation of yeasts

Twenty-five grams of herb brine solutions were taken and homogenized in 225 mL of sterile saline solution (0.85% NaCl) using a stomacher (MAYO, HG-400, Australia). Series of dilutions up to 10^{-5} were prepared. Duplicate aliquots (100 µL) of appropriate dilutions were inoculated onto Potato Dextrose Agar (Oxoid, UK) pH 5.0 supplemented with 100 mg/kg of chloramphenicol (Sigma Aldrich, USA). The plates were incubated at 28°C for 3-5 days. Morphologically different colonies were selected and isolated by streaking plate technique to obtain pure cultures for molecular identification. Isolates were maintained in YPD broth (Sigma Aldrich, USA) with (v/v) 20% added glycerol and as slant in the same medium supplemented with 1.5% agar (Sigma Aldrich, USA) without glycerol.

Molecular identification of isolates

DNA of each strain was extracted using a commercial product of DNeasy Blood and Tissue kit (Qiagen, Cat No./ID: 69504) according to the manufacturer's instructions. The final quantity of DNA was evaluated by the DS-11 FX+ spectrophotometer (Denovix, USA) and diluted to 50 ng using sterile ultra-pure water. Template DNA samples were stored at -20°C till used.

For amplification of the D1/D2 domain of the 26S rRNA gene of the isolates, NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-TCC TCC GTC TAT TGA TAT GC-3′) primer pairs were employed (Kurtzman and Robnett, 1998). PCR reactions were performed in a 50 µL reaction mixture containing 1× PCR reaction buffer, 200 µm dNTPs, 0.4 µM each primer, 1.5-unit *Taq* DNA Polymerase (New England BioLabs, MA), and 50 ng template DNA. The PCR amplification was carried out in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA). It included 3 min of initial denaturation step at 94°C, followed by 35 cycles with 35 s denaturation at 94°C, 40 s annealing at 55°C, and 1 min extension at 72°C with 10 min of final extension at 72°C. Amplification products were visualized on an agarose gel to check the amplification. The amplicons were subjected to bidirectional sequencing in a commercial company (Macrogen Inc., Seoul, Korea).

The sequence data were subjected to the BLAST search in the GenBank database (http://blast.ncbi.nlm.nih.gov/) to match with the closest available reference sequences in the complete National Center for Biotechnology Information (NCBI) nucleotide collection. Accordingly, the accession numbers for the D1/D2 domain of the isolates submitted to NCBI are MT040771 through MT040790. Phylogenetic and molecular evolutionary analyses of 20 isolates from this study and corresponding yeast isolates deposited in the nucleotide database of GenBank (*P. membranifaciens*: DQ409149; KY108894; *K. servazzii*: MH704181; *D. hansenii*: KY107508; *K. marxianus* KT945093; *P. fermentans* KY108803) were aligned with CLUSTAL W (Thompson et al., 1994). Sequences were edited manually using MEGAX (Kumar et al., 2018) for higher accuracy, which is given in detail by Ozer and Bayraktar (2018). A neighbor-joining tree was constructed using Tamura and Nei (1993) model with bootstrap analyses using the heuristic search option conducted with 1,000 replicates.

Growth at different salt concentrations

The yeast strains were grown in 10 mL of YPD broth (Sigma Aldrich, USA) in the

presence of different NaCl concentrations (7.5%, 10%, and 12.5%) for two weeks at 28°C.

Enzymatic characterization

Enzymatic activity of yeast was determined using the API-ZYM test system (BioMérieux, France) thanks to which 19 different enzyme activity can be rapidly analyzed. Previously activated yeast cultures were grown on PDA by streaking plate technique. After the incubation period, single colonies were taken and suspended in distilled water till they reach 5-6 McFarland turbidity. Then the 65 µL of suspensions were inoculated to the wells of API-ZYM strip and incubated at 37°C for around 4-5 hours before the addition of ZYM A and ZYM B reagents to each cupule. The color formation was awaited for around 5 minutes. The strips were held under a 1000 W lamp for 10 seconds to prevent the formation of yellow color due to Fast Blue BB. Enzyme activity was graded from 0 to 5 by comparison of color formed within 5 minutes to the API-ZYM color reaction chart, and the results were expressed on a scale from 0 (no activity) to 5 (maximum activity).

