

Internal transcribed spacer (ITS) sequence-based identification of yeast biota on pomegranate surface and determination of extracellular enzyme profile

TÜLAY TURGUT GENÇ^{1*}, MELİH GÜNAY²

¹Department of Biology, Faculty of Arts and Science, Çanakkale Onsekiz Mart University. Terzioğlu Campus, 17100, Çanakkale, Turkey.
Tel./fax.: +90-286-2180018-ext.1795, *email: tturgutgenc@comu.edu.tr

²Department of Biology, Graduate School of Natural and Applied Sciences, Çanakkale Onsekiz Mart University. Terzioğlu Campus, 17100, Çanakkale, Turkey. Tel. +90-286-2180018, email: melihgunay@comu.edu.tr

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Abstract. *Genç TT, Günay M. 2020. Internal transcribed spacer (ITS) sequence-based identification of yeast biota on pomegranate surface and determination of extracellular enzyme profile. Nusantara Bioscience 12: 59-67.* Yeasts are the most significant organisms to produce fermented products from different types of fruits such as grape, strawberry and pomegranate. The native yeasts on these fruits contribute to beverages' quality and aroma during fermentation. Pomegranate is used in fruit juice and wine production because of high antioxidant characteristic. In order to determine yeast microbiota on the pomegranate fruits collected from Gallipoli (Gelibolu), Çanakkale-Turkey, ITS-5.8S rDNA gene region have been utilized. Also, phylogenetic relationships among identified yeast species were assigned by using sequences of ITS-5.8S rDNA gene region. In addition, extracellular enzyme activity of identified yeast strains was detected by using API-ZYM. *Kluyveromyces lactis*, *Aureobasidium pullulans*, *Hanseniaspora uvarum*, *Candida zeylanoides*, *Kwoniella* sp., and *Metschnikowia pulcherrima* and *Metschnikowia ziziphicola* yeast species were identified on pomegranate surface. Phylogenetic analysis, carried out in ITS-5.8S rDNA gene region of identified yeast strains, revealed the presence of five clades. *Kwoniella* sp., *H. uvarum*, *M. pulcherrima*, and *Kl. lactis* yeast strains revealed high leucine arylamidase activity. Also valine arylamidase activity was determined in *M. pulcherrima* and *Kl. lactis* yeast species. Acid phosphatase activity was determined in *H. uvarum* and *K. lactis* yeast species. Uncultured *Kwoniella* sp. and *H. uvarum* yeast species displayed high β -galactosidase and β -glucosidase activities, respectively.

Keywords: API-ZYM, ITS, PCR-RFLP, pomegranate, yeast microbiota

INTRODUCTION

Yeasts have been used to produce foods (cheese, bread and dairy products etc.) and beverages (beer and wine etc.) for centuries (Braduardi and Porro 2012). They have an important role in the determination of food quality and production of fermented foods. Because of high fermentation ability, yeasts are utilized to fabricate alcohol, beer, wine, cake and to produce secondary metabolites, such as antibiotics and vitamins (Hierro et al. 2004; Lee 1996, Lopez et al. 2001). Some of yeasts also contribute to the developing of flavored beverages. Thus, they can be used as starter culture in the fermentation process.

Enzymes are utilized in different perspectives, such as detergents, textile, biofuels industry, food-producing, animal feeds, and medical fields. Food enzymes are generally applied in baking industry, fruit juice, and dairy products, winemaking. The activity of enzymes during these processes increases their flavor and nutritional value (Wang et al. 2012). Several enzymes, having industrial and biotechnological importance, can be produced by *Saccharomyces cerevisiae* and various non-*Saccharomyces* yeast strains such as *Kluyveromyces lactis*, *Geotrichum candidum*, *Yarrowia lipolytica*, *Rhodotorula* spp., *Kluyveromyces marxianus*, *Komagataella pastoris*,

Debaryomyces hansenii, *Cryptococcus* spp., *Cryptococcus laurentii*, *Sporobolomyces salmonicolor* etc. (Johnson 2013a, b). Hence, the determining of yeast microbiota and the understanding of enzyme activity is important for industrial and biotechnological studies.

Pomegranates (*Punica granatum* L.) contains variety of polyphenols such as ellagic acid and punicalagins, both of which are thought as strong antioxidants. Punicalagins are found only on surface of pomegranate and they have 2-fold of antioxidant ability of green tea and red wine. Pomegranate is used for producing vinegar, wine, liqueur, jam, pomegranate molasses, pomegranate juice, and for a coloring agent of food and enhancing flavor. Due to high sugar content of pomegranate, it can be used to manufacture of pomegranate wine (Bakoyianis et al. 1992, Sevda and Rodrigues 2011). The extracts of pomegranate fruits have antifungal and antimicrobial features (Dağcı and Dıđrak 2005). In addition, pomegranate enhances the longevity, fertility, and growth rate of the nematode *Caenorhabditis elegans* (Kılıçgün et al. 2015). The peel of pomegranate includes phenolic compounds and has high antioxidant activity (Yasoubi et al. 2007).

