

**ÇUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES**

PhD THESIS

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**BIOCONTROL OF FUNGAL PATHOGENS RESPONSIBLE
FOR POSTHARVEST DISEASES IN APPLE AND LEMON
USING ANTAGONISTIC YEASTS**

DEPARTMENT OF FOOD ENGINEERING

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ABSTRACT

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In the present study, the biocontrol potential of epiphytic yeasts against two significant postharvest pathogens, *Penicillium expansum* DSM62841 (blue mold) and *Penicillium digitatum* DSM2750 (green mold) was evaluated *in vitro* and *in vivo*. ‘Starking Delicious’ and ‘Kutdiken’ cultivars were selected as the apple and lemon varieties, respectively. A total of 69 yeasts isolated from different cultivars of apples and lemons were identified by a combination of PCR-RFLP of the 5.8 S rRNA region and sequencing of the D1/D2 domain of the 26 S rRNA gene. After preliminary screening, *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL emerged as promising antagonistic strains due to their strong antagonistic activity against blue and green molds. Besides the experimentation with single and mixed cultures of these yeasts, the effects of different concentrations of the yeasts on biocontrol activity were also investigated. It was discovered that the antagonistic effect of these two yeast strains depends on several mechanisms of action, including competition for space and nutrients, production of VOCs, secretion of extracellular lytic enzymes (β -1,3 glucanase, protease and gelatinase), and inhibition of fungal spore germination. Using antagonistic yeasts, percent (%) inhibition of the mycelial growth of *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750 *in vitro* was achieved by 83.40% and 74.70%, respectively. Apples and lemons were stored in a cold room under industrial conditions for 3 months, and infection rate, disease severity, and lesion diameter of pathogens decreased by yeast treatment compared to the control throughout the storage period. In addition, yeast coated apples and lemons were stored at ambient condition (20 ± 2 °C and 50 ± 5 % RH) for 45 days, and results indicate that yeast application showed positive impact on the quality parameters including pH, titratable acidity, weight loss, brix, texture, and browning during storage. Biodegradation of patulin was achieved successfully by both of the studied yeasts and their mixed culture *in vitro*.

Key Words: Biocontrol, yeast, antagonist, apple, lemon, blue mold, green mold

ÖZ

DOKTORA TEZİ

ELMA VE LIMONDA BOZULMA ETMENİ OLAN FUNGAL PATOJENLERİN ANTAGONİSTİK MAYALAR İLE BİYOKONTROLÜ

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ÇUKUROVA ÜNİVERSİTESİ EN BİLİMLERİ ÜNİVERSİTESİ GIDA MÜHENDİSLİĞİ BÖLÜMÜ

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Bu çalışmada, epifitik mayaların iki önemli hasat sonrası patojene karşı, *Penicillium expansum* DSM62841 (mavi küf) ve *Penicillium digitatum* (yeşil küf) biyokontrol potansiyelleri *in vitro* ve *in vivo* olarak değerlendirilmiştir. Elma çeşidi olarak Starking Delicious, limon cinsi olarak ise Kütüden kullanılmıştır. Farklı elma ve limon çeşitlerinden izole edilen toplam 69 izolatin 5.8 S rRNA bölgelerine PCR-RFLP uygulanmış ve sonrasında 26 S rRNA genine ait D1/D2 bölgesinin sekans dizisi elde edilerek moleküler tanımlamaları yapılmıştır. Ön testlerden sonra, *Aureobasidium pullulans* GE17 ve *Meyerozyma guilliermondii* KL3 suşları mavi ve yeşil küflere karşı göstermiş oldukları güçlü antagonist aktiviteden dolayı umut verici antagonist suşlar olarak seçilmiştir. Denemelerde bu mayalar tekli ve karışık kültür olarak test edilmiş ayrıca maya hücre sayılarının farklı konsantrasyonlarının biyokontrol aktivite üzerine etkisi de incelenmiştir. Bu iki mayanın antagonist etkilerinin alan ve besin için rekabet etme, uçucu organik bileşik üretimi, hücre dışı litik enzim üretimi (β -1,3 glukanaz, proteaz ve jelatinaz) ve fungal spor çimlenmesinin inhibisyonu gibi birden fazla mekanizmaya bağlı olduğu belirlenmiştir. Antagonist maya kullanımı ile *Penicillium expansum* DSM62841 ve *Penicillium digitatum* DSM2750 patojenlerinin misel gelişimi *in vitro* koşullarda sırasıyla %83.40 ve %74.70 oranlarında engellenmiştir. Elma ve limonlar endüstriyel koşullarda soğuk hava odalarında üç ay boyunca depolanmış ve depolama süresi boyunca maya uygulanan meyvelerde bozulma oranı, bozulma şiddeti ve patojen lezyon çapı değerlerinde kontrol grubuna göre düşüş belirlenmiştir. Ek olarak, yüzeyleri maya ile kaplanmış bir şekilde elma ve limonlar oda koşullarında (20 ± 2 °C ve 50 ± 5 % RH) 45 gün süreyle depolanmış ve elde edilen sonuçlara göre maya uygulamasının meyvelerin pH, asitlik, ağırlık kaybı, briks, tekstür ve esmerleşme gibi kalite özelliklerini olumlu etkilediği belirlenmiştir. Çalışmada kullanılan her iki mayanın ve bunların karışık kültürünün patulini *in vitro* koşullarda başarılı bir şekilde parçaladığı belirlenmiştir.

