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Molecular Characterization of the Yeast Isolates Originating from Turkish Autochthonal Product, Brined Grapeleaves

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***Abstract*— Yeast isolates collected from brined grapevine were identified using molecular methods. A total number of 54 isolates were collected. The isolates were grouped into 12 spieces depending upon their phenotypic disparities. RAPD and sequence analyses were used as molecular step. Due to high concentrations of salt and low pH in this product, brined grape leaves did not showed expected fermentation model, salt resistance yeasts were identified. As a result, *Pichia subpelliculosa, Debaryomyces hansenii, Zygosaccharomyces bisporus, Pichia membranifaciens, Hanseniaspora uvarum* were found predominant species.**

***Keywords*— M13, grapevine, yeast, 26s rRNA,**

I. INTRODUCTION

Turkish cuisine has been focus of attention for many people. One of the most famous item of the cuisine is 'brined grapevine' used for preparing 'dolma'. This product is also consumed at Balcan, Middle East and Caucasian countries. Despite consumed by a high number of population, research about the microflora of traditional brined grape leaves are limited. Viticulture is mainly carried out for grape, winemaking and dried grapes. Grapevine (*V. vinifera*) is edible parts of vine leaves which is commonly conserved by brining. Even though consuming of grapevine is not common worldwide, Codex Alimentarius classified vine lives in leafy vegetables [7]. This product is mentioned as fermented vegetable at various articles [3][5][11]. To evaluate this data we identified the microflora of brined grape leaves at our previous research. Hovewer, microflora of brined grape leaves consists of yeast species [8]. This data showed that the product does not have expected fermentation model. Fermented foods may contain yeasts however, lactic acid bacteria are must also be isolated to be classified as fermented. Yeasts are eukaryotic, single celled, aerobic or facultative anaerobic microorganisms which are commonly isolated from sugar-rich environments like fruits, juices, and also other foods. Despite some yeast species such as *Saccharomyces* and *Brettanomyces* are directly related with fermentation, yeasts are commonly known as spoiling microflora because, they can metabolize organic acids and other carbon sources which may be main ingredient of the food. In this research, characterization of the yeasts isolated from brined grapevine was performed.

2. MATERIALS AND METHODS

2.1 ISOLATION OF YEASTS

Potato dextrose Agar (PDA) (Merck) and Yeast Extract Glucose Chloramphenicol Agar (YGC) (Merck) were used for selective isolation of yeasts. By paying attention both microscopic and phenotypic disparities, different colonies was collected and stored in LB Broth (Merck) and 80% Glicerol at -80°C.

2.2 DNA EXTRACTION FOR PCR

Isolation and purification of DNA were performed according to Looke *et al* [15]. Young cell volume 100-200 (OD_{600}=0,4) was measured out and suspended into 1ml distilled water. This suspension was vortexed 3 minutes at 20000g and supernatant was poured out. 100 µl 200mM LiOAc+%1 SDS solution was added on pellet. After vortexing, the mix was incubated 5 minutes at 70^0C. 300µl 96% ethanol was added to suspension. After vortexing, samples were centrifugated 3 minutes 15000g at room temprature. Mixture was washed in 300 µl 70% ice cold ethanol and it was followed by centrifugating mixture 15 seconds at 15000g. Finally ethanol removed and mixture was conserved at -20°C in 50 µl TE solution. 1 µl supernatant was used for PCR.

2.3 RAPD ANALYSIS

RAPD fingerprints was obtained from the yeasts genomic DNA's, using bacteriophage M13 primer. Thus, both costs reduced and also strain diversity determined. Amplification reaction was performed in a total 50 µl mix containing 25 µl PCR master mix (Qiagen), 18 µl water, 4 µl template DNA, 3 µl M13 primer (5'-GAGGAGGGTGGCGGTTCT-3'). Amplification procedure was held as mentioned above: 94˚C 4' first denaturation, 94°C denaturation 30s, annealing at 36°C 45s, 45s elongation at 72°C. After PCR cycle RAPD-PCR was separated by electrophoresis on 1.2% (w/v) agarose gel in SB buffer with DNA size marker (Qiagen) using horizontal electrophoresis system (Biolab). Gels were stained with ethidium bromide, visualized under UV light and digitalized using (Uvitech) [24].