There are different types of identification and classification methods for yeast species. These methods have generally depended on morphological, physiological

features and biochemical characteristics of yeasts species (Kurtzman and Fell 1998; Barnett et al. 2000; Kreger-van Rij 1984), plus the DNA-based molecular techniques such as ribosomal DNA sequencing, DNA-DNA hybridization and RFLPs of chromosomal DNA (Kurtzman and Robnett 1998; Vaughan and Martini 1987; Török et al. 1993; Versavaud and Hallet 1995). The ribosomal DNA genes containing 5S, 5.8S, 18S and 26S (D1/D2 domain) coded regions and non-coded ITS (Internal Transcribed Spacers) and IGS (Intergenic Spacer) regions and their restriction profiles have been used to identify yeast species (Kurtzman and Robnett 1998; Baleiras Couto et al. 2005; Gonzalez et al. 2007; Sabate et al. 2002; Cadez et al. 2002; Diaz and Fell 2000; Naumova et al. 2003; Guillamon et al. 1998; Esteve-Zarzoso et al. 1999).

The aim of this study was to identify yeast microbiota on the pomegranate collected from Gallipoli (Gelibolu), Çanakkale-Turkey, and to determine of extracellular enzyme activity of all yeast strains. For this purpose, we used two molecular techniques, PCR-RFLP analysis and sequencing of ITS-5.8S rDNA gene region and we used API-ZYM test system for characterization of extracellular enzyme activity. Also we constructed phylogenetic tree to analyzed relationships among identified yeast species.

MATERIALS AND METHODS

Sampling and isolating yeast strains

Taking into account of pomegranate ripening period, fruit samples were collected from Koruköy, Gelibolu-Turkey in August, and November in 2008. Pomegranate samples were homogenized in 2% Sodium citrate solution after weighting and spread onto YGC-Agar medium (40 gr/l Yeast Extract Glucose Chloramphenicol Agar) including 0.1% Sodium propionate. After incubation of plates at 30 °C for 2-3 days, the appeared yeast colonies were counted to determine the colony-forming units (CFU/gr) and then transferred to YPD medium (10 gr/l (w/v) Yeast Extract Powder, 20 gr/l (w/v) Bacto-peptone, , 20 gr/l (w/v) Agar, 2% Dextrose) and incubated at same conditions for 2-3 days. The isolated yeast strains were stored in 20% glycerol at -80°C for further use.

Procedures

Genomic DNA isolation and pcr amplification

Yeast strains, grown on YPD medium, were used in genomic DNA isolation method which was previously improved (Sherman et al. 1986). ITS1-5.8S-ITS2 rDNA gene regions of genomic DNA were amplified by using yeast specific primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR amplification was studied by using previously reported conditions (White et al. 1990). PCR reactions were carried out with BIO-RAD Thermal cycler in 25 µl final volume containing 1.25U Recombinant Taq DNA polymerase (Thermo Scientific-EP0402) 10 pmol/µl of each primer, 10 mM of each dNTP, 10X PCR reaction buffer, 3 mM MgCl₂ and 50-100 ng genomic DNA as template. The PCR conditions

were: initial denaturation at 95°C for 1.5 min; 30 cycles of denaturing at 94°C for 2 min; annealing at 60°C for 1 min, an extension at 72°C for 2.5 min; and a final extension step of 5 min at 72°C. The products were electrophoresed in 1.5% agarose gel and visualized. The length of PCR amplicons were calculated using by Gel-Pro Analyzer v4.0 software.

Restriction profiles and DNA sequencing

PCR amplicons were purified using GeneJet PCR Purification Kit (Thermo Scientific-K0702) and then were digested with HaeIII, HinfI, MspI, HhaI and AluI restriction endonucleases according to supplier's instructions. The restriction fragments were analyzed by electrophoresis in 3% agarose gel and photographed. The length of restriction fragments was calculated by using Gel-Pro Analyzer v4.0 software. The yeast strains were grouped with respect to restriction profiles and 10 groups was formed. PCR products of all yeast strains were sequenced by using the Applied Biotechnologies 3500xl Genetic Analyzer. The obtained ITS1-5.8S ITS2 sequences were analyzed using Basic Local Alignment Search Tool (BLAST) on National Center for Biotechnology Information (NCBI) (Altschul et al. 1990). During BLAST analysis, the yeast strains having high sequence similarity were selected as reference yeast strains, mainly belong to the CBS-Knaw Yeast Collection. The resulting sequences were deposited in GenBank (Accession numbers: KR011878, KY366236-KY366245, MK613238-MK613271 for the ITS1-5.8S-ITS2 sequences).

Phylogenetic analysis

Phylogenetic relationship among the yeast strains was analyzed using Maximum Parsimony (MP) in MEGA-X (Kumar et al. 2018). Nucleotide sequences of ITS1-5.8S-ITS2 rDNA gene region of 47 yeast strains and 2 outgroup (*S. cerevisiae* and *P. guilliermondii*) were aligned with ClustalX algorithm in MEGA-X. MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm and bootstrap method in MEGA-X. 1000 bootstrap replicates were used to defined branch support.