RESULTS AND DISCUSSION

Identification of the strains and phylogenetic analyses

A total of 20 yeast isolates obtained from naturally fermented herbs used for the production of Herby cheese were identified by sequencing the D1/D2 domain of 26S rRNA. Among all, eight were ascribed to *Pichia membranifaciens* (40.0%), 6 to *Kazachstania servazzii* (30.0%), 2 to *Debaryomyces hansenii* (%10) 2 to *Kluyveromyces marxianus* (%10) and 2 to *Pichia fermentans* (%10), respectively. The isolates were confirmed by the BLASTn algorithm running on the NCBI website for each strain. The fragments amplified using NL1 and NL4 primers ranged from 552 to 651 bp long, with identity matches of 99-100% with corresponding species from the GenBank. All sequences belonging D1/D2 domain of 26S rRNA obtained in this study have been submitted to the GenBank database with the accession numbers, as indicated in Table 1 with detail information.

Table 1. The detailed information regarding yeasts.

Strain	Source	Identified species	Accession N°
M10.1	*Anthriscus nemorosa* in brine	*Pichia membranifaciens*	MT040779
M11.1	*Allium schoenoprasum* + *Anthriscus nemorosa in brine*	*Pichia membranifaciens*	MT040780
M13.1	*Anthriscus nemorosa* + *Allium schoenoprasum* + *Chaerophyllum macropodum* in whey brine	*Pichia membranifaciens*	MT040782
M13.2	*Anthriscus nemorosa* + *Allium schoenoprasum* + *Chaerophyllum macropodum* in whey brine	*Pichia membranifaciens*	MT040783
M14.1	*Prangos ferulacea* in brine	*Pichia membranifaciens*	MT040784
M15.2	*Allium schoenoprasum* in whey brine	*Pichia membranifaciens*	MT040786
M17.1	*Chaerophyllum macropodum* in whey brine	*Pichia membranifaciens*	MT040787
M18.2	*Allium schoenoprasum* in whey brine	*Pichia membranifaciens*	MT040790
M5.1	*Allium schoenoprasum* in whey brine	*Kazachstania servazzii*	MT040771
M5.2	*Allium schoenoprasum* in whey brine	*Kazachstania servazzii*	MT040772
M6.4	*Anthriscus nemorosa* in whey brine	*Kazachstania servazzii*	MT040775
M9.1	*Allium schoenoprasum* in whey brine	*Kazachstania servazzii*	MT040778
M11.2	*Allium schoenoprasum* + *Anthriscus nemorosa in brine*	*Kazachstania servazzii*	MT040781
M15.1	*Allium schoenoprasum* in whey brine	*Kazachstania servazzii*	MT040785
M6.1	*Anthriscus nemorosa* in whey brine	*Debaryomyces hansenii*	MT040773
M6.3	*Anthriscus nemorosa* in whey brine	*Debaryomyces hansenii*	MT040774
M8.1	*Prangos ferulacea* in whey brine	*Kluyveromyces marxianus*	MT040776
M17.3	*Chaerophyllum macropodum* in whey brine	*Kluyveromyces marxianus*	MT040789
M8.2	*Prangos ferulacea* in whey brine	*Pichia fermentans*	MT040777
M17.2	*Chaerophyllum macropodum* in whey brine	*Pichia fermentans*	MT040788

