KSÜ Tarım ve Doğa Derg 22(Ek Sayı 1): 206-211, 2019
KSU J. Agric Nat 22(Ek Sayı 1): 206-211, 2019
DOI:10.18016/ksutarimdoga.vi.555727

Detection of Extracellular Lipases and Genotypic Identification from Yeast Causing Spoilage of Some Dairy Products Produced in Gaziantep

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ABSTRACT
Yeasts are usually found in high amounts in dairy products, which show their ability to adapt to substrates rich in protein, lipid, sugar and organic acids. A wide distribution of yeast in dairy products is a result of proteolytic and lipolytic activities. Spoilage yeasts and molds can grow in most processed and raw foods, where environmental conditions are not suitable for most bacteria (low pH, low water activity, aw). Nutrients and oxygen in food are the main factors that determine the type of fungal spoilage. In this study, dairy products samples (yoghurt, cream, butter, curd cheese, Antep cheese) were collected from local markets in Gaziantep province. In our study, a total of 20 yeast strains were isolated from dairy products and investigated for lipase activities in solid media containing tributyrin. Twenty yeast isolates identified by amplification and sequencing of the ITS region using ITS1 and ITS4 primers Yeasts were identified as *Kluyveromyces marxianus* (8 isolates), *Candida intermedia* (8 isolates), *Pichia fermentans* (2 isolates), *Yarrowia lipolytica* (1 isolate), *Kluyveromyces lactis* (1 isolate).

Research Article

Article History
Received : 18.04.2019
Accepted : 31.05.2019

Keywords
Spoilage yeast
Extracellular lipase
Genotypic identification

Gaziantep İline Ait Bazı Süt Ürünlerinde Bozulmaya Neden Olan Mayalardan Ekstraselüler Lipaz Enzimi Aranması ve Lipaz Aktivitesine Sahip Suşların Genotipik İdentifikasyonu

ÖZET
Mayalar genellikle süt ürünlerinde yüksek miktarda bulunurlar; bu da onların protein, lipid, şeker ve organik asitlerce zengin substratlara iyi bir şekilde adapte olabilme yeteneklerini gösterir. Bozulmaya neden olan mayalar ve küfler, çoğu bakteri için çevresel koşulların uygun olmadığı (düşük pH, düşük su aktivitesi, aw) işlenmiş ve çiğ gıdalarda gelişim gösterebilmektedir. Mayaların süt ürünlerinde geniş bir dağılıma sahip olmaları proteolitik ve lipolitik aktivitelerinin bir sonucudur. Besin maddeleri ve oksijen gıdalardaki fungal bozulma tipini belirleyen ana faktörlerdir. Bu çalışmada Gaziantep ilindeki yerel marketlerden çeşitli süt ürünleri örnekleri (yoğurt, krema, tereyağı, lor peyniri, Antep peyniri) alındı. Toplam 20 maya suşu izole edildi ve tributirinden oluşan katı ortamdaki lipaz aktiviteleri açısından araştırıldı. ITS1 ve ITS4 primerleri kullanılarak ITS bölgesinin amplifikasyonu ve sekanslanması ile mayalardan 8 izolat *Kluyveromyces marxianus*, 8 izolat *Candida intermedia*, 2 izolat *Pichia fermentans*, 1 izolat *Yarrowia lipolytica*, 1 izolat *Kluvyeromyces lactis* olarak tanımlanmıştır.

Araştırma Makalesi

Makale Tarihçesi
GelişTarihi : 18.04.2019
Kabul Tarihi : 31.05.2019

Anahtar Kelimeler
Bozulmaya neden olan mayalar
Ekstraselüler lipaz
Genotipik identifikasyon

To Cite : Tokak S, Kılıç Hİ, Yalçın HT, Duran T 2019. Detection of Extracellular Lipases and Genotypic Identification from Yeast Causing Spoilage of Some Dairy Products Produced in Gaziantep. KSU J. Agric Nat. 22 (Suppl 1): 206-211. DOI: 10.18016/ksutarimdoga.vi.555727.

INTRODUCTION
Yeast is one of the main reasons for the deterioration of low pH yogurt and fermented milk products, which provide a selective environment for their growth (Fleet, 1990; Rohm et al., 1992). Yeasty, fermented off-flavors and gassy appearance are usually detected

when yeasts grow up to 10^5–10^6 CFU/g. One of the indirect causes of the degradation of dairy products is microbial enzymes such as proteases, phospholipases and lipases, some of which remain active in food even after the enzyme-producing microbes have been destroyed. Thermally resistant lipases are associated with sour taste development in UHT milk. UHT milk, butter, some cheeses and milk powder products even affect by residual lipases (Fox et al., 1976). The release of short-chain fatty acids into dairy products results in sour taste and odor formation, while the release of long-chain fatty acids causes a soapy taste.