Extracellular enzyme profiles

Extracellular enzyme profiles of yeast strains were determined by using API-ZYM kit system (bioMérieux, France), which is a minimized and semi-quantitative test system. API-ZYM is utilized for screening 19 different enzyme activities (Alkaline phosphatase, Esterase (C 4), Esterase Lipase (C 8), Lipase (C 14), Leucine arylamidase, Valine arylamidase, Cysteine arylamidase, Trypsin, α -chymotrypsin, Acid phosphatase, Naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase). All yeast strains were grown in YPD culture at 30 °C for 12 hours (till the exponential stage) with constant shaking (120 rpm/rev). The 65 mL from the saturated yeast culture was transferred to each microwells of API-ZYM strip. The API-ZYM strips were incubated at 30 °C for 4 hours. Then, ZYM A and ZYM B reagents were added to each cupule

and all the strips were incubated at room temperature for 5 minutes. Enzyme profiles of yeast strains were defined by color scalar of API-ZYM kit system. Enzyme activities were carried out in duplicate for each strain to confirm the results.

RESULTS AND DISCUSSION

Results

Pomegranate samples were collected from Koruköy, Gelibolu-Turkey in 2008 during ripening period. After incubation on YGC medium, 47 yeast strains were isolated and colony-forming units (CFU/gr) were calculated as $3.7 \times 10^3 \text{ gr}^{-1}$. The isolated yeast strains were classified into 8 groups according to their colony morphologies such as colony form (circular, irregular, etc.), colony elevation (raised, convex, flat, etc.) and colony margin (entire, undulate, filiform, etc.) (data not shown).

After genomic DNA extraction, the yeast strains were classified into four groups according to PCR amplicons of ITS1-5.8S-ITS2 rDNA gene regions (Table 1). The first group composed of 6 yeast strains with nearly 400bp in PCR amplicon length. In the second and third groups contained 21 and 8 yeast strains having PCR length between 600 to 700bp. The last group represented 12 yeast strains giving a PCR amplicon with 750-800 bp in length. All yeast members of PCR groups were digested with five restriction endonucleases (HinfI, HaeIII, MspI, AluI, and HhaI) and analysis of PCR-RFLP results was given in Table 1. The yeast strains present in Group I, Group III, and Group IV displayed two different restriction profiles while Group II gave 4 different RFLP profiles. The yeast strain P-48 was distinguished from other strains in Group I with HaeIII digestion. All yeast strains in this group were not cleaved by AluI restriction enzyme. Third PCR group was separated into two restriction profiles by the action of four restriction enzymes (HinfI, HaeIII, MspI, and HhaI) except AluI. And the members of last PCR group, Group IV, did not have a cleavage site for MspI. The yeast strains in this group showed different profiles with respect to digestion of HinfI, HaeIII, AluI, and HhaI restriction enzymes except for MspI. Group II yeast strains showed 4 restriction profiles with respect to HaeIII, MspI, AluI, and HhaI digestions. HinfI restriction enzyme gave same restriction profile in all strains of Group IV. According to PCR-RFLP results, we obtained 10 different restriction profiles that can represent ten different yeast species. These results showed that the restriction enzymes used in RFLP analysis were suitable to discriminate yeast strains.

ITS1-5.8S-ITS2 rDNA gene region of all yeast strains was sequenced and BLAST analysis was carried out of yeast strains. The resulting sequences were submitted to the GenBank database and accession numbers were obtained. The yeast species which show sequence similarity, were given in Table 2 along with reference accession number on GenBank Database. According to the BLAST analysis of ITS1-5.8S-ITS2 rDNA gene region, while 28 yeast strains showed 96 to 99% similarity with their reference yeast species, 18 yeast strains gave range from 80%-83%