P. membranifaciens strains were found to be the dominant species along with *K. servazzii*, which is a basionym of *Saccharomyces servazzii*. The prevalence of yeast species differs according to the type of cheese, its origin, raw materials used, and ripening conditions. Most of the researchers reveals that *D. hansenii* was mostly isolated yeast species among traditionally produced raw milk cheeses, especially those manufactured from raw ewes' or goats' milk. As, it can grow in high salt concentrations (Aponte et al., 2010; Karasu-Yalcin et al., 2017; Padilla et al., 2014). Similar to our results, Mei et al. (2014) found that *K. servazzii* was the dominant species along with *S. cerevisiae* in a Camembert-type cheese type by culture-independent PCR-denaturating gel electrophoresis (PCR-DGGE). However, Lavoie et al. (2012) reported that *K. servazzii* could be found in non-dominant microflora of milk and cheese along with *P. membranifaciens*. *K. servazzii* were mostly isolated from alcoholic beverages (Moon et al., 2014; Spitaels et al., 2014) and sourdough (Lhomme et al., 2015). It is noteworthy that, the presence of *K. servazzii* in naturally fermented herbs could be attributed to the assimilation of lactic acid produced by LAB present in the brine. *K. marxianus* is frequently isolated from whey, brine, and different type of cheeses due to its ability to ferment lactose (Cardoso et al., 2015; Haastrup et al., 2018; Lane and Morissey, 2010). Apart from *D. hansenii* and *K. marxianus*, many researchers also reported *Yarrowia lipolytica*, and *Galactomyces candidum* species to have been isolated from different kinds of artisanal cheeses as a result of molecular identification (Ceugniez et al., 2017; Dugat-Bony et al., 2016). Similar to our results, Álvarez-Martín et al. (2007) reported that *P. fermentans* strains constituted the non-dominant microflora of Spanish blue-veined Cabrales cheese.

In some of the other studies in which molecular techniques were not applied for identification, some species belonging to *Candida* genus and *Cryptococcus laurenti* were reported (Al-Otaibi 2012; Karasu-Yalcin et al., 2017). Even in some cases, identification using molecular methods in which Internally Transcript Sequence (ITS) region is amplified by PCR makes some challenges to differentiate between species. Partial amplification of large subunit is often of choice (Aydin et al., 2020). Nnon-molecular techniques may not give the best results owing to the inherent phenotypic and genetic heterogeneity in populations, or the limited ability of phenotypic tests used to identify species.

Phylogenetic analysis based upon the D1/D2 domain of 26S rRNA sequences of isolates was carried out employing the neighbor-joining analyses with 1,000 replicates revealing polymorphism between species (Figure 1). The percentage of replicate trees where the associated taxa clustered together in the bootstrap test is indicated adjacent to the branches. The phylogenetic tree divided all isolates into two major groups, which are shown in Figure 1 as Cluster-I and Cluster-II. In the first cluster, there are two sub-clusters formed with high bootstrap values within the genus of *Pichia*. The second cluster, however, was divided into three sub-clusters between *D. hansenii*, *K. marxianus*, and *K. servazzizi* species.

Growth abilities at differen NaCl concentrations

All isolates were able to grow in the presence of salt with concentrations of 7.5 and 10%, apart from *P. fermentans* strains. Only three strains of *P. membranifaciens* (M13.1, M13.2, M14.1) and both of *D. hansenii* were able to grow at 12.5% NaCl concentration. Taking into account that salt content can reach up to 9.07% in Van herby cheese (Tarakci et al., 2004), all of the strains, except for *P. fermentans* M8.2 and M17.2, could be present in ripened Van herby cheese, which has high salt concentrations particularly, according to population dynamics.

Enzymatic activities

Enzyme profiles of the strains are given in detail in Table 2 and Figure 2. Nearly most of the isolates exhibited very low α-glucosidase, β-glucosidase, α-mannosidase, and α-fucosidase activities while none of the isolates showed β-glucuronidase and N-acetyl-β-glucosaminidase activities. *Kluyveromyces marxianus* M8.1, *D. hansenii* M6.1, and M6.3 had vigorous β-glucosidase

activity, which is a crucial enzyme for the conversion of galactose into glucose-6-phosphate using Leloir pathway with lactose permease. The high ß-glucosidase activity among *K. marxianus* and *D. hansenii* strains were reported in yeast isolated from feta cheese (Psomas et al., 2001). Since the presence or availability of either lactose or galactose in cheese is generally limited, these strains may be essential for residual lactose metabolism during ripening of the herby cheese (Frohlich-Wyder et al., 2019).