Anahtar Kelimeler: Biyokontrol, maya, antagonist, elma, limon, mavi küf, yeşil küf

EXTENDED SUMMARY

Before and after harvest, various spoilages occur in fresh fruits and vegetables. A great majority of these deteriorations causing economically significant losses are caused by fungal diseases. As a result of fungal diseases, quality problems are observed in fruits and vegetables and their shelf life is shortened. In addition, the ratio of postharvest losses in fruits and vegetables is increasing due to transportation, processing, and storage under inappropriate conditions. While these losses constitute 25 % of the total fruits and vegetables produced in developed countries, it is over 50 % in developing countries ([Spadaro and Droby, 2016](#); [Agirman and Erten, 2020](#)). According to the recent report published by FAO, the amount of food produced is increasing rapidly with the continuous increase of the global population. While the world population has doubled in the last 50 years, food production has tripled ([FAO, IFAD, UNICEF, WFP and WHO, 2019](#)). Despite this, in 2018, more than 821 million people around the world were reported to be starving and malnourished ([Güneş and Turmuş, 2020](#)). For these reasons, it is essential for humanity to increase food production depending on the increased world population and to deliver the produced food to the consumer with as few losses as possible.

Today, use of synthetic fungicides is the primary method to control postharvest diseases of fruits and vegetables worldwide. However, the development of fungicide-resistant biotypes of pathogens and strong consumer demand to reduce the use of harmful chemicals in their food supply urgently led to a shift in global trend to seek safer, high-efficiency, low-residue, nontoxic, environmentally and economically friendly alternative biocontrol methods. Among the different approaches that have emerged for postharvest disease management in fruits and vegetables and to reduce the use of synthetic fungicides, biocontrol with antagonistic yeasts has emerged as a promising method.

The main purpose of this study is to ensure the biocontrol of fungal pathogens, which are the main cause of postharvest diseases in apples and lemons, with epiphytic antagonistic yeasts and to minimize the losses in these fruits after harvest. For this purpose, the yeasts used in the study were isolated from the surfaces of apple and lemon, and identified by molecular methods. The efficacy of identified epiphytic yeasts against the target pathogens used in the study was determined by applying a preliminary test. Among all isolates, the most successful two yeast strains were selected for subsequent analyses. Afterwards, the biocontrol potential and possible action mechanisms of selected yeasts were determined by various *in vitro* analyses. Within the scope of *in vitro* analyses, yeast strains were used as single and mixed cultures. In addition, studies were carried out on different concentrations (10^6 and 10^8 cells/mL) of yeasts to determine the cell number exhibiting the highest activity against fungal pathogens. Dual culture assay, production of volatile organic compounds (VOCs), secretion of extracellular lytic enzymes (protease, chitinase, pectinase, gelatinase and β -1,3 glucanase), iron competition, effect of yeasts on spore germination of fungal pathogens, and percent inhibition of mycelial growth were performed as *in vitro* analyses. There are many studies in the literature that have been reported to be successful in laboratory conditions but not on fruit. Based on this information, *in vivo* (on apple and lemon) analyses were carried out after *in vitro* analyses. In this context, apples and lemons were subjected to long-term storage in both standard room conditions and in cold rooms where industrial conditions were provided. The effect of yeast treatment on the quality parameters (pH, total acidity, brix, browning index, texture, weight loss, and infection rate) of apples and lemons during storage under standard room conditions was investigated. On the other hand, infection rate, disease severity, and lesion diameter values were followed regularly during the storage of fruits in cold rooms. Additionally, the ability of yeasts to colonize in the wounded parts of the fruits and the effect of VOCs produced by the yeasts on pathogen growth were

examined. Finally, the ability of the yeasts used in this study to degrade patulin, an important toxin produced by *Penicillium* spp. in apples, was investigated *in vitro*.