Lipases (EC 3.1.1.3) are hydrolase class enzymes that are primarily responsible for the hydrolysis of acylglycerols. Lipases are frequently encountered in nature and are responsible for the biological transformation of lipids (triacylglycerol). It is distinguished from esterases by its unique ability to function on aqueous and non-aqueous interfaces (Verger, 1997; Schmid and Verger, 1998). Lipases are produced by organisms ranging from microbes to animals and microbial lipases are produced by bacteria and fungi and secreted out of the cell. Yeasts area traditional source because of their relatively simple growth conditions (Prakash et al., 2013; Sanchez and Demain 2011; Singhania et al., 2010). Lipases have promising applications in many areas such as product processing in organic chemistry, detergent production, synthesis of biosurfactants, oleochemistry industry, dairy industry, agrochemical industry, paper production, food, cosmetics and pharmaceuticals. The development of lipase-based technologies for the synthesis of novel compounds is rapidly increasing the use of these enzymes (Liese et al., 2000). Given their biodiversity, their rapid growth rate and their susceptibility to genetic manipulation make microorganisms the most important source of enzymes (Demain and Adrio, 2008; Adrio and Demain, 2014). In this study, we aimed to isolate and identify the causing spoilage yeasts that have lipase activity in dairy products.

MATERIAL and METHODS

Sampling and Isolation

Dairy products (yoghurt, cream, butter, curd cheese, Antep cheese) used in this study were obtained from various markets and dairies in Gaziantep. The samples were transported to the laboratory under aseptic conditions and diluted by homogenizing in sterile peptoned water. Since the products we obtained in the study were fresh and unspoiled, all samples were kept at room temperature for 2-4 days in order to accelerate the deterioration process of all samples. Yeast isolation was performed on Yeast Malt (YM) agar composed of (g/L); 3 g yeast extract, 3 g malt extract, 5g peptone, 10 g glucose and 15 g agar. After 3 days incubation at 27 0C, yeasts showing different colony morphology such as color, shape and size were picked and purified by streaking at least three times on YM agar plates (Yalcin and Ucar, 2009; Corbaci et al., 2012). Lipase enzyme activities were tested on Potato Dextrose Agar (PDA, g/L: potato extract, 4; dextrose, 20; agar, 15) plates supplemented with tributyrin (1%). After 3 days of incubation at 27 ℃, the hydrolysis zone around the yeast colonies was examined and the largest zone was selected for further investigation.

Genomic DNA Isolation of the Yeast Strains

DNA extraction was performed according to the method described by Liu et al (2000). Overnight culture of pure isolates was then transferred to eppendorf growing in YPG broth and centrifuged at 7000 g for 5 minutes at 4 ° C to collect cells. 500 ml of lysis buffer was added to the pellet and the tube was vortexed until a homogeneous mixture was obtained. The mixture was vortexed after adding 150 ml of potassium acetate and incubated at 65 ℃ for 10 minutes. The mixture was centrifuged at 12000 g for 4 minutes at 4 ℃ and the supernatant transferred to sterile tube. The genomic DNA in the aqueous phase was precipitated with equal volume of isopropanol and then washed with 70% cold ethanol. The DNA was dried at room temperature, then dissolved in nuclease-free water and stored at -20 ℃ until use.

Amplification and Sequencing of ITS Region

PCR amplification reactions were performed under the following conditions as described in other sources by using the Thermal Cycler. PCR amplicons of the ITS1-5.8-ITS2 region were produced using ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers. Amplification 40 PCR cycles were performed in 2 minutes of denaturation at 95 ℃, annealing at 58 ℃ for 2 minutes, extension at 72 ℃ for 2 minutes and final extension at 10 minutes. The DNA sequence analysis reaction was performed according to the recommendations of the BigDye® Terminator V3.1 Cycle Sequencing DNA sequence assay kit. The DNA sequence analysis reaction was performed in both directions with both forward and reverse primer.

Phylogenetic Analysis of Molecularly Characterized Isolates

Sequence comparisons were performed using the Basic Local Aligment Search Tool (BLAST) in the NCBI GenBank database. As a result of the comparison, species and genus level identification was made and the sequence data of the identified strains were obtained by accesion numbers in NCBI-GenBank. Tamura-Nei neighbor joining method was used for the construction of phylogenetic tree by MEGA software version 7.0 (Figure 2) (Tamura and Nei, 1993; Kumar

et al., 2016). Confidence levels of the clades were estimated from bootstrap analysis (1,000 replicates) (Limtong et al., 2012).