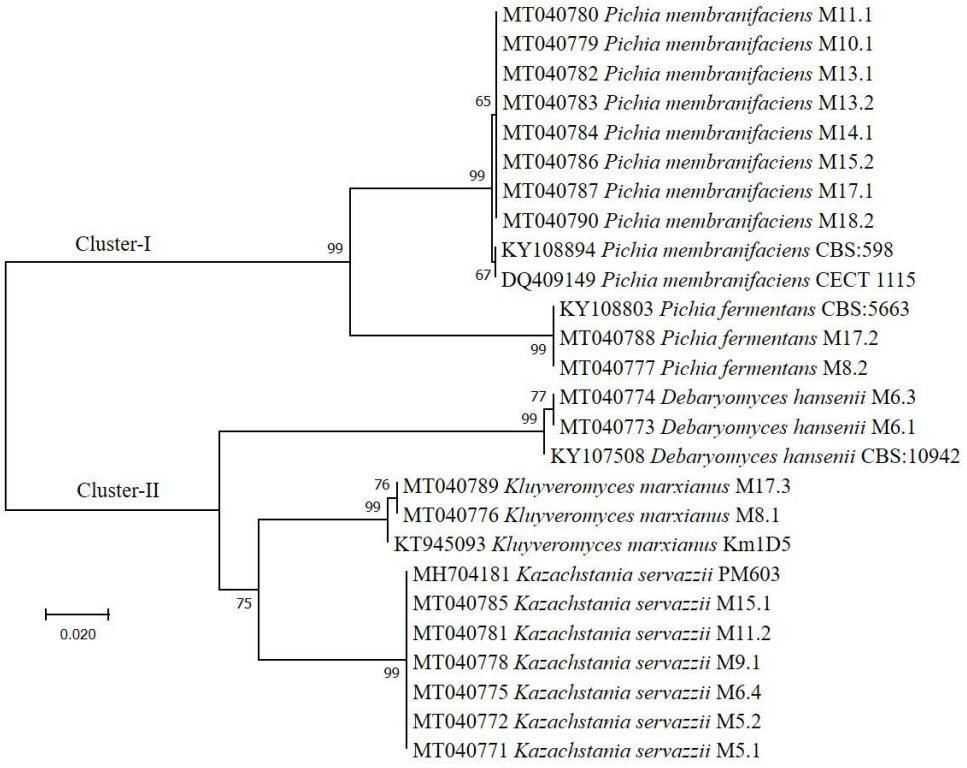

Figure 1. Phylogenetic tree of yeasts generated by neighbor-joining analysis with bootstrap of 1,000.

All isolates had proteolytic enzyme activities of arylamidases (aminopeptidases) which catalyze the hydrolysis of N-terminal amino acids from peptide, amide, and arylamides (Dodor and Tabatabai, 2007). These enzymes were reported to prevent the bitter taste that occurred during the ripening in mold-ripened soft cheeses since they are essential tools in the liberation of amino acids and the development of the desired flavor (Gobbetti et al., 2015). Besides, some of aldehyde and alcohol derivatives are usually formed from the degradation of valine, methionine, leucine, and methionine (Zeng et al., 2014). High leucine arylamidase activities were also reported in Erzincan Tulum cheese and Mihalic cheese by Karasu-Yalcin et al. (2012, 2017). Our results reveal high valine and cystine arylamidase activities which differs from the isolates obtained from Tulum and Mihalic cheeses. According to the results reported by Kesenkas and Akbulut (2006), *K. marxianus* and *D. hansenii* were found to stimulate the growth of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* in the early stage of the ripening period, where yeasts were used as adjunct cultures, probably by liberating of peptides. Eventhough most of the production of Van herby cheese is made from raw milk in households, the controlled production includes pasteurazation and starter culture addition, in which *L. lactis* subsp. *lactis* and *L. lactis* subsp.

cremoris cultures are used (Ocak et al., 2015). *K. marxianus* M8.1, *D. hansenii* M6.1 and M6.3 strains may have a special role in stimulating the growth of these two starters during the early stages of ripening.

Table 2. Enzyme activity assay obtained by API-ZYM kit.