sequence similarity with *Kwoniella bestiolae* (*Cryptococcus bestiolae*) (Ref. Acc. Numbers KY103928.1). Therefore, the yeast strains present in third (P-1, P-4, P-5, P-6, P-7, P-9, P-11, P-12, P-19, P-22, P-26, P-39, P-41 and P-42), fifth (P-15), sixth (P-30, P-31) and eight (P-14) restriction profiles were determined as an uncultured *Kwoniella* sp. The yeast strains, P-3, P-21, P-24, and P-27, localized in fourth restriction profile with respect to PCR-RFLP results. Despite the fact that P-21 yeast strain has different colony morphology than their group members, according to the sequence analysis of P-3, P-21, P-24, and P-27 strains, they showed similarity with *Aureobasidium pullulans* (Ref. Acc. No. KT898651.1). During BLAST analysis, the four yeast strains cannot give any match with *A. pullulans* yeast strain that belongs to the CBS yeast database. So, the results of four yeast strains were obtained from the NCBI-BLAST database giving highest sequence similarity (98-100 % identity). The sequence analysis of 6 yeast strains having first and second restriction profile, P-47, P-49 and P-55a yeast strains gave 98% sequence similarity with *M. ziziphicola* (Ref. Acc. No. KY104214.1). P-25a, P-53, and P-48 yeast strains showed sequence similarity with *M. pulcherrima* %97, %92, and %98 respectively (Ref. Acc. No. KY104205.1). We also observed that all six yeast strains produced pulcherrimin pigment. The yeast strains belonging to seventh (P-20, P-25b, P-29, P-32, P-33, P-34 and P-35) and ninth (P-8, P-23, P-36, P-37, P-38, P-44, P-46, P-50, P-51, P-52 and P-55b) restriction profiles were identified as *Candida zeylanoides* (Ref. Acc. No. KY102548.1) and *Hanseniaspora uvarum* (Ref. Acc. No. KY103563.1), respectively. P-18 strain demonstrated 99% similarity with *Kluyveromyces lactis* (Ref. Acc. No. KY103775.1) yeast species. These results also supported the idea that the yeast strains with different restriction profiles may be the same species, as in the case of *Kwoniella* sp. yeast strains. The percent distribution of yeast species on the pomegranate surface was determined as: 38.30% *Kwoniella* sp., 23.40% *H. uvarum*, 14.9% *C. zeylanoides*, 6.38% *M. pulcherrima* and *M. ziziphicola*, 8.51% *A. pullulans* and 2.13% *K. lactis*. It was observed that *Kwoniella* sp. and *H. uvarum* yeast species were dominant on surface yeast microbiota of pomegranate.

The phylogenetic analysis of all yeast strains was carried out by using MEGA-X phylogenetic analysis tool. ITS1-5.8S-ITS2 rDNA gene sequences of yeast strains were aligned by the ClustalX v1.6 algorithm and Maximum parsimony tree was constructed. All of analysis was done by using MEGA-X. 1000 bootstrap replicates were used to defined branch support. The percentage of trees is shown next to the branch and frequencies under 50% are not given. When the MP tree is analyzed, we obtained five clades. The first clade is composed of 24 yeast strains and divided into two cluster as *Kwoniella* sp. (19) with reference yeast strain (CBS:10118) and *A. pullulans* (5) with reference yeast strains (KT898651.1). The second clade includes *H. uvarum* yeast strains (12) with reference strain (CBS:104). Third clade consist of *Kl. lactis* and reference strain (CBS:739). The fourth clade consist of 8 yeast strains belongs to *Metschnikowia* genus, *M. pulcherrima* (3), *M. ziziphicola* (3) and their reference

strains (for *M. pulcherrima*, CBS:5833; for *M. ziziphicola*, CBS:10358). The fifth clade includes *C. zeylanoides* yeast strains (8) with reference strain (CBS:641), only one strain (KY366245) was separated from others in the clade (Figure 1).

The extracellular enzymatic activities of all isolated yeast strains were screened by using API-ZYM (Bio Mérieux, France) kit system. The level of enzyme activity was determined with respect to API-ZYM color reaction chart from zero (no activity) to five (highest activity), and results were given in Table 3. Trypsin, α -chymotrypsin, α -galactosidase, β -glucuronidase, N-acetyl- β -glucosaminidase, α -mannosidase, and α -fucosidase enzyme activities were not detected in the yeast strains. All yeast strains showed intermediate level of esterase lipase and leucine arylamidase enzyme activities. All strains of *M. pulcherrima*, *A. pullulans*, and *K. lactis* showed similar patterns in their enzyme activities. But the strains of *Kwoniella* sp., *H. uvarum* and *C. zeylanoides* exhibited two different enzymatic patterns. The strains of *Kwoniella* sp., P-1, P-5, P-6, P-7, P-9, P-11, P-19, P-26, P-31, P-41, P-42, possessed high β -galactosidase activity and the strains of *H. uvarum*, P-8, P-36, P-44, P-46, P-50, P-51, P-52, showed high β -glucosidase activity. However, the other strains of *Kwoniella* sp., P-4, P-12, P-14, P-15, P-22, P-30 and P-39, and *H. uvarum*, P-23, P-37, P-38 and P-55b, did not have β -galactosidase and β -glucosidase activity, respectively. *M. pulcherrima* yeast strains have moderate level of α - and β -glucosidase activity.

Discussion

Pomegranate juice can positively affect the protein synthesis of *S. cerevisiae* and significantly decrease oxidative damage (Aslan et al. 2014). The fruits have a high nutritional value because of polyphenol content. Both the fruit and its extract have antibacterial, anticancer, and antioxidant activity as well as stress protectant ability

(Santos et al. 2012). Therefore, in this study, we determined the yeast microbiota present on pomegranate surface, PCR-RFLP, and ITS1-5.8S-ITS2 rDNA gene sequence analysis was used for yeast identification and classification. We also detected extracellular enzyme profiles using API-ZYM test system.

