ISSN 2449-8955 European Journal of Biological Research *Research Article*

DOI: http://dx.doi.org/10.5281/zenodo.5539705

Diversity and extracellular enzyme profiles of yeasts on organic and fungicide treated strawberries

Tülay Turgut Genç [1,*], Melih Günay [2]

[1] Çanakkale Onsekiz Mart University, Faculty of Arts and Science, Department of Biology, Çanakkale, Turkey

[2] Çanakkale Onsekiz Mart University, Graduate School of Natural and Applied Sciences, Çanakkale, Turkey

* Corresponding author e-mail: tturgutgenc@comu.edu.tr

Received: 18 July 2021; **Revised submission:** 17 August 2021; **Accepted:** 27 September 2021

https://jbrodka.com/index.php/ejbr

ABSTRACT: Since yeasts can survive under variable environmental conditions using different food sources they have a wide distribution in nature. Fruits are suitable living spaces for yeasts and other microorganisms due to their high and different sugar contents. Strawberry fruit as well as other fruits are very sensitive to pathogenic fungi. Due to their residues on fruits, limitations on the use of fungicides have led to increased use of microorganisms with antagonistic effects as biological control agents. The biological agents to be used are selected mainly from the microorganisms found in the natural microbiota of the fruit. Therefore, in this study yeast biota on strawberry fruit collected from fungicide treated (Klorzon and Topas) and organic fields was determined using molecular identification methods. In addition, extracellular enzyme profiles of the identified yeast species were determined by the APIZYM-based system. There was no difference in the diversity of yeast species on strawberries collected from fungicide treated and organic fields, but the yeast density on organic strawberries was greater than fungicide treated fruits. The identified yeast species on fruits were determined as *Metschnikowia pulcherrima* (61.7%), *Hanseniaspora uvarum* (34.0%) and *Wickerhamomyces pijperi* (4.3%). *W. pijperi* yeast species was reported on strawberry fruit in our study first time. It was determined that *H. uvarum* and *W. pijperi* yeast species showed no α-glucosidase enzyme activity. All yeast strains showed industrially important β-glucosidase enzyme activity.

Keywords: Strawberry; Fungicide treatment; D1/D2 rDNA; RFLP; Extracellular enzyme; Yeast.

1. INTRODUCTION

Fruits are suitable environments for microbial growth due to their high sugar and nutrient content. Although the low pH and the amount of water in the fruits often inhibit the growth of bacteria preferring neutral pH to some extent, fruits are vulnerable to fungal organisms. Regardless of their pathogenicity, all fungi can damage the fruit, and these damages on fruits before or after harvest can cause economic losses as well as threaten human health. It is known that some pathogenic fungi cause allergic infections [1, 2]. Yeast and yeast-like organisms found in the natural microbiota of plants and fruits are necessary for epiphytic microbial balance, and disruption of this balance causes the formation of different diseases. Citrus fruit washed with water decays more quickly than unwashed fruit which occurs as a result of the loss of the natural epiphytic microbial balance [3]. Determining the natural microbial flora of fruits contributes to the

development of different biological control agents for different fruits. Thus, it may be possible to preserve the fruit for a long time using sprays containing appropriate microbial flora before and after harvest. Fruits can be consumed naturally or used in manufacturing the different products such as wine, vinegar, jam, ice cream. In particular, the natural microbiota of fruits is important not only in making the primary aroma of the wine but also in the amount of alcohol and the formation of permanent aroma in the following process. For this reason, it is of great importance for wine producers to know the microbiota of fruits.

Strawberry (*Fragaria* spp.), one of the most widely grown fruits in the world, is a small pinkish-red fruit covered with seeds. Strawberry is widely used in ice cream, dairy products (fruit-flavored yoghurt and milk), candies and chocolates, bakery products, frozen foods, jam, vinegar and winemaking [4, 5]. Strawberry fruit is open to microbial contamination as it has a soft surface with indented-protruding [6]. Especially *Colletotrichum acutatum* and *Botrytis cinerea* are the most important fungus species that damage strawberry fruit [7]. *C. acutatum* and *B. cinerea* causes anthracnose fruit rot and grey mold diseases, respectively. Fungicides containing tetraconazole or penconazole active components are used against these diseases in strawberries. The commercial combination of active ingredients, such as the combination of cyprodinil with fludioxonil or the combination of fenhexamid with captan, were often the most effective in the treatments of anthracnose fruit rot and grey mold diseases [7]. It is known that the long-term use of specific fungicides caused an increase in the density of resistant isolates in the population and the effect of the fungicide decreased in subsequent applications [8].

It is very important to determine the fruit surface microbiota in the production of biological sprays to be developed against such fungi and other microorganisms that damage the fruit. It was reported that the intensity of yeast species on strawberry fruit was lower (at 3%) than other fruits (raspberries and blueberries), the main reason being that the yeast cells could not pass the epidermis [6]. The indigenous yeast population on fruits can be affected by many different factors such as geographical location, climatological conditions, soil structure, pesticide handling, fruit variety, degree of maturity, and also parts of the fruit plant [9-12]. For example, some fungus species (*Botrytis cinerea*, *Rhizoctonia fragariae*, *Acremonium* spp., *Alternaria* spp., *Cladosporium* spp., *Aspergillus* spp., *Penicillium* sp., and *Phoma* spp.,) were observed in fruit as well as in other parts of the plant. However, *Rhizopus stolonifer* and *Sclerotinia minor* fungus species exist only in the fruit part of the plant [11]. The intensity of yeast on ripe strawberry fruits was 10 times higher than the intensity of yeast on unripe fruit [12]. In addition, the natural yeast flora of strawberries was 10 times more sensitive to fungicides such as Switch and Signum than mould fungi such as *C. acutatum* and *B. cinerea* [12]. This result indicates that fungicides developed against strawberry pathogens are predominantly more effective on yeast flora. Despite the restrictions and prohibitions introduced in the use of fungicides in recent years, these fungicides are still in use and cause to change the profile of microbial flora, especially on the fruits. For this reason, it is necessary to determine the natural surface flora of fruits produced without using fungicides.

