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# Molecular Identification of Yeast Strains with High Fermentation Capacity Isolated from Kırkağaç Melon Varieties

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## Abstract

The yield of fermentation process, bioethanol, is one of the important product obtained from renewable energy sources. Yeasts are commonly used in ethanol production due to their high ethanol tolerance, high ethanol productivity, and their ability to ferment different kinds of simple sugars. *Saccharomyces cerevisiae* yeast species is the most commonly used in industrial ethanol production. Therefore, isolation and identification of new yeast strains with high fermentation capacity is very important for the bioethanol industry. The aim of this study was isolation of novel and industrially applicable yeast species that can be used in bioethanol production. Yeast strains were isolated from the Dalaman, Altınbaş and Kıvırcık sarı dilim melon varieties that are unique to Kırkağaç district of Manisa province. The fermentation ability of yeast strains was evaluated and the selected yeast strains were identified by PCR-RFLP analysis of 26S and ITS1-5.8S-ITS2 rDNA gene regions. Sequence analysis of the 26S rDNA-D1/D2 gene region was used for molecular identification of yeast strains. The phylogenetic analysis of identified yeast strains was carried out using the MEGA XI phylogenetic analysis tool. It was determined that 14 of the 53 yeast strains isolated were able to ferment glucose and sucrose, and four of them were able to ferment lactose. The yeast strains having the fermentation capacity were identified as *Pichia kluyverii*, *Lachancea thermotolerans*, *Pichia kudriavzevii*, *Saccharomyces cf. cerevisiae/paradoxus* and *Saccharomycescerevisiae*. These yeast strains have the potential to be used in the bioethanol industry after other physiological and biochemical tests are complete

**Keywords:** Fermentation, Melon, PCR-RFLP, Ribosomal RNA

## 1. Introduction

Yeasts are unicellular eukaryotic microorganisms with a wide range of distribution in nature. Identification of yeast species with high fermentation ability from different habitats is very important for the fermentation industry and especially for bioethanol production. Yeasts can be isolated from a variety of natural habitats as well as from different plant parts such as leaves, flowers and fruits [1, 2, 3, 4]. Since yeasts like sugary environments, especially fruits with high sugar content are preferred habitats for yeasts. Some yeast species show general distribution on fruits, while the others have a specific distribution on certain fruits. Melon (*Cucumis melo* L.) plant is belonging to the *Cucurbitaceae* family. The origin of the melon is thought to be Africa. [5, 6]. The members of *Cucurbitaceae* family are used in medicine and pharmaceutical industry due to their anti-tumor effects glycosides. Similarly, the fruits of

some species in this family (such as *Citrullus colocynthis* and *Cucumis melo*) have been reported to have antimicrobial activity [7, 8]. The nutritional value of the melon is quite high and it contains different sugars in varying proportions depending on the melon variety. For this reason, melon fruit is a very suitable habitat preferred by all microorganisms, including yeasts. *Fusarium equiseti*, *Fusarium xysporum*, *Fusarium proliferatum*, *Fusarium solani*, *Macrophomina phaseoli*, *Rhizoctoniasolani*, *Alternaria* sp., *Aspergillus* sp., *Pythium* sp. and *Rhizopus* sp. were isolated and identified from melon samples [9, 10, 11]. Some of these fungal pathogens cause drying in melons [9, 10, 11]. *Acidovorax citrulli* is a Gram-negative bacterium causes bacterial fruit spot disease in melons [12, 13, 14]. *Rhodotorula aurantiaca*, *Pichia anomala* and *Rhodotorula glutinis* yeast species isolated from melon fruit were determined to be effective on *A. citrulli* [15]. It has been reported that spray drugs containing various combinations of these yeast species may be suitable for biological control instead of chemical drugs [15].

*S. cerevisiae* have been used in alcohol production especially in the brewery and wine industries as well as obtaining biofuels from renewable energy sources [16]. In addition, some other non-*saccharomyces* yeast species such as *Pichia stipites* and *Kluyveromyces fagilis*, were reported as good ethanol producers from different types of sugars [17, 18, 19]. The thermotolerant yeast *K. marxianus* can ferment both hexose and pentose sugars [20]. Yeast strains that have been used in bioethanol production were summarized in a review [21].*Pichia kudriavzevii* yeast strains isolated from apple, watermelon, melon, papaya and pineapple fruits, can be used for bioethanol production at a higher rate than the reference *S. cerevisiae* yeast strain [22]. In recent years, since the production of bioethanol especially from fruit and vegetable wastes has been emphasized, the Isolation and identification of new yeast strains with high fermentation capacity from different fruits and vegetables has gained importance. Therefore, the aim of this study is to isolate and identify new yeast strains that have the potential to be used in bioethanol production.