ITS1-5.8S-ITS2 gene region of yeast strains was amplified and cleaved by using *HinfI*, *HaeIII*, *MspI*, *AluI*, and *HhaI* restriction enzymes. ITS1-5.8S-ITS2 gene region of all isolated yeast strains were sequenced and analyzed in BLAST tool on NCBI. *M. pulcherrima*, *M. ziziphicola*, *Kwoniella* sp., *A. pullulans*, *C. zeylanoides*, *H. uvarum* and *K. lactis* yeast species were identified on the microbiota of pomegranate surface. *Kwoniella* sp. and *H. uvarum* yeast species were dominated half of the yeast biota on fruit surface. The yeast species belong to genera *Kloeckera* and *Rhodotorula*, which were predominant genera in other fruits, were not recorded on the surface of pomegranate.

While the *HinfI* restriction profile of *H. uvarum* strains was similar to previous results, *HhaI* restriction profile was different (Guillamon et al. 1998; Rodriguez-Vico et al. 2003; Romano et al. 2009; Baffi et al. 2010; Gibson et al. 2011). The *HhaI*, *HaeIII* and *HinfI* profiles of *C. zeylanoides* yeast strains showed similar restriction patterns with other researches (Guillamon et al. 1998; Esteve-Zarzoso et al. 1999). Similarly, *HinfI*, *HaeIII* and *HhaI* profiles of *M. pulcherrima*, *A. pullulans* and *K. lactis* yeast species revealed the same restriction pattern previously reported (Guillamon et al. 1998; Esteve-Zarzoso et al. 1999; Rodriguez-Vico et al. 2003; Romano et al. 2009; Baffi et al. 2010; Gibson et al. 2011). We also determined that the restriction profiles of both *Kwoniella* sp. and *M. pulcherrima* species members were different, but, they were determined as same species with respect to in the sequence analysis of ITS1-5.8S-ITS2 gene region. These results that the same yeast species could possess different restriction profiles as reported before.

Table 1. PCR-RFLP profiles of yeast strains

PCR Group no.	Rest. Profile	Yeast Strain	<i>HinfI</i> (bp)	<i>HaeIII</i> (bp)	<i>MspI</i> (bp)	<i>AluI</i> (bp)	<i>HhaI</i> (bp)
Group I (~400 bp)	1	P-25a, P-47, P-49, P-53, P-55a	195/191	287/106	217/117/50	-	217/95
	2	P-48	195/191	389/284/106	217/117/50	-	217/95
Group II (~600-700 bp)	3	P-1, P-4, P-5, P-6, P-7, P-9, P-11, P-12, P-19, P-22, P-26, P-39, P-41, P-42	296/185/145	-	198/152/143/100	-	325/215
	4	P-3, P-21, P-24, P-27	294/177/138	465/155	284/188/150	390/208	190/185/103/91
	5	P-15	294/177/138	-	284/188/150	-	331/210/107
	6	P-30, P-31	294/177/138	-	200/150/143/100	-	331/210/107
Group III (~600-700 bp)	7	P-20, P-25b, P-29, P-32, P-33, P-34, P-35	321/313	432/142/82	-	-	304/298
	8	P-14	320/215/95	-	197/145/145/99	-	289/177/140
Group IV (~750-800bp)	9	P-8, P-23, P-36, P-37, P-38, P-44, P-46, P-50, P-51, P-52, P-55b	354/191/166	-	-	585/185	339/114/ 108/99
	10	P-18	306/189/109/90	677/62	-	428/188/136	303/202/109

The developing molecular identification methods allow researchers to identify more accurate and rapid identification of yeast species than traditional methods. The PCR-RFLP and sequence analysis of ITS1-5.8S-ITS2 gene region has been generally utilized for this purpose. Although, the sequence analysis of ITS1-5.8S-ITS2 gene region is available for determination of yeast microbiota, it could not be useful for some yeast strains. For example, in this study, 18 yeast strains showed low similarity with *Kwoniella* sp. (80-83%). It is thought that above 95%

nucleotide identity could be convenient to identify yeast strains, but below 95% nucleotide identity could not be sufficient. Therefore, additional identification methods should be utilized to determine *Kwoniella* sp. strains, such as biochemical analysis and morphological features. HinfI, HaeIII, and HhaI (CfoI) endonucleases have been commonly used for identification and differentiation of yeast strains. In this study, we used additional two different restriction enzymes, MspI and AluI, for discrimination of yeast strains such as *Kwoniella* sp. yeast species.