Microbial enzymes are utilized in many fields such as agricultural, chemical industry, food processing industry, textile industry, pharmaceuticals, wood processing industry, analytical applications, cosmetics, and environmental pollution control, such as bioremediation and biodegradation [13]. In the food industry, these enzymes are used in mainly dairy products, wine production and bakery. Microbial enzymes are effective in enhancing the flavor and nutrient values of the products during the fermentation process [14].

This research aims to determine yeast biota on strawberry surfaces and to identify the yeast species having industrially important extracellular enzyme activities. The yeast diversity was determined on strawberry fruits collected from fungicide treated and organic gardens and a totally 47 of yeast strains were

isolated. These yeast strains were identified as *M. pulcherrima, H. uvarum* and *W. pijperi* according to sequence analysis of the 26S rDNA gene region. The distribution of *W. pijperi* yeast species on strawberry fruit was determined for the first time in our study. The evolutionary history was inferred with the Maximum Parsimony method using MEGAX software [15, 16]. The extracellular enzyme profiles of yeast strains were determined with the API-ZYM kit system. All yeast strains were displayed a high industrially important β-glucosidase activity.

2. MATERIALS AND METHODS

2.1. Fruit sampling and isolation

Strawberry samples were collected from two different fruit gardens located in Gelibolu peninsula (40° 51' 50" N 26° 37' 20" E) in Çanakkale, Turkey, during harvest (between June and October) in 2009 vintage. While different fungicides are applied in the first strawberry garden, no chemical is applied in the second garden since organic farming is carried out. In the fungicide-applied strawberry garden, Klorzon 10 EC (tetraconazole 100 g/l) and Topas 100 EC (penconazole 100 g/l) was applied twice with 7-day intervals and twice with 10-day intervals, respectively. From each garden, 250 g of healthy strawberry samples were randomly and aseptically collected. Samples were transported in cold boxes to the laboratory and analyzed within 24 h of harvest. About 10 g of each sample was aseptically homogenized in 100 ml of distilled water. Homogenates were serially diluted with sterile distilled water and 100 µl from each dilution was plated in duplicate on YGC medium (5 g/l yeast extract, 20 g/l glucose, 0.1 g/l chloramphenicol, 14.9 g/l agar) supplemented with 0.1% sodium propionate. After incubation at 30 °C for 3 days the colonies were counted out in duplicates. According to colony morphology and frequency, yeast colonies were isolated and restreaked on YEPD (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose) medium for purification. All isolated yeast strains were stored at 4 °C on YEPD slants and also -80 °C for future analysis.

2.2. DNA extraction and PCR-RFLP analysis

Genomic DNA extraction of yeast strains was carried out by a previously developed DNA extraction procedure [17]. ITS1-5.8S-ITS2 rDNA gene regions of yeast strains amplified using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and 26S rDNA gene regions were amplified using NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') primers as previously reported conditions [18, 19]. PCR products were electrophoresed and the length of PCR amplicons was calculated by Gel-Pro Analyzer v4.0 software. PCR products of ITS1-5.8S-ITS2 rDNA and 26S rDNA gene regions were purified GeneJet PCR Purification Kit (Thermo Scientific, K0702) and were digested with Hae III, Hha I and Hinf I restriction endonucleases, according to supplier's instructions. The length of restriction fragments was calculated by using Gel-Pro Analyzer v4.0 software The yeast strains were classified concerning restriction patterns.

2.3. Phylogenetic analysis

PCR products of selected nine yeast strains were sequenced by utilizing the Applied Biotechnologies 3500xl Genetic Analyzer. The attained 26S rDNA gene sequences were analyzed by using BLAST (Basic Local Alignment Search Tool) online tool on NCBI (National Center for Biotechnology Information) webserver. All sequences of 26S rDNA regions were uploaded to GenBank. 26S rDNA sequences of selected yeast strains were studied by using MEGAX (Molecular Evolutionary Genetics Analysis) software [16]. The

nucleotide sequences of 26S rDNA gene regions of nine yeast strains and *Saccharomyces cerevisiae* as an outgroup were aligned with ClustalW (v1.6) algorithm in MEGA-X. The maximum parsimony tree was constructed by using a bootstrap method and Subtree-Pruning-Regrafting (SPR) parameters for the determination of phylogenetic relationships of yeast strains [15, 20]. 1000 bootstrap replicates were used to defined branch support and bootstrap values above 50% were given.

2.4. Extracellular enzyme profile

Extracellular enzyme profiles of identified yeast strains were determined by using the API-ZYM kit system (Bio-Mérieux, France). API-ZYM kit system is a minimized and semi-quantitative test system and 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, ß-galactosidase, ß-glucuronidase, α-glucosidase, ß-glucosidase, N-acetyl-ß-glucosaminidase, α-mannosidase, α-fucosidase). All yeast strains were grown in a YEPD-Agar medium at 30 °C for 12 hours with constant shaking (120 rpm/rev). The 65 µl from the saturated yeast culture were transferred to each microwell of the API-ZYM strip. The API-ZYM strips were incubated at 37 °C for 4 hours. After that, 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 the color scalar of the API-ZYM kit system (0-5 scalar).