2.4 ITS-PCR AND GEL ELECTROPHORESIS

The fingerprints obtained from the isolates were grouped into their disparities. One sample was chosen for all groups and

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26S rRNA D1/D2 section (600bp) of all chosen represantives were amplified. PCR mix contained 15 µl master mix (Qiagen),

0,5 µl NL-1 5'-GCATATCAATAAGCGGAGGAAAAG-3'), 0,5L NL-4 (5'-GGTCCGTGTTTCAAGACGG-3'), 3 µl template DNA, 11 µl water. PCR program was performed as followed: 95°C 15 minutes first denaturation, 35 cycles of 96°C 30 seconds denaturation, 50°C 15s annealing, 68°C 2 minutes elongation and finally 72°C 10 minutes elongation. PCR products were separated on %1 agarose gel [23,12]. After separation amplicons were sequenced followed by purification according to Lõoke *et. al* 2012 [14]. Sequencing process was executed by service procurement. Outcomes evaluated by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) program [10].

3. RESULTS AND DISCUSSION

Yeasts can be found in soil and grapes as natural flora. It is known that grape microflora mainly consists of yeast species and various lactic acid bacteria such as *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus*. Although this microflora is thought to be able to transferred to the vine leaves, it cannot develop at low salt content in the leaves and high salt rates such as 9-12% when pickled.

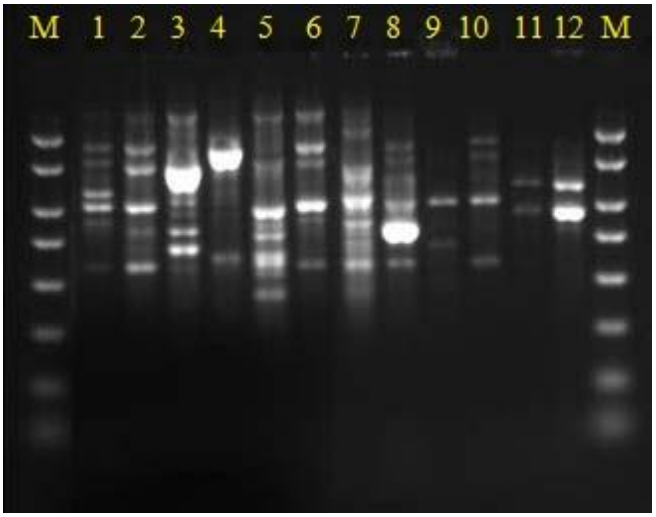
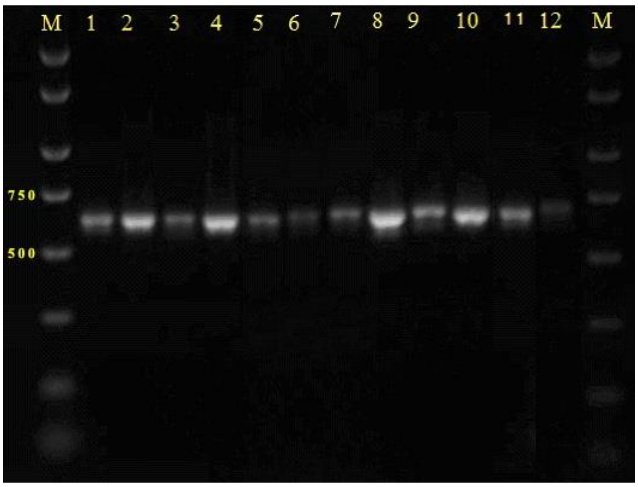