Isolate	Control	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Pm 10.1	0	4	5	3	0	5	4	4	0	0	5	5	0	0	0	0	0	0	0	0
Pm 11.1	0	4	4	3	1	5	4	4	1	1	5	5	1	1	0	0	0	0	1	0
Pm 13.1	0	4	5	3	1	5	4	3	1	1	5	5	1	1	0	0	0	0	0	0
Pm 13.2	0	2	4	3	1	5	3	3	1	1	5	5	0	1	0	1	1	0	1	1
Pm 14.1	0	1	4	3	0	5	4	4	0	0	5	5	0	0	0	0	0	0	0	0
Pm 15.2	0	3	5	3	2	5	4	4	0	0	5	5	0	0	0	0	0	0	0	0
Pm 17.1	0	3	5	3	1	4	5	5	1	1	5	5	1	1	0	0	0	0	0	0
Pm 18.2	0	5	4	2	1	5	4	4	0	0	5	5	0	0	0	0	0	0	0	0
Ks 5.1	0	2	5	4	1	5	3	3	1	1	0	3	0	0	0	0	0	0	1	0
Ks 5.2	0	1	5	4	1	5	3	3	1	1	2	3	0	0	0	0	0	0	0	1
Ks 6.4	0	1	5	3	1	5	3	3	1	1	1	3	2	1	0	0	0	0	1	0
Ks 9.1	0	1	4	3	1	5	3	3	1	1	1	3	2	1	0	0	0	0	0	0
Ks 11.2	0	1	5	3	1	5	3	3	1	1	2	3	0	0	0	1	0	0	1	0
Ks 15.1	0	0	5	4	0	5	4	4	0	0	2	2	0	0	0	0	0	0	0	0
Dh 6.1	0	4	5	3	0	5	2	3	0	0	4	2	0	4	0	1	0	0	0	0
Dh 6.3	0	5	4	3	1	5	3	2	0	0	5	2	0	4	0	0	0	0	0	0
Km 8.1	0	5	4	3	1	5	4	3	1	1	5	4	1	5	0	1	4	0	1	1
Km17.3	0	1	4	3	0	5	5	5	0	0	5	5	0	0	0	0	0	0	0	0
Pf M8.2	0	1	4	3	0	5	4	3	0	0	5	5	0	0	0	0	0	0	0	0
Pf 17.2	0	2	4	3	0	5	4	4	0	0	5	5	0	0	0	0	0	0	0	0

Pm: *Pichia membranifaciens*, Ks: *Kazachstania servazzii*, Dh: *Debaryomyces hansenii*, Km: *Kluyveromyces marxianus*, Pf: *Pichia fermentans* 1: Alkaline phosphatase, 2: Esterase (C4), 3: Esterase lipase (C8), 4: Lipase (C14) 5: Leucine arylamidase, 6: Valine arylamidase, 7: Cystine arylamidase, 8: Trypsin, 9: α-chymotrypsin, 10: Acid phosphatase, 11: Naphtol-AS-BI-phosphohydrolise, 12: α-galactosidase, 13: β- galactosidase, 14: β-glucuronidase, 15: α-glucosidase, 16: β-glucosidase, 17: N-acetyl- β-glucoaminidase, 18: α-mannosidase, 19: α-fucosidase.