## 2. Materials and Methods

### 2.1. Yeast strains

Kırkağaç melon samples (Dalaman, Altınbaş and Kıvırcık sarı dilim) were collected aseptically from Kırkağaç district of Manisa -Turkey (39°05'55.7" North Latitude, 27°41'59.6" East longitude) in July and August in 2020. Melons samples were weighed and homogenized in 2% sodium citrate solution, were spread onto YGC-Agar medium (40 gr/L Yeast Extract Glucose Chloramphenicol Agar) including 0.1% sodium propionate. Plates were incubated at 30°C for 2-3 days, the growing yeast colonies were counted to determine the colony forming units (CFU/gr). Yeast strains, having different colony morphology, were selected randomly and transferred to YPD medium (10 gr/L Yeast Extract, 20 gr/L Bacto-peptone, 20 gr/L Agar, 2% Dextrose). After incubation of plates at 30°C for 2-3 days, the isolated yeast strains were stored at -80°C for further use.

### 2.2. Fermentation capacity

The carbohydrate utilization test was performed using a YP medium supplemented with 1.6% bromothymol blue as pH indicator and fermentable carbon sources (2% dextrose, 2% sucrose, 2%, lactose and 2% starch). The Durham tubes were also placed into the media to detect the gas production [23]. The yeast strains were inoculated to cultures and incubated at 30 °C up to 30 days. The color

change in the growth medium from green to yellow and the $CO_2$ in Durham tubes were accepted as a fermentation positive. All tests were assayed in at least triplicate.

### 2.3. Genomic DNA isolation and PCR amplification

Genomic DNA extraction of yeast strains was carried out by a previously developed DNA extraction procedure [24]. D1/D2 rDNA gene region of yeast strains were amplified by NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') primers [25]. ITS1-5.8S-ITS2 rDNA gene regions of yeast strains were amplified with ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers [26]. PCR products were electrophoresed in 1.5% agarose gel and the length of PCR amplicons was calculated by Gel-Pro Analyzer v4.0 software.

### 2.4. Restriction profiles and DNA sequencing

PCR products of ITS1-5.8S-ITS2 and D1/D2 rDNA gene regions were purified using GeneJet PCR Purification Kit (Thermo Scientific-K0702) and then digested with HinfI, HaeIII and HhaI restriction enzymes according to the supplier's instructions. The restriction fragments were electrophoresed in 3% agarose gel. The length of restriction fragments was calculated by using Gel-Pro Analyzer v4.0 software. The yeast strains were grouped according to restriction profiles. Yeast strains having different restriction profiles were selected randomly and PCR products D1/D2 rDNA were sequenced with the Applied Biotechnologies 3500xl Genetic Analyzer. The obtained D1/D2 rDNA sequences were analyzed using Basic Local Alignment Search Tool (BLAST) on National Center for Biotechnology Information (NCBI) [27]. D1/D2 rDNA gene sequences of the selected yeast strains were submitted to the GenBank database to get the accession numbers.

## 3. Result and Discussion

The melon samples were collected from Kırkağaç, Manisa-Turkey. Depending on the colony morphology differences 26, 13 and 14 yeast strains were isolated from Dalaman (MD), Altınbaş (MA) and Kıvırcık sarı dilim (MK) melon varieties, respectively. The fungal load of melon samples was determined as $3.6X10^4$ CFU/g for Dalaman, $1.5X10^5$ CFU/g for Altınbaş melon and $1.3X10^4$ CFU/g for Kıvırcık sarı dilim melon. The isolated yeast strains were classified into 6 groups in Altınbaş and Dalaman melons and 4 groups in Kıvırcık sarı dilim melon, according to their colony morphologies (Table 1).