Apple and lemon fruits were selected as raw materials in this thesis, since these two fruits have a significant position for Turkey's agriculture and economy due to their great export and production rate. According to the latest updated data by FAOSTAT in 2019, Turkey ranked third in the world for apple production with the amount of 3.62 million tons, while in sixth place for lemon production as 950 thousand tons (FAOSTAT, 2019). “Starking Delicious” was chosen as the apple variety due to the fact that it creates 87 % of apple exports and 55 % of the total apple production in Turkey, and is also widely known and consumed as an international apple variety all over the world. “Kutdiken” cultivar was selected as the lemon variety in this thesis due to the fact that it is the most widely produced lemon cultivar in Turkey with high yield and quality, and also suitable for long term storage of up to nine months. There are also other reasons which caused the preference of this lemon type, such as it is a local variety unique to Turkey, as well as having a high as high cultivation ratio in the Çukurova region where this study was conducted. Patulin producer fungus *Penicillium expansum* (blue mold) (Liu et al, 2013) and *Penicillium digitatum* (green mold) (Pérez-Alfonso et al, 2012), which are the main spoilage factors in apples and lemons and commonly cause deterioration in many different fruits and vegetables, were selected as fungal pathogens intended to be controlled in this study.

Epiphytic yeasts, isolated from the surface of apples and lemons, were identified according to the sequence alignment of the D1/D2 region of the 26S rRNA gene. A total of 69 yeast isolates were identified and it was determined that these yeasts belong to five different genera and six different species. These species are as follows: *Saccharomyces cerevisiae*, *Saccharomyces paradoxus*, *Aureobasidium pullulans*, *Meyerozyma guilliermondii*, *Rhodotorula mucilaginosa* and *Wickerhamomyces anomalus*. As a result of preliminary tests, among 69 isolates, *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3

yeast strains were found as the most effective strains against two important postharvest pathogens (*Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750) in apples and lemons, and the study was continued with these two strains. As a result of *in vitro* analysis, it was determined that the antagonistic activities of these two yeast strains appeared by the combination of more than one mechanism. These mechanisms include competition for space and nutrients, production of volatile organic compounds (VOCs), secretion of extracellular lytic enzymes, and inhibition of spore germination of fungal pathogens. The competition for iron element was not detected in either yeast strain. Using the *Aureobasidium pullulans* GE17 alone resulted in the inhibition of the mycelial growth of *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750 by 83.40 % and 74.70 % respectively. Spore germination of both pathogens was reduced between 86 % to 95 % as a result of using mixed culture of these two yeasts at a concentration of 10^8 cells/mL. It was clearly determined that the biocontrol activity of yeast strains against blue and green molds increased in parallel to increase in the concentration of yeast cell numbers. According to the results of *in vitro* analyses, it was determined that utilization of *Meyerozyma guilliermondii* KL3 strain alone did not exhibit sufficient activity against target pathogens in this study. However, utilization of *Aureobasidium pullulans* GE17 strain alone and in combination with *Meyerozyma guilliermondii* KL3 exhibited powerful antagonistic activity against *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750.

Strains of *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 successfully degraded the patulin, which is the toxic metabolite proven to be harmful on human health and produced by various moulds including *Penicillium*, *in vitro*. After 40 h of incubation at 28 °C, the highest biodegradation of initial patulin concentration (7 µg/mL) was achieved by utilization of mixed culture at the level of 99.93 %, followed by strain KL3 (99.74 %) and GE17 (96.12 %).

It was determined that both yeast strains were colonized quite successfully in the wounded tissue of apples. After one week of incubation, the number of

yeasts were determined as 10.14 log cfu/wound and 9.51 log cfu/wound in apples inoculated with *Aureobasidium pullulans* GE17 alone and mixed culture, respectively. On the other hand, colonization ability of mixed culture was found to be better in wounded lemons compared to the single culture of *Aureobasidium pullulans* GE17. At the end of one-week incubation, the average yeast count in the injured parts of lemons treated with mixed culture and *Aureobasidium pullulans* GE17 alone were determined as 8.31 log cfu/wound and 4.83 log cfu/wound, respectively.