Figure 1. M: Marker, 1: *Pichia subpelliculosa (8), 2: Pichia subpelliculosa (13), 3: Debaryomyces hansenii (15), 4: Zygosaccharomyces bisporus 17), 5: Pichia membranifaciens (19), 6: Pichia subpelliculosa (22), 7: Pichia membranifaciens (33), 8: Pichia subpelliculosa (34), 9: Debaryomyces hansenii (57), 10: Pichia subpelliculosa (71), 11: Hanseniaspora uvarum (77), 12: Hanseniaspora uvarum (78)*

By paying attention both microscopic and phenotypic disparities, different colonies was collected. A total number of 54 isolates were obtained from the samples. When fingerprints of the samples examined, these 54 isolates were grouped into 12 candidate species. RAPD profiles are shown at Figure 1. *Pichia* was found as the predominant species. Prior to sequence analyze 26S rRNA D1/D2 sections of these 12 samples were amplified and separated on gel electrophoresis in order to ensure sequencing expected 600kb DNA fragments.



A. Figure 2. 26S rRNA D1/D2 sections of the samples

Food spoilage yeasts are commonly resistant to osmotic stress caused by salt, sugar and acidity. For instance the yeast *Debaryomyces hansenii* which was isolated from saline environments such as sea water, concentrated brines, salty food, is one of the most halotolerant species. It can grow in media containing as high as 4 M NaCl, which is almost 25% salt concentration [21]. Yeast population in food varies depending on the production method. The yeast flora of pickles with a high salt concentration of 15% and above and other vegetable brines with a salt concentration of 5% and below differs. In traditional production method salt concentrations vary a wide range due to non-standart production. The salt concentration of brined grapevine samples in this study reached up to %24 salt concentrations.

Stage	Prevalent microorganisms
1-Initation	Various Gram (+) and Gram (-) bacteria
2-Primary fermantation	Lactic acid bacteria and yeasts
3-Secondary fermantation	Yeasts
4-Post-fermantation	Aerobic: Surface growth of oxidative yeasts, molds and bacteria, Anerobic: None

TABLE 1. Sequence of microbial types during natural fermentation of brined vegetables [2]

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As can be seen from the Table 1, fermentation in vegetables is carried out by various lactic acid bacteria (LAB) and yeast species. LAB's involved in food fermentation are; *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Weisella* (Wessels 2004). Important yeasts are *Saccharomyces* spp., *Kluyveromyces* spp. fermentative yeasts such as and *Hansenula* spp., *Pichia* spp., *Candida* spp. and oxidative yeasts such as *Debaryomyces hansenii*. In addition to these microorganisms, mesophile aerobic bacteria, coliform bacteria and different yeasts can also contribute vegetable fermentation [12] Two main yeast groups play a role in fermentation. The first of these are yeasts that make alcoholic fermentation in brine and form the desired aromatic by-products as long as they are fermentable sugars. When the sugars are consumed, the yeast that oxidizes the acids produced by the bacteria and develops on the surface during post fermentation is the second group.

	Molecular identification result	Rate of genetic similarity	Reference strain no	Number of represented isolates
1	*Pichia subpelliculosa*	%99	EF550340.1	4
2	*Pichia subpelliculosa*	%99	EF550340.1	7
3	*Debaryomyces hansenii*	%99	HM988696.1	2
4	*Zygosaccharomyces bisporus*	%95	AB375304.1	2
5	*Pichia membranifaciens*	%99	EU019218.1	3
6	*Pichia subpelliculosa*	%98	JQ419775.1	19
7	*Pichia membranifaciens*	% 99	EU019218.1	3
8	*Pichia subpelliculosa*	%99	JQ419771.1	3
9	*Debaryomyces hansenii*	%95	EU285526.1	2
10	*Pichia subpelliculosa*	%99	JQ419771.1	2
11	*Hanseniaspora uvarum*	%99	JQ678687.1	2
12	*Hanseniaspora uvarum*	%99	EU004081.1	5

TABLE 2. Molecular identification results of the isolates

Spoilage in fermented vegetables is often attributed to yeasts [23] Degradation can be in the form of softening in the plant structure after enzymatic destruction of pectins or gas fermentation and bloating. Although many yeasts (eg *S. cerevisiae*, *Lachancea kluyveri*, and *P. anomala*) have enzymes such as pectinesterase and polygalacturonase, which have a destructive effect on plant tissues, most do not have all pectinolytic enzymes. However, when oxidative yeasts increase in numbers on the surface, they facilitate the degradation by decomposition of lactic acid and make the product more available for other microorganisms [6].