Results

In our study, a total of 67 isolates which had different colony morphology (color, shape and size) were obtained for the isolation of yeast from dairy products. After isolation, screening was performed for the production of lipase in the Tributyrin agar medium (Figure 1). Of these isolates, 20 isolates (2 isolates from Antep cheese, 6 isolates from cream, 2 isolates from curd cheese, 8 isolates from butter and 2 isolates from yoghurt) were selected for identification. Sequence analysis of the ITS region was performed for the molecular level typing of isolates having lipase activity. After the sequence analysis, it was determined that the species obtained by BLASTN search were 4 genera compared to GenBank database. 8 of the species were *Candida intermedia* (1 species was from Antep cheese, 4 species were from cream, 1 species was from curd cheese and 2 species were from butter), 8 were *Kluyveromyces marxianus* (1 species was from cream, 5 species were from butter and 2 species weere from yoghurt), 2 were *Pichia fermentans* (1 species was from curd cheese and 1 species was from butter) 1 was *Yarrowia lipolytica* (from cream) and 1 was *Kluyveromyces lactis* (from Antep cheese) (Table 1).

Table 1. Molecular identification results of the lipolytic yeast isolates (A: Antep cheese, K: Cream, L: Curd cheese, T: Butter, Y: Yoghurt)

Isolate no	Species	GenBank acc. number	Homology (%)
A1	*Kluyveromyces lactis*	KP132318.1	99
A6	*Candida intermedia*	KF728822.1	99
K3	*Yarrowia lipolytica*	KF851353.1	91
K4	*Candida intermedia*	KF728807.1	99
K7	*Candida intermedia*	KF728822.1	99
K13	*Candida intermedia*	KF728822.1	99
K14	*Candida intermedia*	KF728807.1	100
K15	*Kluyveromyces marxianus*	KM921925.1	99
L3	*Candida intermedia*	KF728807.1	99
L14	*Pichia fermentans*	NR_130688.1	98
T1	*Kluyveromyces marxianus*	KY103813.1	98
T3	*Kluyveromyces marxianus*	KY103813.1	99
T6	*Kluyveromyces marxianus*	KM921933.1	99
T13	*Kluyveromyces marxianus*	KM921925.1	99
T14	*Candida intermedia*	KY495771.1	99
T15	*Kluyveromyces marxianus*	KX303813.1	97
T19	*Candida intermedia*	KY495771.1	99
T20	*Pichia fermentans*	KY104545.1	99
Y2	*Kluyveromyces marxianus*	KM921925.1	99
Y6	*Kluyveromyces marxianus*	KY103813.1	99

Figure 1. Detection of lipase activities of the yeast isolates on solid medium

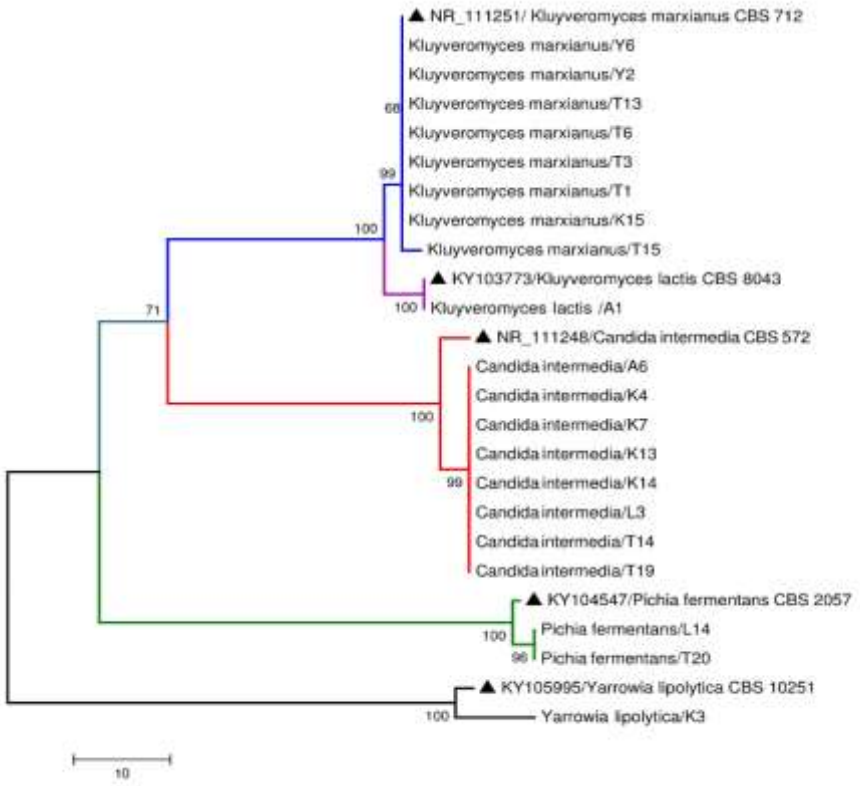

Figure 2. Phylogenetic tree representing the placement of the identified lipolytic yeasts.