The fermentation capacity of the yeast strains was analyzed in dextrose, sucrose, lactose and starch carbon sources. It was observed that all isolated yeast strains were not capable of fermenting starch. Fermentation tests of 14 yeast strains (MD1, MD2 MD8, MD9, MD11, MD13, MD23, MD26, MA3, MA4, MA8, MA9, MA12 and MK12) showed positive results on dextrose and sucrose, while only four yeast strains (MD2, MD26, MA3 and MA4) were determined to have the capacity to ferment lactose. Therefore, genomic DNA of these yeast strains were isolated and used for molecular identification.

The amplification of D1/D2 rDNA gene region of yeast strains resulted in one PCR group with the length of ~600-650bp. The amplification of ITS1-5.8S-ITS2 rDNA gene region of yeast strains resulted in four PCR groups with the length of ~450bp (MD1, MD8, MD11, MD13, MD23, MA827, MA9, MA12 and MK12 yeast strains) ~500bp (MD9 yeast strain), ~700bp (MD26 and MA4 yeast strains) and

~850bp (MD2 and MA3 yeast strains). When the yeast strains present in PCR groups and the colony morphology groups were compared, the yeast strains MD9 and MD23 having different colony morphologies localized within the same PCR group. Similarly, although MA8, MA9 and MA12 yeast strains showed the same PCR length of the ITS1-5.8S-ITS2 rDNA gene, it was observed that the MA12 yeast strain had different colony morphology from the other two yeast strains.

**Table 1.** Grouping of Yeast Strains According to Colony Morphologies

| **Melon Variety** | **Yeast strains** |
|---|---|
| **Dalaman** | MD-1, MD-4, MD-6, MD-7, MD-8, MD-9, MD-11, MD-13, MD-19, MD-22, MD-23 |
| | MD-2, MD-12, MD-20 |
| | MD-3, MD-14, MD-15, MD-21, MD-25 |
| | MD-24 |
| | MD-5, MD-17, MD-18, MD-26 |
| | MD-10, MD-16 |
| **Altınbaş** | MA-12 |
| | MA-2, MA-3, MA-4 |
| | MA-1, MA-5, MA-6, MA-7, MA-13 |
| | MA-10 |
| | MA-8, MA-9 |
| | MA-11 |
| **Kıvırcık sarı dilim** | MK-1, MK-4, MK-5, MK-8, MK-12 |
| | MK-3, MK-6, MK-9, MK-14 |
| | MK-2, MK-7, MK-13 |
| | MK-10, MK-11 |

Restriction profiles of ribosomal DNA gene regions have been used for interspecies and intraspecies identification of yeast species isolated from different environments [1, 29, 30, 31, 32, 33]. The amplification results of 26S and ITS1-5.8S-ITS2 rDNA regions were digested with HinfI, HaeIII and HhaI restriction enzymes and the results were given in Table 2 and Table 3, respectively. The yeast strains were grouped into four groups according to their 26S rDNA PCR restriction profiles. 26S rDNA gene region of four yeast strains (MD2, MD26, MA3 and MA4) were not digested with Hha I restriction enzyme. It was observed that the yeast strains in the first ITS1-5.8S-ITS2 rDNA PCR group were in the same group according to their restriction profiles. However, the yeast strains in the second and third ITS1-5.8S-ITS2 rDNA PCR groups were showed different restriction profiles. It was determined that the nine yeast strains had the same restriction profile, while the other five yeast strains had different restriction patterns, and so they were localized in different profile groups. In general, it is thought that each different profile obtained as a result of PCR-RFLP analysis indicates a different yeast strain [34].

Therefore, it can be considered that 14 yeast strains having the fermentation ability in glucose, sucrose or lactose may belong to 6 different yeast species.

The 26S rDNA amplification products of all yeast strains were sequenced and analyzed with the BLAST tool on the NCBI web server. The nucleotide sequences of the 26S rDNA gene region were submitted to the GenBank Database on NCBI and attained accession numbers (Table 4). According to the BLAST analysis of 26S rDNA gene region, all yeast strains displayed 95-100% similarity with their reference yeast strains. It was determined that all yeast strains in the first 26S group (9 yeast strains) were identified as of *Pichia kluyverii*. MD2 and MA3 yeast strains present in the second group were identified as *Saccharomyces cerevisiae* and *Saccharomyces cf. cerevisiae/paradoxus*, respectively. The other two yeast strains present in the third group (MD26 and MA4) were identified as *Kluyveromyces marxianus* and *Lachancea thermotolerans*, respectively. MD9 yeast strains showed 98-99% sequence similarity with *P. kudriavzevii* reference yeast strain (KY108856.1).