3. RESULTS AND DISCUSSION

3.1. Yeast identification and diversity

Fruit samples were collected from two different strawberry gardens wherein one different fungicides, Klorozon and Topas, were applied but in the other one, no chemicals were applied which make organic farming. Depending on the colony morphology differences 26 yeast strains from the organic farming garden (Garden 1, G1) and 21 yeast strains from the fungicides applied garden (Garden 2, G2) were selected randomly for future identifications (Table 1). The total yeast counts in Garden 1 and Garden 2 were calculated as 1.5 x 10^7 and 1.2 x 10^3 CFU/ml, respectively. According to European Commission Health & Consumer Protection Directorate, the acceptable limits of yeast and mould count in fruits like strawberries can be less than 10^3 CFU/g or ml [21]. In our counts, although the yeast concentration was close to the acceptable limits in Garden 2, it was observed that the yeast counts in Garden 1 were well above the acceptable limits. The main reason for this situation is that the organic farming application was carried out in the first garden and thus the collected strawberry samples were not exposed to fungicides.

Table 1. Isolated yeast strains from strawberry fruit.

Garden No	Yeast strains	CFU/ml
G-1	S-1, S-2, S-3, S-4, S-5, S-6, S-7, S-8, S-9, S-10, S-11, S-12, S-13, S-14, S-15, S-16, S-17, S-18, S-19, S-20, S-21, S-22, S-23, S-24, S-25, S-26	1.5 x 10^7
G-2	S-27, S-28, S-29, S-30, S-31, S-32, S-33, S-34, S-35, S-36, S-37, S-38, S-39, S-40, S-41, S-42, S-43, S-44, S-45, S-46, S-47	1.2 x 10^3

G-1: Organic strawberry field; G-2: fungicide treated strawberry field.

The effect of fungicides on epiphytic yeasts of grapes, grasses and strawberries were determined previously. The fungicide treatment of grapes (including cyprodinil + fludioxonil) and grasses (including

phyllosphere) resulted in a dramatic reduction of yeast density as compared with the untreated control [22,23]. However, in another research, the yeast counts on fungicide treated grapes (including iprodione, pyrimethanil, and cyprodinil + fludioxonil) were found higher than on control samples [24]. The yeast counts on strawberry samples treated with Switch (cyprodinil + fludioxonil) or Signum (boscalid + pyraclostrobin) were found to be similar to the control [12]. In our research, the yeast counts in untreated samples were greater than in fungicide treated samples. Different sampling and isolation strategies, sampling period and fruit ripening can cause these kinds of variations as indicated before [12, 25].

The morphology of colonies was determined at 25 °C on YEPD after growth for 3 days. The isolated forty-seven yeast strains were classified into four groups according to their colony morphology features (Table 2).

Table 2. Grouping of isolated yeast strains according to colony morphologies.

Group No	Yeast strains
1	S-1, S-3, S-4, S-5, S-6, S-7, S-9, S-11, S-12, S-20, S-27, S-33, S-38, S-39, S-40
2	S-14, S-15, S-16, S-18, S-22, S-24, S-26, S-29, S-30, S-31, S-32, S-37, S-43, S-47
3	S-17, S-19, S-21, S-23, S-25, S-28, S-34, S-35, S-36, S-45, S-46
4	S-2, S-8, S-10, S-13, S-41, S-42, S-44

Restriction analyzes of ITS1-5.8S-ITS2 and 26S rDNA gene regions are used to identify yeast strains isolated from different foods and to determine the differences between strains [26-28]. The amplification results of ITS1-5.8S-ITS2 and 26S rDNA regions were given in Table 3 and Table 4, respectively. It was observed that PCR products of ITS1-5.8S-ITS2 were formed in two groups with the length of ~400bp (29 yeast strains) and ~650bp (18 yeast strains). Similarly, PCR products of 26S rDNA were grouped in two with the length of ~550bp (29 yeast strains) and ~650bp (18 yeast strains). Because of the variability within the ribosomal DNA regions, the restriction fragment length polymorphism (RFLP) analysis of these regions is useful for interspecies and intraspecies level identification of yeasts [29, 30]. Therefore, in this study, the amplified rDNA regions of 26S rDNA and ITS1-5.8S-ITS2 rDNA regions were cut with Hae III, Hha I and Hinf I restriction enzymes and regrouped again according to the restriction fragment lengths. It was observed that the restriction profiles of yeast strains present in the first and second ITS1-5.8S-ITS2 rDNA PCR groups were similar (Table 3). The yeast strains in the first 26S rDNA PCR group displayed similar restriction patterns while the second PCR group showed three restriction profiles (Table 4). The restriction patterns of Hinf I, Hae III, and Hha I restriction enzymes in this group were similar to previously reported profiles of *Metschnikowia pulcherrima* yeast species [31, 32]. Eleven yeast strains (S-17, S-19, S-21, S-23, S-25, S-28, S-34, S-35, S-36, S-45 and S-46) were not digested with Hha I restriction enzyme and thus it has a distinct profile from other yeast strains. It was observed that two yeast strains (S-8 and S-13) have differed from other yeast strains (S-2, S-10, S-41, S-42 and S-44) for the Hae III restriction pattern. Generally, the restriction patterns of yeast strains with HaeIII, HinfI and HhaI enzymes are similar to previous studies [27, 32-35].