The effect of VOCs produced by yeasts on the lesion diameter of pathogens, inhibition (%) against mycelial growth of pathogens, disease severity, and infection rate in cv. Starking Delicious apples and cv. Kutdiken lemons were evaluated. According to the data obtained, higher % inhibition against mycelial growth of both pathogens, lower disease severity values, and lower infection ratios were determined as a result of the application of mixed culture compared to the single culture of *Aureobasidium pullulans* GE17 alone in apples. Similar results were obtained in lemons, and a low efficacy was observed as a result of using the single culture of *Aureobasidium pullulans* GE17. However, utilization of mixed culture led to a significant decrease in the infection rate and disease severity values, and also a remarkable increase in the inhibition ratio on the mycelial growth of green and blue moulds. It was determined that VOCs produced by the use of mixed culture in lemons inhibited the mycelial growth of *Penicillium digitatum* DSM2750 by 73.30% and *Penicillium expansum* DSM62841 by 75.28% compared to the control group.

Apple and lemon fruits were spray coated with the solution of single culture, mixed culture, or sterile distilled water (control) and stored in standard room conditions (20 ± 2 ° C and $50 \pm 5\%$ RH) for 45 days. During storage quality parameters of fruits were followed by five day intervals. At the end of the storage period, 28.80 % of the apples were infected in the control group, while this ratio was 11.11 % in the apples treated with mixed culture, and only 8.33 % in fruits

treated with single culture of *Aureobasidium pullulans* GE17. Similarly, as a result of coating the lemon surfaces with yeast, the infection rate decreased from 8.23 % (control) to 2.35 % (mixed culture) and 0.58 % (*Aureobasidium pullulans* GE17 alone) after 45 days of storage. Additionally, it was clearly understood from the results that yeast treatment helped to preserve quality characteristics (pH, total acidity, brix, weight loss, browning index, and texture) in both apple and lemon fruits during 45 days of storage in standard room conditions ($20 \pm 2^{\circ}\text{C}$ and $50 \pm 5\%$ RH) compared to the samples without yeast treatment. It has been observed that the inoculation of yeasts as a single or mixed culture on the surfaces of apples and lemons positively affected the quality characteristics of fruits.

cv. Starking Delicious apples and cv. Kutdiken lemons were stored in a cold room under industrial storage conditions (apple: 4°C and 95 % RH; lemon: 10°C and 90 % RH) for 3 months and disease severity, infection rate, and lesion diameter values were measured by 15 day intervals. After two months of storage in apples, close results were obtained for treatment of *Aureobasidium pullulans* GE17 alone and mixed culture. Both treatments inhibited the growth of blue mold and green mold by approximately 50 %. In lemons, it was detected that the single culture application of *Aureobasidium pullulans* GE17 was more effective in controlling two pathogens studied than mixed culture under the cold room storage conditions. During the period when infection rate reached 100 % in the control group lemons, ratio of infected fruit in the lemons inoculated with single culture of *Aureobasidium pullulans* GE17 together *Penicillium digitatum* DSM2750 or *Penicillium expansum* DSM62841 were 60 % and 45 %, respectively. During the same period, ratios of infected fruits inoculated with mixed culture for the groups of green and blue moulds were 65 % and 75 %, respectively.

GENİŞLETİLMİŞ ÖZET

Hasat öncesi ve sonrasında taze meyve ve sebzelerde çeşitli bozulmalar meydana gelmektedir. Ekonomik olarak önemli kayıplara yol açan bu bozulmaların büyük bir çoğunluğu fungal hastalıklardan kaynaklanmaktadır. Fungal hastalıklar sonucu meyve ve sebzelerde kalite sorunları görülmekte ve buna bağlı olarak raf ömürleri kısalmaktadır. Ayrıca, meyve ve sebzelerde taşıma, işleme ve depolamanın uygun şartlar altında yapılmaması sonucu hasat sonrası meydana gelen kayıpların oranı artmaktadır. Bu kayıplar gelişmiş ülkelerde üretilen toplam meyve-sebzenin %25'ini oluştururken, gelişmekte olan ülkelerde ise bu oranın %50'nin üzerinde olduğu bildirilmiştir (Spadaro ve Droby, 2016; Agirman ve Erten, 2020). FAO tarafından 2019 yılında yayınlanan rapora göre küresel nüfusun sürekli artmasıyla birlikte üretilen gıda miktarı da hızla artmaktadır. Son 50 yıl içerisinde dünya nüfusu iki kat artarken, gıda üretimi ise üç kat artmıştır (FAO, IFAD, UNICEF, WFP ve WHO, 2019). Buna rağmen, 2018 yılında dünyada yaklaşık 821 milyondan fazla insanın açlık çektiği ve yetersiz beslendiği bildirilmiştir (Güneş ve Turmuş, 2020). Bu nedenlerden dolayı artan dünya nüfusuna bağlı olarak gıda üretiminin artırılması ve üretilen gıdaların olabildiğince düşük kayıplarla tüketiciye ulaştırılması insanlık açısından elzemdir.