**Table 2.** PCR-RFLP Results of 26S rDNA Gene Region

| **Yeast strains** | **HinfI** | **HaeIII** | **HhaI** |
|---|---|---|---|
| MD-1, MD-8, MD-11, MD-13, MD-23, MA-8, MA-9, MA-12, MK-12 | 220-210-175 | 400-120-100 | 450-175 |
| MD-9 | 300-300 | 290-200-125 | 250-280-150 |
| MD-2, MA-3 | 215-215-200 | 300-190-150 | - |
| MD-26, MA-4 | 450-150 | 475-150 | - |

**Table 3.** PCR-RFLP Results of ITS1-5.8S-ITS2 rDNA Gene Region

| **Yeast strains** | **HinfI** | **HaeIII** | **HhaI** |
|---|---|---|---|
| MD-1, MD-8, MD-11, MD-13, MD-23, MA-8, MA-9, MA-12, MK-12 | 245-210 | 375-100 | 450-175-120 |
| MD-2 | 385-380-120 | 325-245-170-155 | 380-380-125 |
| MD-26 | 275-200-125-80 | 650-100 | 320-200-190 |
| MD-9 | 225-180-80 | 400-150 | 225-210-80 |
| MA-3 | 350-350-125-80 | 340-260-190-90 | 350-350-150 |
| MA-4 | 350-340 | 325-240-100 | 340-340 |

The phylogenetic analyses were conducted in MEGA XI using 26S rDNA gene sequences of yeast strains [28].*Schizosaccharomyces pombe* yeast species was selected as an outgroup. The evolutionary history was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [35]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The

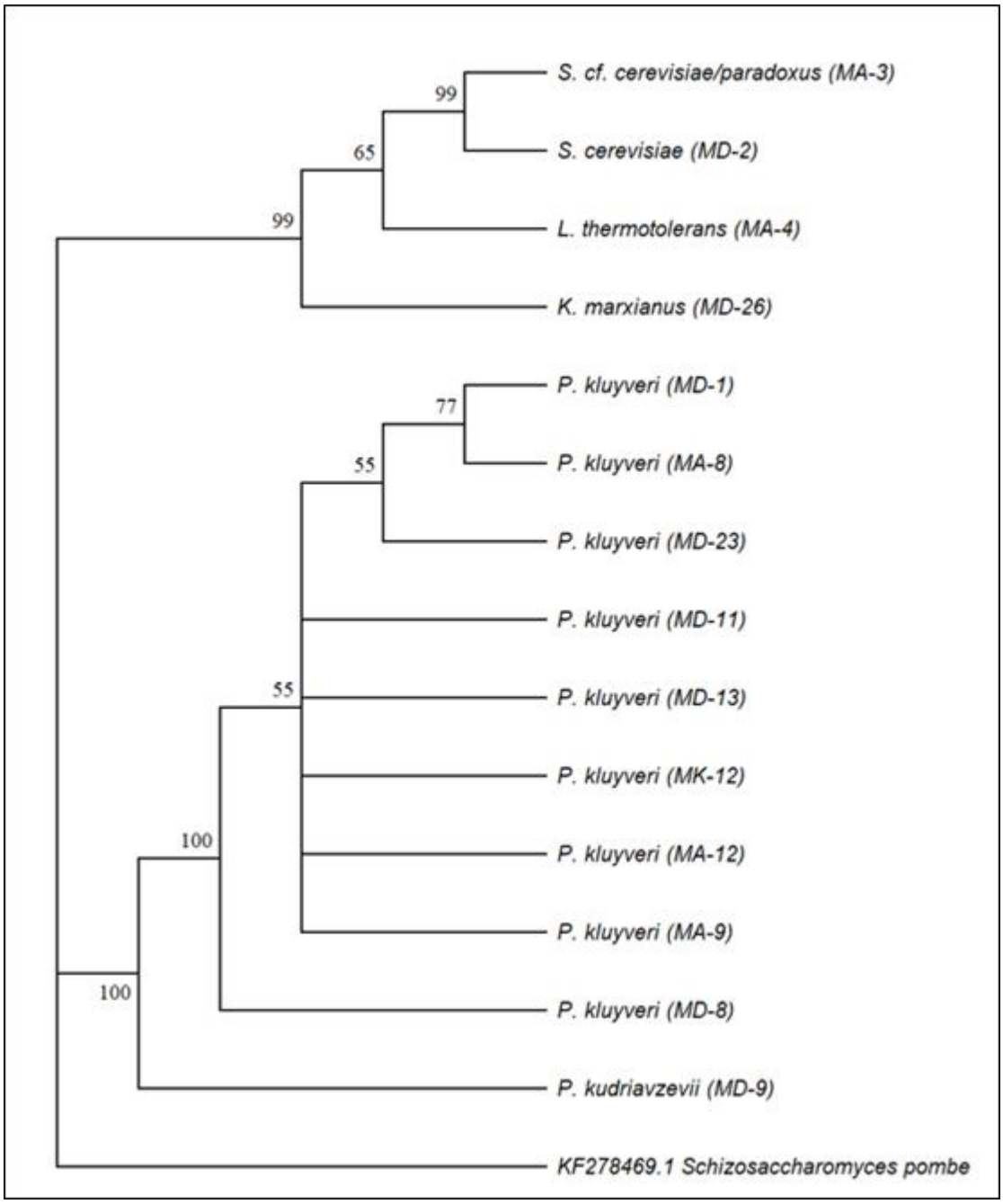
percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [35]. The Maximum parsimony tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates) [36]. This analysis involved 15 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. There were a total of 632 positions in the final dataset. According to the result of the maximum parsimony tree of 26S region, 14 yeast species were divided into two main clades (Figure 1). The first clade including 2 yeast species was separated into 2 sub-clades: *P. kudriavzevii* and *P. kluyverii*. The second clade consists of four yeast species and is divided into 2 sub-clades. The first sub-clade contains *S. cf.cerevisiae/paradoxus*, *S. cerevisiae* and *L. thermotolerans* yeast species, and the second sub-clade includes *K. marxianus* yeast species.