When the yeast strains present in colony morphology groups were compared with the PCR-RFLP group, the yeast strains present in the first and second colony morphology groups localized in the first group of ITS1-5.8S-ITS2 and 26S rDNA. All yeast strains in the third morphology group localized in the same 26S rDNA group. The yeast strains in the fourth morphology group were divided into two different groups: two yeast strains (S-8, S-13) in group 3 and five yeast strains (S-2, S-10, S-41, S-42, S-44) in group 4. No

difference in morphology and PCR-RFLP groups was observed in the distribution of yeast strains isolated from strawberries collected from organic and fungicide-treated gardens.

Table 3. PCR-RFLP results of ITS1-5.8S-ITS2 rDNA gene region.

PCR*	Profile number	Yeast strains	Restriction Fragment*		
			Hae III	Hha I	Hinf I
~400	1	S-1, S-3, S-4, S-5, S-6, S-7, S-9, S-11, S-12, S-14, S-15, S-16, S-18, S-20, S-22, S-24, S-26, S-27, S-29, S-30, S-31, S-32, S-33, S-37, S-38, S-39, S-40, S-43, S-47	270-110	210-95-95	190-190
~650	2	S-2, S-8, S-10, S-13, S-17, S-19, S-21, S-23, S-25, S-28, S-34, S-35, S-36, S-41, S-42, S-44, S-45, S-46	-	315-310-125	325-175-160-65

PCR and restriction products were given as base pair (bp).

Table 4. PCR-RFLP results of 26S rDNA gene region.

PCR*	Profile number	Yeast strains	Restriction Fragment*		
			Hae III	Hha I	Hinf I
~550	1	S-1, S-3, S-4, S-5, S-6, S-7, S-9, S-11, S-12, S-14, S-15, S-16, S-18, S-20, S-22, S-24, S-26, S-27, S-29, S-30, S-31, S-32, S-33, S-37, S-38 S-39, S-40, S-43, S-47	285-115-110-75	390-150-60	340-230
~650	2	S-17, S-19, S-21, S-23, S-25, S-28, S-34, S-35, S-36, S-45, S-46	440-130-125	-	400-190
	3	S-2, S-10, S-41, S-42, S-44	440-130-125	530-65	430-215-50
	4	S-8, S-13	370-150-140	530-65	430-215-50

*PCR and restriction products were given as base pair (bp).

Employing PCR-RFLP analysis, four different restriction profiles were attained. We assumed that each restriction profile may represent different yeast species. At least one yeast strain from the groups formed according to morphological differences and PCR-RFLP profiles was randomly selected and used for sequencing. Therefore, nine yeast strains (S-3, S-5, S-8, S-10, S-19, S-20, S-22, S-24 and S-33) were sequenced and analyzed by the BLAST tool on the NCBI web server. The nucleotide sequences of the 26S rDNA gene region were submitted to GenBank Database on NCBI and attained accession numbers for all sequences (Table 5).

According to the BLAST analysis of the 26S rDNA gene region, all sequenced yeast strains displayed 96-99% similarity with their reference yeast strains except S-19. This yeast strain showed 87.0% and 81.71% similarity with the reference strains of KT922724.1 and KY107833.1 (CBS: 2585), respectively. S-3, S-5, S-20, S-22, S-24 and S-33 yeast strains were identified as *M. pulcherrima*. Therefore, 29 yeast strains present in the first PCR-RFLP group of ITS1-5.8S-ITS2 and 26S rDNA can be identified as *M. pulcherrima.* Similarly, the sixteen yeast strains present in the second and third PCR-RFLP group of 26S rDNA can be defined as *Hanseniaspora uvarum*. Since the S-8 yeast strain was identified as *Wickerhamomyces pijperi* according to the BLAST result, it can be assumed that the S-13 yeast strain is the same species. These results showed that the restriction enzymes used for RFLP analysis were suitable for the discrimination of yeast strains.

The predominant yeast species associated with strawberry fruits were *M. pulcherrima* (61.7%), *H. uvarum* (34.0%) and *W. pijperi* (4.3%). The percent distribution of *M. pulcherrima* yeast species on strawberry fruits collected from Garden 1 and Garden 2 was similar: 65.4% in the organic garden and 57.1%

in the fungicides applied garden. On the other hand, it was determined that the *H. uvarum* yeast population (42.9%) on strawberry fruits collected from the fungicides applied garden was higher than the organic garden (26.9%). *W. pijperi* yeast species was identified only on strawberry samples collected from the Organic garden. It is observed that the yeast strains belonging to *M. pulcherrima* and *H. uvarum* were dominant on strawberry fruits.

Table 5. BLAST results of 26S rDNA gene region.

Yeast Strains	Similarity (%)	Identified yeast strains (Ref. Acc. Number)	GenBank Accession Number
S-3	96.70%	*M. pulcherrima* (KY108490.1)	MZ401466
S-5	96.08%	*M. pulcherrima* (KY108490.1)	MZ401467
S-8	99.57%	*W. pijperi* (KY110127.1)	MZ401468
S-10	99.82%	*H. uvarum* (KY107833.1)	MZ401469
S-19	81.71%	*H. uvarum* (KY107833.1)	MZ401470
S-20	97.77%	*M. pulcherrima* (KY108498.1)	MZ401471
S-22	96.81%	*M. pulcherrima* (KY108490.1)	MZ401472
S-24	98.59%	*M. pulcherrima* (KY108497.1)	MZ401473
S-33	96.35%	*M. pulcherrima* (KY108490.1)	MZ401474

It was shown that the application of fungicides did not affect the diversity of the epiphytic yeast community on strawberries [12]. Similarly, in our research the diversity of yeast species on strawberries was similar, but the density of some yeast species on fruits collected from fungicide treated and untreated gardens was different.