The five species isolated from product, selected as representative of the the samples with microsatelite markers are, respectively; *Pichia subpelliculosa, Debaryomyces hansenii, Zygosaccharomyces bisporus, Pichia membranifaciens, Hanseniaspora uvarum.* Species mentioned above could withstand high salt concentration. In addition to it, the results relate with the species isolated from fermented vegetables (Harris 1998). As a result of the experiment, it was determined that the dominant genus in the product was *Pichia* (75%) and the dominant species was *Pichia subpelliculosa* (64.81%) (Table2).

Katsaboxakis [14] mentioned the presence of yeast in vine leaves produced by different methods. However, there is no information about yeast species. They attributed absence of lactic acid bacteria to the low pH value of the leaves. Although there is no precise data on the microflora of the product, it is observed that the genetic diagnosis results overlap with the yeast species that can be found in similar products when compared with other fermented foods of fruit and vegetable origin [9], [4]). *Pichia* is the second largest yeast genus known for over 100 species. *Pichia subpelliculosa*, an amylolytic yeast, was the most dominant type of the research also known by the name *Hansenula subpelliculosa* [22]) and its fermentation characteristic is very similar to *Saccharomyces cerevisiae* [17]. It can be isolated with *Debaryomyces hansenii* in Cachaça production, one of the local beverages of Brazil. It is thought that *P.subpelliculosa*, which develops on the surface of soy products and breaks down lactic acid, may also have an oxidative effect on pickled leaves. Because it is one of the main species isolated in cucumber pickles with a high salt concentration [4].

Debaryomyces hansenii is a type of spoiling yeast that can often be seen in foods such as cheese, meat, wine, beer, fruit, and also in foods with high salt concentrations such as pickles [4] *D. hansenii* is common in nature because of salt tolerance, proteolytic and lipolytic enzyme capacity, metabolism of milk, fat and proteins, ability to grow low temperature and water activity [27]. Because *D. hansenii* is commonly found in salty products because of its ability to develop in 24% NaCl and low water activity such as 0.65 [19]. *D. hansenii* was isolated in 7.4% of leaf samples with salt concentrations up to 25%.

Hanseniaspora uvarum can be isolated from apple, grape, lemon cherry and plums which is one of main deterioration factors in wines. *H. uvarum* can also be isolated from pickled, cocoa, olives and salads. It can develop at a maximum of 0.94 aw (salt). This yeast type can be isolated from 1.34% of all foods [4]

Pichia membranifaciens is also known as *Candida valida* in telemorph, which constitutes 11.1% of the samples in the experiment, can develop in low pH foods because of can 1% acetic acid resistance. Various studies on biocontrol about this yeast exists, which can be isolated from 4,32% of all foods.

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Zygosaccharomyces has an osmophilic character and is a yeast type that has been reported to be isolated especially from foods with a high sugar concentration. On the other hand, isolation from foods with high salt concentrations is also been reported. [18]. investigated high salt tolerant yeast flora in a pickle-like local product made from umami fruit,. In the study, 4 different RAPD fingerprints of the isolates were observed and *Zygosaccharomyces bisporus* and *Pichia subpeliculosa* species identified after the sequence of the 26S rRNA D1 / D2 region.

4. CONCLUSION

Molecular techniques have become ordinary and common in microbiology and identification processes. RAPD-PCR reduces the analysis costs because species which showed same fingerprint does not need to be sequenced. While foodborne yeasts are not considered as pathogens, unlike fermented milks like kefir, common spoilaging features of them are reducing the nutritional value of foods and changing its characteristics. Hence, identification of yeasts in foods became requirement in order to struggle with them. Identification of the yeasts will help to perceive the spoilage mechanism and extend the shelf life.

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