Table 2. BLAST results of ITS1-5.8S-ITS2 rDNA gene region

Yeast strains	Identified yeast strains (reference accession no.)	Identity (%)	GenBank accession no.	References
P-1	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.58	KY366236	Vu et al. 2016
P-3	<i>A. pullulans</i> (KT898651.1; Strain Code: A85)	99.61	MK613238	Carvalho et al. 2016
P-4	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.33	MK613239	Vu et al. 2016
P-5	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.33	MK613240	Vu et al. 2016
P-6	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.26	MK613241	Vu et al. 2016
P-7	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.40	MK613242	Vu et al. 2016
P-8	<i>H. uvarum</i> (KY103563.1; CBS:104)	99.71	MK613243	Vu et al. 2016
P-9	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.33	MK613244	Vu et al. 2016
P-11	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.22	MK613245	Vu et al. 2016
P-12	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.26	MK613246	Vu et al. 2016
P-14	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.58	KY366237	Vu et al. 2016
P-15	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.75	KY366238	Vu et al. 2016
P-18	<i>Kl. lactis</i> (KY103775.1; CBS: 739)	99.26	KY366239	Vu et al. 2016
P-19	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.29	MK613247	Vu et al. 2016
P-20	<i>C. zeylanoides</i> (KY102548.1; CBS:641)	100	MK613248	Vu et al. 2016
P-21	<i>A. pullulans</i> (KT898651.1; Strain Code: A85)	98.26	KY366240	Carvalho et al. 2016
P-22	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.33	MK613249	Vu et al. 2016
P-23	<i>H. uvarum</i> (KY103563.1; CBS:104)	98.70	KY366241	Vu et al. 2016
P-24	<i>A. pullulans</i> (KT898651.1; Strain Code: A85)	98.88	KY366242	Carvalho et al. 2016
P-25a	<i>M. pulcherrima</i> (KY104205.1; CBS:5833)	96.91	KY366243	Vu et al. 2016
P-25b	<i>C. zeylanoides</i> (KY102548.1; CBS:641)	100	MK613250	Vu et al. 2016
P-26	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.29	MK613251	Vu et al. 2016
P-27	<i>A. pullulans</i> (KT898651.1; Strain Code: A85)	100	MK613252	Carvalho et al. 2016
P-29	<i>C. zeylanoides</i> (KY102548.1; CBS:641)	100	MK613253	Vu et al. 2016
P-30	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.31	KY366244	Vu et al. 2016
P-31	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.33	MK613254	Vu et al. 2016
P-32	<i>C. zeylanoides</i> (KY102548.1; CBS:641)	99.29	KY366245	Vu et al. 2016
P-33	<i>C. zeylanoides</i> (KY102548.1; CBS:641)	100	MK613255	Vu et al. 2016
P-34	<i>C. zeylanoides</i> (KY102548.1; CBS:641)	99.65	MK613256	Vu et al. 2016
P-35	<i>C. zeylanoides</i> (KY102548.1; CBS:641)	99.65	MK613257	Vu et al. 2016
P-36	<i>H. uvarum</i> (KY103563.1; CBS:104)	99.42	KY366246	Vu et al. 2016
P-37	<i>H. uvarum</i> (KY103563.1; CBS:104)	99.56	MK613258	Vu et al. 2016
P-38	<i>H. uvarum</i> (KY103563.1; CBS:104)	99.71	MK613259	Vu et al. 2016
P-39	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.29	MK613260	Vu et al. 2016
P-41	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	82.15	MK613261	Vu et al. 2016
P-42	<i>K. bestiolae</i> (KY103928.1; CBS: 10118)	80.48	MK613262	Vu et al. 2016
P-44	<i>H. uvarum</i> (KY103563.1; CBS:104)	99.56	MK613263	Vu et al. 2016
P-46	<i>H. uvarum</i> (KY103563.1; CBS:104)	99	MK613264	Vu et al. 2016
P-47	<i>M. ziziphicola</i> (KY104214.1; CBS:10358)	97.60	MK613265	Vu et al. 2016
P-48	<i>M. pulcherrima</i> (KY104205.1; CBS:5833)	91.61	KR011878	Vu et al. 2016
P-49	<i>M. ziziphicola</i> (KY104214.1; CBS:10358)	98	MK613266	Vu et al. 2016
P-50	<i>H. uvarum</i> (KY103552.1)	98.58	KY366247	Vu et al. 2016
P-51	<i>H. uvarum</i> (KY103563.1; CBS:104)	99	MK613267	Vu et al. 2016
P-52	<i>H. uvarum</i> (KY103563.1; CBS:104)	99.42	MK613268	Vu et al. 2016
P-53	<i>M. pulcherrima</i> (KY104205.1; CBS:5833)	98	MK613269	Vu et al. 2016
P-55a	<i>M. ziziphicola</i> (KY104214.1; CBS:10358)	97.90	MK613270	Vu et al. 2016
P-55b	<i>H. uvarum</i> (KY103563.1; CBS:104)	99.56	MK613271	Vu et al. 2016

Note: Columns indicates the isolated yeast strain code, the BLAST results with the reference strains given in parenthesis, the % of identity with the BLAST reference strain, the GenBank accession number and references.