Previously reported that 32 different yeast species were distributed on soft-grained fruits (raspberry, blackberry, strawberry, etc.) [26]. In our study, only *M. pulcherrima* and *H. uvarum* yeast species were identified on strawberry fruit, but the other yeast species cannot be determined. However, *W. pijperi* yeast species, which was previously reported in blackberry juice, pineapple and grape grains, was not recorded on strawberry fruits [36]. Thus *W. pijperi* yeast strain was reported on strawberry fruit for the first time in our study. *M. pulcherrima* and *H. uvarum* yeast species are used as biocontrol agents. It has been reported that they have protective properties against *Botrytis cinerea*-induced diseases that occur after harvest in some fruits [37-40].

3.2. Phylogenetic analysis

The evolutionary history was inferred using the Maximum Parsimony method [15]. The phylogenetic analysis of sequenced yeast strains was carried out by using the MEGAX phylogenetic analysis tool [16]. The D1/D2 domain of the 26S rDNA gene sequences of yeast strains were aligned by the ClustalX v1.6 algorithm and the maximum parsimony tree was constructed by using default parameters (Figure 1). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Therefore, 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. The MP 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) [20]. This analysis involved 15 nucleotide sequences belonging to nine isolated yeast strains, three reference strains and one outgroup strain. *S. cerevisiae* yeast

species was selected as an outgroup. The included codon positions were 1st + 2nd + 3rd + Noncoding. There were a total of 1071 positions in the final dataset. Consistency Index for all sites (CI) and parsimony informative sites (iCI) were 0.846154 and 0.772021, respectively. The Retention Index for all sites (RI) and parsimony informative sites (iRI) were 0.840387 and 0.840387, respectively. Rescaled Index for all sites (RC) and parsimony informative sites (iRC) were 0.711097 and 0.648796, respectively. According to the maximum parsimony tree, nine yeast strains were separated into two main clades. It was determined that the first clade consisted of two subclades including *M. pulcherrima* yeast strains in the first subclade and *H. uvarum* yeast strains in the second subclade. The second clade in the maximum parsimony tree contained *W. pijperi* yeast species.

3.3. Extracellular enzyme profiles

The ability of the yeasts to the breakdown peptides, phosphomonoesters, lipids, mucopolysaccharides, polysaccharides, chitin, cellulose, starch, and galactans may be evaluated simply with API ZYM assay [41]. Characterization of extracellular hydrolytic enzyme activities is important for industrial applications of the yeast species. In addition, it is suitable for assessing microbial, biochemical and functional diversity of microorganisms like yeast. Thus, characterizing these enzyme activities can be utilized to define and discriminate the yeast strains within the species [42]. In this study, the extracellular enzyme profile of all isolated yeast strains was determined using the API-ZYM kit system. The activity of the enzymes was expressed in nanomoles of the hydrolyzed substrate according to the intensity of the color reaction on a five-step scale: 0 means no reaction, 1 means 5 nanomoles, 2 means 10 nanomoles, 3 means 20 nanomoles, 4 means 30 nanomoles, 5 means 40 nanomoles and more [43].

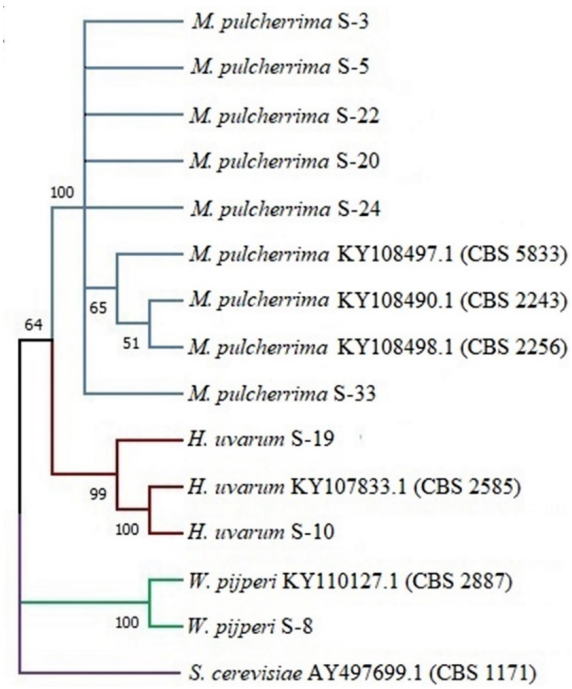

Figure 1. Maximum-parsimony phylogenetic tree of yeast species obtained with sequences of 26S rDNA regions. MP tree was constructed by using the bootstrap method and Subtree-Prunning-Regrafting (SPR) parameters in MEGAX software. 1000 bootstrap replicates were used to define branch support and above 50% bootstrap values were given. *S. cerevisiae* yeast was selected as an outgroup.

According to APIZYM test results, the extracellular enzyme profile of yeast strains was given in Table 6, and the enzyme profile of one yeast strain representing each group was presented in Figure 2. It was found that none of the isolates had an activity of lipase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Therefore, these enzymes were not included in Table 6 and Figure 2. Although high levels of leucine arylamidase and β-glucosidase activity were found in all yeast strains, there were differences in other enzyme activities.