**Table 4.**Blast Analysis of 26S rDNA Gene Region

| Yeast strains | Identified Yeast Strains (Ref. Acc. Number) | GenBank Accession Number |
|---|---|---|
| **MD-1** | *P. kluyveri* (KY108826.1) | MW836759 |
| **MD-8** | *P. kluyveri* (KY108826.1) | MW836762 |
| **MD-11** | *P. kluyveri* (KY108826.1) | MW836771 |
| **MD-13** | *P. kluyveri* (KY108826.1) | MW836772 |
| **MD-23** | *P. kluyveri* (KY108826.1) | MW836775 |
| **MA-8** | *P. kluyveri* (KY108826.1) | MW836765 |
| **MA-9** | *P. kluyveri* (KY108826.1) | MW836766 |
| **MA-12** | *P. kluyveri* (KY108826.1) | MW836776 |
| **MK-12** | *P. kluyveri* (KY108826.1) | MW836769 |
| **MD-9** | *P. kudriavzevii* (KY108856.1) | MW836763 |
| **MD-2** | *S. cerevisiae* (KY109314.1) | MW836760 |
| **MA-3** | *S. cf. cerevisiae/paradoxus* (KY109345.1) | MW836757 |
| **MD-26** | *K. marxianus* (KY108106.1) | MW836777 |
| **MA-4** | *L. thermotolerans* (XR 00243225.1) | MW836758 |

The non-*Saccharomyces* yeast strains are generally used for various fields, such as biomedical studies, biocontrol agents, bioremediation and fermentation process [37, 38]. Many different yeast species having high ethanol tolerance and fermentation capacity have been isolated and identified from different sources. In recent years, the isolation and identification of new yeast strains that can be used in the bioethanol industry has gained an importance. The isolated strains of *P. kluyverii*, *L. thermotolerans* and *P. kudriavzevii* yeast species are very important in this respect. These yeast strains have the potential to be used in the bioethanol industry as well as other commercial applications in the future.

**Figure 1.**Maximum Parsimony Tree of Yeast Strains Isolated from Kırkağaç Melon Varieties

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