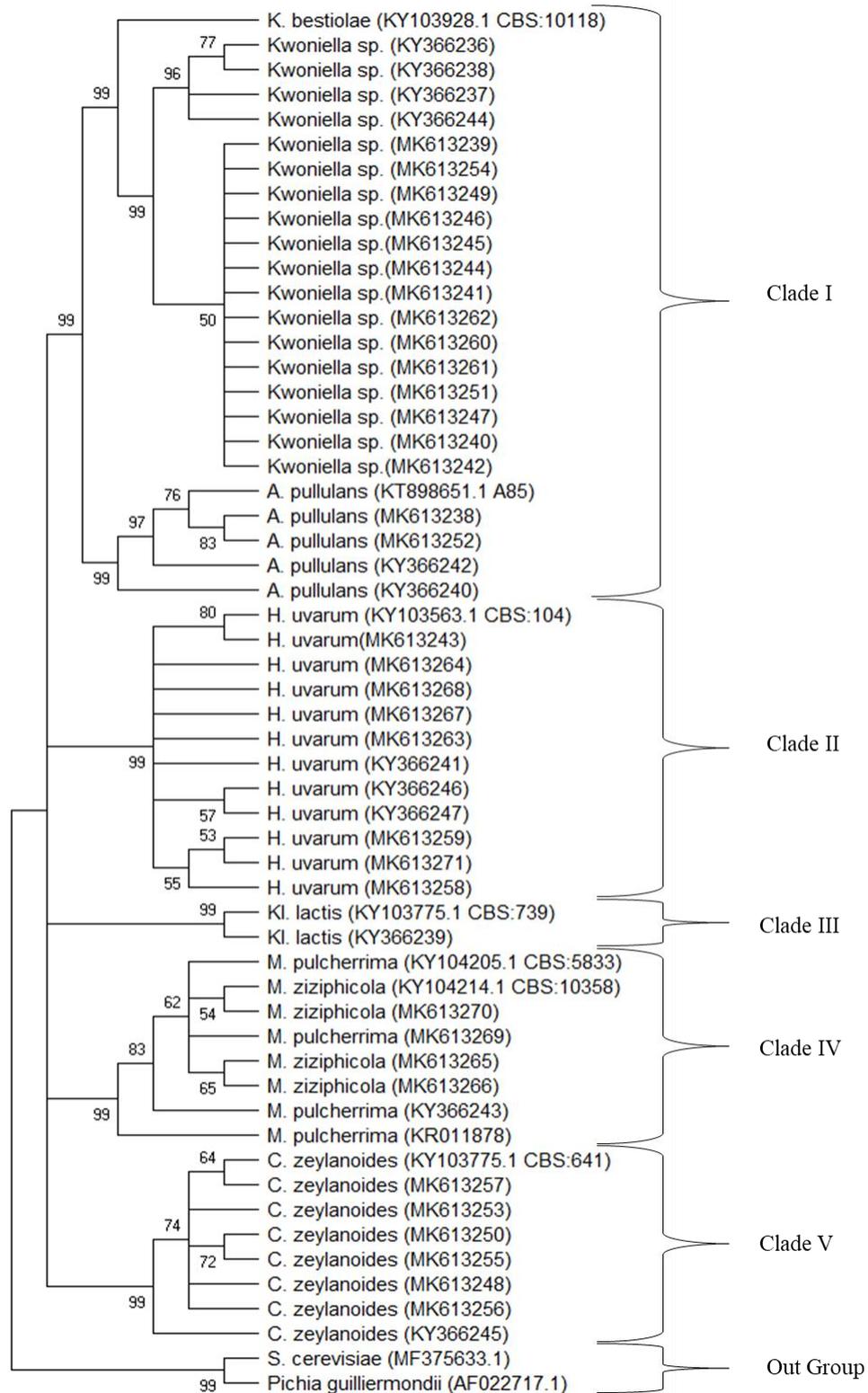


Figure 1. The evolutionary history was inferred using the Maximum Parsimony method. The most parsimonious tree with length = 2305 is shown. The consistency index is (0.656399), the retention index is (0.904739), and the composite index is 0.593870 (0.578885) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar 2000). The analysis involved 54 nucleotide sequences including reference strains' sequences and 2 outgroups in this study, which included 3 *M. pulcherrima*, 3 *M. ziziphicola*, 18 *Kwonella* sp., 4 *A. pullulans*, 1 *K. lactis*, 11 *H. uvarum*, and 7 *C. zeylanoides* yeast strains. *S. cerevisiae* and *P. guilliermondii* strains were selected as outgroup.

Table 3. Extracellular enzyme activity of yeast strains that isolated from pomegranate fruits

Yeast species	C	1	2	3	4	5	6	7	8	9	10	11	12
<i>Kwoniella</i> sp. (P-1, P-5, P-6, P-7, P-9, P-11, P-19, P-26, P-31, P-41, P-42)	0	1	3	2	0	4	0	0	1	1	5	0	0
<i>Kwoniella</i> sp. (P-4, P-12, P-14, P-15, P-22, P-30, P-39)	0	0	2	1	0	1	0	0	2	1	0	0	0
<i>H. uvarum</i> (P-23, P-37, P-38, P-55b)	0	5	0	1	0	4	0	0	0	0	0	0	0
<i>H. uvarum</i> (P-8, P-36, P-44, P-46, P-50, P-51, P-52,)	0	1	2	1	0	4	1	0	1	1	0	0	5
<i>C. zeylanoides</i> (P-20, P-25b)	0	0	2	2	0	2	0	0	2	0	0	0	0
<i>C. zeylanoides</i> (P-29, P-32, P-33, P-34, P-35)	0	0	2	2	0	2	1	0	2	2	0	0	0
<i>M. pulcherrima</i> (P-25a, P-47, P-49, P-53, P-55a)	0	3	3	2	0	4	4	2	3	2	0	3	2
<i>A. pullulans</i> (P-3, P-21, P-24, P-27)	0	0	2	2	0	2	0	0	1	0	0	0	0
<i>K. lactis</i> (P-18)	0	5	2	2	1	5	4	1	3	2	1	0	1