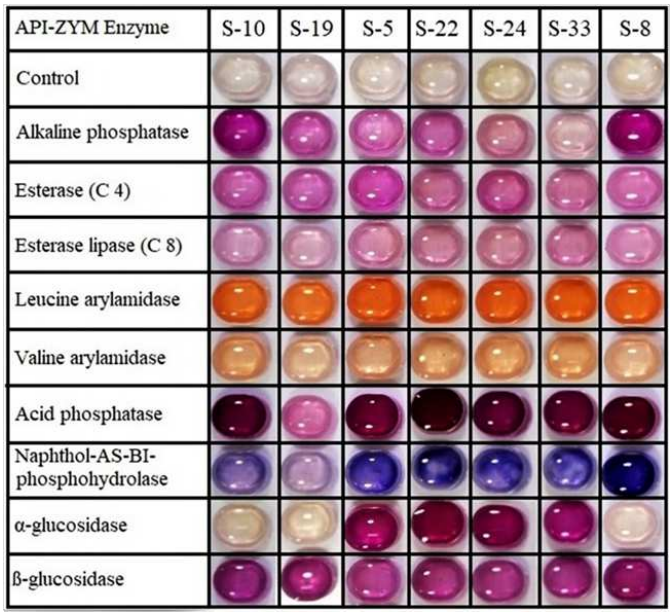

Figure 2. The extracellular enzyme activities of yeast strains. Seven yeast strains representing each enzyme profile were selected and given.

Table 6. Extracellular enzyme profile of yeast strains determined with API ZYM test.

Yeast strains	C	1	2	3	4	5	6	7	8	9
M. pulcherrima (S-1, S-3, S-4, S-5, S-6, S-7, S-11, S-27, S-29, S-32, S-38, S-47)	0	3	4	2	5	2	5	4	4	3
M. pulcherrima (S-9, S-14, S-15, S-16, S-18, S-22, S-37)	0	3	2	2	5	2	5	4	5	3
M. pulcherrima (S-12, S-20, S-24, S-31, S-40)	0	2	3	2	5	2	5	4	5	3
M. pulcherrima (S-26, S-30, S-33, S-39, S-43)	0	1	2	2	5	2	5	4	4	4
H. uvarum (S-2, S-10, S-41, S-42, S-44)	0	5	3	2	5	2	5	2	0	4
H. uvarum (S-17, S-19, S-21, S-23, S-25, S-28, S-34, S-35, S-36, S-45, S-46)	0	3	3	1	5	1	2	1	0	4
W. pijperi (S-8, S-13)	0	5	2	2	5	1	5	5	0	4

C, Control; 1: Alkaline Phosphatase; 2: Esterase (C4); 3: Esterase Lipase (C8); 4: Leucine arylamidase; 5: Valine arylamidase; 6: Acid Phosphatase; 7: Naphthol-AS-BI-phosphohydrolase; 8: α-glucosidase; 9: β-glucosidase.

W. pijperi yeast species (S-8 and S-13) showed the same extracellular enzyme profile. Although these yeast strains showed high alkaline phosphatase, acid phosphatase naphthol-AS-BI-phosphohydrolase and β-glucosidase enzyme activity, it was determined that they did not contain α-glucosidase enzyme activity as

seen in *H. uvarum* yeast species. All *H. uvarum* yeast strains showed high leucine arylamidase and β-glucosidase activity but no α-glucosidase activity was recorded. The alkaline phosphatase, acid phosphatase and naphthol-AS-BI-phosphohydrolase enzyme activities of yeast strains (S-17, S-19, S-21, S-23, S-25, S-28, S-34, S-35, S-36, S-45, S-46) were lower than other *H. uvarum* yeast strains (S-2, S-10, S-41, S-42, S-44). Interestingly, it was observed that the groups formed according to the extracellular enzyme profiles of *H. uvarum* yeast strains were overlapped with the group of 26S rDNA PCR-RFLP.

Even though all *M. pulcherrima* yeast strains showed a single restriction profile according to PCR-RFLP results, it was determined that yeast strains had four different enzyme profiles according to extracellular enzyme results. All *M. pulcherrima* yeast strains showed high leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase activity. However, slight differences were observed in the alkaline phosphatase, esterase, esterase lipase and β-glucosidase enzyme activities of yeast strains. In our results, *M. pulcherrima* yeast strains showed different enzyme activities even if they were identified as the same species, like *H. uvarum* strains.

The β-glucosidase enzyme (EC 3.2.1.21) is an industrial enzyme used to break the β-1-4 glycosidic bond in oligosaccharides or glycosidic compounds. β-glucosidases are also used in fruit juice and wine production, as well as the sweetening, aroma formation and quality enhancement of wines. Leucine arylamidase enzyme (EC 3.4.11.2) is an enzyme belonging to the aminopeptidase group and hydrolyzes the N-terminal ends of amino acids. Like the β-glucosidase enzyme, leucine arylamidase is also used in wine production to increase the aroma and taste quality of wines [44-47].

4. CONCLUSION

Due to the absence of antibiotics or mycotoxins production in yeast, yeasts have been used alone or integrated with other control methods in the biological control of some fungal diseases in fruits. [12,48-50]. It is important to determine yeast diversity which including potential yeast strains for the biological control, of fruits. Therefore, in this study, yeast diversity and extracellular enzyme profiles of yeast strains were determined on strawberry fruits collected from the organic and fungicide applied fields. It was determined that yeast density in strawberry fruits collected from organic farming fields was higher than the fungicide treated strawberries. *W. pijperi* yeast species was recorded only in organic strawberry fruits, and the distribution of other yeast species was determined to be similar. The results indicate that the fungicide treatment has no drastic effect on yeast species with high density, but it causes the elimination of rarely found yeast species in biota. In addition, the fungicide treatment did not affect the extracellular enzyme profile of yeast strains. All isolated yeast strains showed industrially important high β-glucosidase activity. Although *M. pulcherrima* and *H. uvarum* yeast strains were defined as the same species by 26S rDNA sequencing analysis, it was determined that yeast strains revealed different extracellular enzyme activities within the species. As indicated before, the API-ZYM system could be useful for the identification of yeast strains of the genus *Metschnikowia* and *Hanseniaspora* and the differentiation of the enzymotypes for epidemiological purposes. In the future, for the selection of industrial yeast species, it will be appropriate to determine the biochemical and metabolic differences of yeast strains besides the molecular differences.