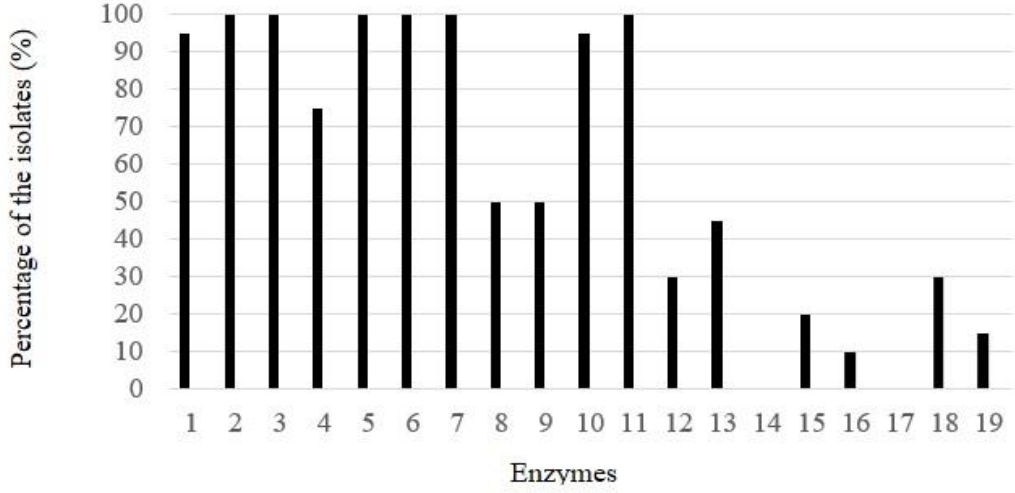

Figure 2. Percentage of yeast isolates having 19 different enzyme activities.
1: Alkaline phosphatase, 2: Esterase (C4), 3: Esterase lipase (C8), 4: Lipase (C14) 5: Leucine arylamidase, 6: Valine arylamidase, 7: Cystine arylamidase, 8: Trypsin, 9: α-chymotrypsin, 10: Acid phosphatase, 11: Naphtol-AS-BI-phosphohydrolise, 12: α-galactosidase, 13: β- galactosidase, 14: β-glucuronidase, 15: α-glucosidase, 16: β-glucosidase, 17: N-acetyl- β-glucoaminidase, 18: α-mannosidase, 19: α-fucosidase

Either weak or no trypsin and α-chymotrypsin activities were observed. Strains displaying low proteinase and relatively strong peptidase activities are essential for the cheese technology. They improve the texture defects in the cheese and have debittering effects (Mathara et al., 2004). Such isolates could have a technological importance for the production of Van herby cheese. According to alkaline and acid phosphatase results, most of the strains exhibited higher acid phosphatase activities. The reason for this is thought to arise from the fact that acid phosphatase has higher thermal activity as well as having lower optimum working pH (Stepaniak, 2004). It acts synergistically with proteolytic enzymes and hydrolyzes casein molecules. This synergetic action gives rise to the extensive production of small peptides and free amino acids. It contributes significantly to the cheese ripening in the point of view of aroma production (Akuzawa and Fox, 2004). On the other hand, all isolates displayed esterase (C4) and esterase lipase (C8) activities, while lipase (C14) activity was observed very weekly for most of the isolates. Lipases are carboxylesterases which catalyze the hydrolysis of the ester linkages on lipids; thus, it results in the liberation of free fatty acids (Decimo et al., 2017). Many researchers have reported different amount of lipolytic strains even for the same species (Karasu-Yalcin et al., 2017; Landel et al., 2006). This discrepancy is thought to arise not only from the strains difference but also from the difference in the application methods. It is hard to evaluate lipolytic activities on an agar medium in which tributyrin and Tween 20 is often used for lipase production. Since lipolysis affects the aroma dramatically, strains displaying moderate or high lipase, esterase, and lipase esterase activities have the potential to be used as adjunct starter cultures.

All strains were evaluated to have multiple and diverse enzymatic activities. Although low protease activity is the case for most of the adjunct culture to prevent the bitter taste, acid phosphatase and arylamidase have crucial roles during the ripening period. Taking into account these parameters, *P. membranifaciens* M13.1, M13.2, and M14.1 had strong acid phosphate and arylamidase activities as well as growing well under high salt concentrations. In addition to high acid phosphatase and arylamidase activities, ß-galactosidase activitiy should also be taken into account for the conversion of galactose to glucose-6-phosphate. Accordingly, *K. marxianus* M8.1, *D. hansenii* M6.1 and M6.3 were found to have ß-galactosidase activity with high acid phosphatase and moderate arylamydase activities.

CONCLUSIONS

Yeast microflora of spontaneously fermented herbs used for the production of Van herby cheese was identified and enzymatic characterization of the isolates was evaluated as a tool for technological characterization. It has been revealed that fermented herb brines included the species of *P. membranifaciens*, *K. servazzii*, *D. hansenii*, *K. marxianus*, and *P. fermentans*, as a result of partial large subunit sequencing. The isolates were found to have variable enzyme activities including, phospahatase, ß-galactosidase, lipases, esterase, arylamydases, which could be crucial during the ripening of Van herby cheese. Selected strains can be used in adjunct culture combines for herby cheese production, however, to this end, more studies are required in a proper herby cheese food matrix.

ACKNOWLEDGEMENTS

The authors would like to thank Prof. Dr. Hayri Coşkun and Prof. Dr. Yusuf Tunçtürk for supplying natural herbs from Van province of Turkey.

CONFLICT OF INTEREST

The author(s) declares no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHORS CONTRIBUTIONS

Erkan Güneş and İbrahim Çakır performed isolation and enzymatic assay of the isolates. Furkan Aydın made the molecular analyses and wrote the manuscript. All authors read and approved the final manuscript.

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