Note: C. Control; 1. Alkaline phosphatase; 2. Esterase (C4); 3. Esterase lipase (C8); 4. Lipase (C14); 5. Leucine arylamidase; 6. Valine arylamidase; 7. Cystine arylamidase; 8. Acid phosphatase; 9. Naphthol-AS-BI-phosphohydrolase; 10. β -Galactosidase; 11. α -Glucosidase; 12. β -Glucosidase. Due to trypsin, α -chymotrypsin, α -galactosidase, β -glucuronidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase enzymes have not shown activity, these enzymes are not shown in this table

β -galactosidase and β -glucosidase enzymes hydrolyze glycosidic bonds in oligosaccharides, polysaccharides and heteroglycosides (Karasova et al. 2002). These enzymes have been found various organisms, like bacteria, yeast, fungi. β -galactosidases are responsible from the breakdown of lactose into glucose and galactose (Shaima et al. 2017). The deficiency of β -galactosidase enzyme can cause lactose intolerance that is threatened by using β -galactosidase supplemented foods (Vasiljevic and Jelen 2001). The enzyme of β -glucosidase (EC 3.2.1.21) hydrolyzed β -1-4 glycosidic bonds in cellulose molecules. β -glucosidase enzymes have generally used for various applications like biofuel production, splits of bitter components for juice extraction and contributes to aroma production of wine (Li et al. 2013; Gueguen et al. 1998; Harhangi et al. 2002). The β -glucosidase enzyme has enormous potential for food processing and utilized to increase the flavor of tea and fruit juice (Keerti et al. 2014). Arylamidases (aminopeptidases, EC 3.4.11.2) are the proteolytic enzymes and hydrolyze N-terminal amino acids (Dodor and Tabatabai 2007). Cystine arylamidase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α - and β -glucosidase enzymes have a significant role in enhancing the aroma and flavor of wine (Delfini and Formica 2001; Nikolaou et al. 2007; Nikolaou et al. 2006; Fleet et al. 1997). As shown that extracellular enzymes are important for various fields such as wine industry and medical applications. For this reason, identified yeast strains having high enzyme activities could be used for different industrial applications.

Kwoniella sp., *H. uvarum*, *M. pulcherrima* and *Kl. lactis* yeast strains showed high leucine arylamidase activity. Also valine arylamidase activity was determined in *M. pulcherrima* and *Kl. lactis* yeast species, acid

phosphatase activity in *H. uvarum* and *Kl. lactis* yeast species. *Kwoniella* sp. and *H. uvarum* yeast species displayed high β -galactosidase and β -glucosidase activities, respectively. The obtained results in this study showed that the same yeast species could be different extracellular enzyme profiles. Therefore, variety of extracellular enzyme profiles of yeast strains could be used for discrimination of same yeast strains. Eleven yeast strains belonging to *Cryptococcus* genus were investigated for determining the extracellular enzyme profile and the results showed that the members of *Cr. laurentii* and *Cr. neoformans* yeast species have different enzyme profiles (Garcia-Martos et al. 2001).

M. pulcherrima yeast species are used as biocontrol agent to the agricultural areas. It has also shown protective features for post-harvest pathogens, especially, causing by *Botrytis cinerea* (De Curtis et al. 1996; Piano et al. 1997; Spadaro et al. 2002). *H. uvarum* which is also a potential biocontrol agent, had an antagonistic effect for inhibiting spore formation of *B. cinerea* (Liu 2010a, b). *A. pullulans* yeast species have been used as indicator of environmental pollution (Deshpande et al. 1992). They have also synthesized an extracellular polysaccharide, pullulan, used in food and pharmaceutical industry (Bernier 1958; Sena et al. 2006; Cheng et al. 2011). *C. zeylanoides* yeast strains could display a remarkable ability of removing color of sulphonic and non-sulphonic dyes, at acidic pH and synthetic medium (Ramalho et al. 1999). Therefore, *M. pulcherrima* and *A. pullulans* yeast species are industrially important yeasts due to having high alpha-and beta- β -glucosidase activities, respectively. In addition, *Cr. bestiolae* yeast species is also good candidate for industrially production of β -galactosidase enzyme. Extracellular enzymes are used for different areas such as wine industry, medical applications, textile industry,

baking industry. For this reason, the determining of yeast strains which have high enzyme activity is highly significant for producer.

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