Authors' Contributions: TTG: sampled the fruits, designed the experiments and wrote the article. MG: made all the laboratory experiments. Both authors read and approved the final manuscript.

Conflict of Interest: The authors have no conflict of interest to declare.

Acknowledgment: This work was supported by Çanakkale Onsekiz Mart University the Scientific Research Coordination Unit, Project number: FHD-2019-2689.

REFERENCES

1. Kurup VP. Fungal allergens. Curr Allergy Asthma Rep. 2003; 3: 416-423.
2. Mons E. Occupational asthma in greenhouse workers. Curr Opin Pulm Med. 2004; 10(2): 147-150.
3. Droby S, Chalutz E, Hofstein R, Wilson CL, Wisniewski ME, Fridlender B, et al. Pilot testing of *Pichia guilliermondii*: a biocontrol agent of postharvest diseases of citrus fruit. Biol Control. 1993; 3: 47-52.
4. Dermesonlouoglou EK, Bimpilas A, Andreou V, Katsaros GJ, Giannakourou MC, Taoukis PS. Process optimization and kinetic modelling of quality of fresh-cut strawberry cubes pretreated by high pressure and osmosis. J Food Proces Preserv. 2016; 41: 13137.
5. Dermesonlouoglou EK, Giannakourou M, Taoukis PS. Kinetic study of the effect of the osmotic dehydration pre-treatment with alternative osmotic solutes to the shelf life of frozen strawberry. Food Bioprod Process. 2016; 99: 212-221.
6. Tournas VH, Katsoudas E. Mould and yeast flora in fresh berries, grapes and citrus fruits. Int J Food Microbiol. 2005; 105: 11-17.
7. Wedge DE, Smith BJ, Quebedeaux JP, Constantin RJ. Fungicide management strategies for control of strawberry fruit rot diseases in Louisiana and Mississippi. Crop Prot. 2007; 26: 1449-1458.
8. Menzel CM, Gomez A, Smith LA. Control of grey mould and stem-end rot in strawberry plants growing in a subtropical environment. Australas Plant Pathol. 2016; 45: 489-498.
9. De la Torre M, Millan M, Perez-Juan P, Morales J, Ortega J. Indigenous yeasts associated with two Vitis vinifera grape varieties cultured in southern Spain. Microbios. 1999; 100: 27-40.
10. Guerra E, Sordi G, Mannazzu I, Clementi F, Fatichenti F. Occurrence of wine yeasts on grapes subjected to different pesticide treatments. Ital J Food Sci. 1999; 3(11): 221-230.
11. Rigotti S, Viret O, Gindrat D. Fungi from symptomless strawberry plants in Switzerland. Phytopathol Mediterr. 2003; 42: 85-88.
12. Debode J, Van Hemelrijck W, Creemers P, Maes M. Effect of fungicides on epiphytic yeasts associated with strawberry. Microbiologyopen. 2013; 2(3): 482-491.
13. Liu X, Kokare C. Microbial enzymes of use in industry. In: Brahmachari G, ed. Biotechnology of microbial enzymes. Academic Press, Cambridge, 2017.
14. Li S, Yang X, Yang S, Zhu M, Wang X. Technology prospecting on enzymes: application, marketing and engineering. Comput Struct Biotechnol J. 2012; 9(2): e201209017.
15. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evol. 1985; 39: 783-791.
16. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018; 35: 1547-1549.
17. Kristjuhan A, Lõoke M, Kristjuhan K. Extraction of Genomic DNA from Yeasts for PCR-Based Applications. Biotechniques. 2011; 50(5): 325-328.
18. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR Protocols: A Guide to Methods and Applications. San Diego, California, Academic Press, 1990: 315-322.
19. Kurtzman CP, Robnett CJ. Identification and Phylogeny of Ascomycetous Yeasts from Analysis of Nuclear Large Subunit (26S) Ribosomal DNA Partial Sequences. Antonie Leeuwenhoek. 1998; 73: 331-371.