DISCUSSION

Microbial enzymes, such as phospholipase and lipase, have indirect effects on the deterioration of dairy products, and these enzymes remain active even uneffected by enzyme-producing microorganisms (Shah, 1994; Sorhaug and Stepaniak, 1997). For example; The production of phospholipase in raw milk results in the formation of bitter taste due to the conversion of natural lipases of milk into fatty acids. Temperature resistant lipases are associated with sour taste development in UHT milk (Fox et al., 1976). UHT milk, butter, some cheeses and milk powder products such as residual lipases even can be affected. The release of short-chain fatty acids results in sour taste and odor formation, while the release of long-chain fatty acids produces stable taste.

Spoilage of cheese products, in addition to processing and maturation, it is caused by the development of thermodouric species which survive in temperature applications. Low pH in cheese accelerates the development of yeasts. The organisms that cause degradation in cheese include *Geotrichum candidum*, *Pichia spp.* and *Candida spp.* (Johnson et al., 1990). In our study, yeasts that cause deterioration of antep cheese and curd cheese were determined as *Candida intermedia*, *Kluyveromyces lactis* and *Pichia fermentans*. Dairy products such as yoghurt and sour cream are affected by yeast development. These products are usually protected by cooling and show acidic properties, these conditions accelerate the development of yeasts. *Candida*, *Pichia*, *Kluyveromyces*, *Rhodotorula*, *Debaryomyces* and *Torulopsis* have been associated with yoghurt spoilage (Suriyarachchi and Fleet, 1981; Kosse et al., 1997). *Kluyveromyces marxianus* from yoghurt is isolated in accordance with the studies, *Candida intermedia*, *Kluyveromyces marxianus* and *Yarrowia lipolytica* species were isolated from cream. Although butter initially contains organisms contained in raw milk, pasteurisation, salt addition, and preservatives are inhibited by microbial growth, resulting in contamination after production. The organisms associated with deterioration in butter samples are *Candida*, *Cryptococcus*, *Geotrichum*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Trichosporon* and *Yarrowia* (Lopandic et al., 2006; Sagdic et al., 2010). Yeasts isolated from butter in our study were *Candida intermedia*, *Kluyveromyces marxianus* and *Pichia fermentans*.

Lipolytic yeasts are found in many habitats contaminated with oil, including oil contaminated soil, oily vegetable waste, dairy products and degraded foods (Thakur, 2012). Several sources of lipase-producing yeasts have been compiled by several authors (Palekar et al., 2000; Vakhlu and Kour, 2006; Ciafardini et al., 2006; Potumarthi et al., 2008; Thakur, 2014). Some yeast sources *Candida antarctica*, *Candida rugosa*, *Candida tropicalis*, *Candida curvata*, *Candida cylindraceae*, *Candida deformans*, *Candida parapsilosis*, *Candida utilis*,

Candida valida, *Candida viswanathii*, *Galactomyces geotricum*, *Arxula adeninivorans*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, *Trichosporon fermantans*, *Trichosporon asahii*, *Rhodotorula mucilaginosa* and *Aureobasidium pullulans* (Ciafardini et al., 2006; Potumarthi et al., 2008; Lukaszewicz et al., 2013). In a study conducted in our country, two yeast species (*Pichia fermentans* and *Candida zeylanoides*) with lipolytic activity from butter were isolated (Corbaci et al., 2017). In our study, although the yeast species with lipase activity is consistent with other studies, the species of *Candida intermedia* isolated is reported for the first time in our country. In our study, 4 species with high lipase activity have been determined and *Candida intermedia* is important for the first time in Turkey.

CONCLUSION

As a result, the data showed that although microbial diversity in dairy products is still not discovered, there are only a few genera and species that make up the microbiota. These species are important in terms of contributing to the degradation of the product and the production of heat-resistant enzymes and these species should also be considered. The deterioration potential of certain species or species depends on the combination of species occurrence, individual cell count, density of enzyme activity, and heat stability of secreted enzymes. No doubt, in the light of this information, further research is required to prevent microbial spoilage in dairy products.

ACKNOWLEDGEMENTS

This work was supported by the Scientific Research Unit of Gaziantep University (FEF. 15.03). We would like to sincerely thank the Scientific Research Unit of Gaziantep University for providing funding and necessary logistics for this research work.

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