20. Nei M, Kumar S. Molecular Evolution and Phylogenetics. Oxford University Press, New York, 2000.
21. European Commission. European Commission Health & Consumer Protection Directorate general. OECD Issue Paper on Microbial Contaminant Limits for Microbial Pest Control Products. 2012; 65: 43-53.
22. Comitini F, Ciani M. Influence of fungicide treatments on the occurrence of yeast flora associated with wine grapes. Ann. Microbiol. 2008; 58: 489-493.
23. Buck JW., Burpee LL. The effects of fungicides on the phylloplane yeast populations of creeping bentgrass. Can J Microbiol. 2002; 48: 522-529.
24. Cadez N, Zupan J, Raspor P. The effect of fungicides on yeast communities associated with grape berries. FEMS Yeast Res. 2010; 10: 619-630.
25. Fonseca A, Inacio J. Phylloplane yeasts. C. Rosa and G. Peter, eds. Biodiversity and ecophysiology of yeasts. The yeast handbook. Springer, Berlin, 2006: 263-301.
26. Babjeva I, Reshetova I. Yeast Resources in Natural Habitats at Polar Circle Latitude. Food Technol Biotech. 1998; 36(1): 1-5.
27. Baffi MA, Bezerra CS, Arévalo-Villena M, Briones-Pérez AI, Gomes E, Da Silva R. Isolation and molecular identification of wine yeasts from a Brazilian vineyard. Ann Microbiol. 2010; 61: 75-78.
28. Carvalho CM, Meirinho S, Estevinho MLF, Choupina A. Yeast species associated with honey: Different identification methods. Arch Zootec. 2010; 59(225): 103-113.
29. Kurtzman CP, Fell JW. Summary of species characteristics. In: Kurtzman CP & Fell JW (Eds) The Yeasts, A Taxonomic Study, 4th edn. Elsevier Science BV, Amsterdam, 1998: 915-947.
30. Pincus DH, Orenga S, Chatellier S. Yeast identification past, present, and future methods. Med Mycol. 2007; 45: 97-121.
31. Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A. Identification of Yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int J Syst Evol Microbiol. 1999; 49: 329-337.
32. Rodrıguez-Vico F, Clemente-Jimenez JM, Mingorance-Cazorla L, Martinez-Rodriguez S, Las Heras-Vazquez FJ. Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. Food Microbiol. 2003; 21: 149-155.
33. Guillamon JM, Sabate J, Barrio E, Cano J, Querol A. Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. Arch Microbiol. 1998; 169(5): 387-392.
34. Romano P, Capece A, Siesto G, Romaniello R. Restriction analysis of rDNA regions to differentiate non-*Saccharomyces* wine species in mixed cultures. Int J Eng Res Technol. 2009; 1(4): 068-071.
35. Gibson BR, Pham T, Wimalasena T, Box WG, Koivuranta K, Storgards E, Smart KA. Evaluation of ITS PCR and RFLP for differentiation and identification of brewing yeast and brewery 'wild' yeast contaminants. J Inst Brew. 2011; 117(4): 556–568.
36. Mambuscay LA, Lopez WA, Cuervo RA, Argote FE, Osorio-Cadavid E. Identification of yeast in the native pineapple juice, blackberry and grape [in Spanish]. Biotecnol Sector Agropecuario Agroindustr. 2013; 2: 136-144.
37. De Curtis F, Torriani S, Rossi F, De Cicco V. Selection and use of *Metschnikowia pulcherrima* as a biological control agent for postharvest rots of peaches and table grapes. Ann Microbiol. 1996; 46: 45-55.
38. Piano S, Neyrotti V, Migheli Q, Gullino ML. Biocontrol capability of *Metschnikowia pulcherrima* against *Botrytis postharvest* rot of apple. Postharvest Biol Tec. 1997; 11: 131-140.
39. Spadaro D, Vola R, Piano S, Gullino ML. Mechanisms of action and efficacy of four isolates of the yeast *Metschnikowia pulcherrima* active against postharvest pathogens on apples. Postharvest Biol Tec. 2002; 24(2): 123-134.

40. Hua L, Yong C, Zhanquan Z, Boqiang L, Guozheng Q, Shiping T. Pathogenic mechanisms and control strategies of *Botrytis cinerea* causing post-harvest decay in fruits and vegetables. Food Quality Safety. 2018; 2(3): 1-9.
41. Bending GD, Turner MK, Jones JE. Interactions between crop residue and soil organic matter quality and the functional diversity of soil microbial communities. Soil Biol Biochem. 2002; 34: 1073-1082.
42. García-Martos P, Marín P, Hernández-Molina JM, García-Agudo L, Aoufi S, Mira J. Extracellular enzymatic activity in 11 *Cryptococcus* species. Mycopathologia. 2001; 150(1): 1-4.
43. Baldrian P, Kolařík M, Stursová M, Kopecký J, Valášková V, Větrovský T, Zifčáková L, Snajdr J, Rídl J, Vlček C, Voříšková J. Active and total microbial communities in forest soil are largely different and highly stratified during decomposition. The ISME J. 2012; 6(2): 248-258.
44. Fleet GH, Charoenchai C, Henschke PA, Todd BEN. Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. Aust J Grape Wine Res. 1997; 3(1): 2-8.
45. Dodor DE, Tabatabai MA. Effects of cropping systems and microbial biomass on arylamidase activity in soils. Biol Fert Soils. 2002; 35: 253-261.
46. Nikolaou E, Soufleros EH, Bouloumpasi E, Tzanetakis N. Selection of indigenous *Saccharomyces cerevisiae* strains according to their oenological characteristics and vinification results. Food Microbiol. 2006; 23(2): 205-211.
47. Nikolaou A, Meriç S, Fatta D. Occurrence patterns of pharmaceuticals in water and wastewater environments. Anal Bioanal Chem. 2007; 387: 1225-1234.
48. Guetsky R, Elad Y, Stienberg D, Dinoor A. Establishment, survival and activity of the biocontrol agents *Pichia guilliermondii* and *Bacillus mycoides* applied as a mixture on strawberry plants. Biocontrol Sci Tech. 2002; 12: 705-714.
49. Wszelaki AL, Mitcham EJ. Effect of combinations of hot water dips, biological control and controlled atmospheres for control of gray mold on harvested strawberries. Postharvest Biol Tec. 2003; 27: 255-264.
50. Zhang HY, Wang L, Dong Y, Jiang S, Cao H, Meng RJ. Postharvest biological control of gray mold decay of strawberry with *Rhodotorula glutinis*. Biol Control. 2007; 40: 287-292.