Günümüzde dünya genelinde meyve ve sebzelerin hasat sonrası hastalıklarını kontrol etmek amacıyla temel olarak sentetik fungusitler kullanılmaktadır. Fakat fungusit direnci kazanan patojenlerin gelişmesi, zararlı kimyasalların gıda zincirinde azaltılmasına yönelik dünya genelinde giderek artan tüketici eğilimi acil bir şekilde verimliliği yüksek, kalıntı oranı düşük, toksik olmayan çevre ve ekonomi dostu alternatif biyokontrol metotlarının bulunmasına yol açmıştır. Hasat sonrası meyve ve sebzelerdeki çürümeyi yönetmek ve sentetik fungusit kullanımını azaltmak amacıyla ortaya çıkan farklı yaklaşımlar arasında antagonistik mayalar ile biyokontrol yöntemi umut verici bir gelişme olarak ortaya çıkmış ve önem kazanmıştır.

Bu çalışmada temel amaç elma ve limonda bozulma etmeni olan fungal patojenlerin antagonistik mayalar ile biyokontrolünün sağlanması ve hasat sonrası bu meyvelerde meydana gelen kayıpların en düşük seviyeye düşürülmesidir. Bu amaçla çalışmada kullanılacak olan mayalar öncelikle elma ve limon yüzeylerinden izole edilerek moleküler yöntemlerle tanımlanmış ve tanımlanan bu epifitik mayalara ön test uygulanarak çalışmada kullanılacak olan hedef patojenlere karşı etkinlikleri belirlenmiştir. Ön denemeler sonucu başarılı bulunan iki maya suşu ile çeşitli analizler gerçekleştirilerek öncelikle *in vitro* koşullarda bu mayaların biyokontrol aktiviteleri ve sahip oldukları aksiyon mekanizmaları belirlenmeye çalışılmıştır. *İn vitro* analizler kapsamında maya suşları tekli kültür ve karışık kültür şeklinde kullanılmış, ayrıca mayaların farklı konsantrasyonları (10^6 ve 10^8 cells/ml) üzerine çalışmalar yapılarak en iyi aktivite sergiledikleri hücre sayısı belirlenmeye çalışılmıştır. *İn vitro* analizler olarak ikili kültür denemesi, uçucu organik bileşiklerin biyokontrol aktivitesi üzerine etkisi, hücre dışı litik enzim (proteaz, kitinaz, pektinaz, jelatinaz ve β -1,3 glukanaaz) aktivitesi, demir emilim mekanizması, mayaların fungal patojenlerin spor çimlenmesi üzerine etkisi ve fungal patojenlere karşı mayaların sergilediği inhibisyon yüzdesi belirlenmiştir. Literatürde laboratuvar koşullarda başarılı olan ancak meyve üzerinde aynı etkiyi sergilemediği bildirilen birçok çalışma mevcuttur. Bu bilgiye dayanarak *in vitro* analizlerden sonra canlı (elma ve limon) üzerinde *in vivo* analizlere geçilmiştir. Bu kapsamda elma ve limonlar hem standart oda koşullarında hem de endüstriyel koşulların sağlandığı soğuk hava depolarında uzun süreli depolama işlemine tabi tutulmuştur. Bu kapsamda standart oda koşullarında depolama boyunca, maya uygulamasının elma ve limonların kalite özellikleri (pH, toplam asitlik, brik, esmerleşme indeksi, tekstür, ağırlık kaybı ve bozulma oranı) üzerine etkisi araştırılmıştır. Soğuk oda koşullarında ise meyvelerde bozulma oranı, bozulma şiddeti ve lezyon çapı değerleri depolama boyunca takip edilmiştir. Ayrıca meyvelerin yaralı bölgelerinde mayaların kolonize olma yetenekleri ve mayalar tarafından üretilen uçucu organik bileşiklerin patojenler üzerine etkisi

incelenmiştir. Bunların dışında çalışmada kullanılan mayaların elmada bulunan ve *Penicillium* spp. türleri tarafından üretilen önemli bir toksin olan patulini parçalayabilme yetenekleri *in vitro* olarak araştırılmıştır.

Çalışmada meyve olarak elma ve limonun seçilme amacı bu iki meyvenin Türkiye tarımı ve ekonomisinde ihracat ve üretim oranı açısından önemli bir yere sahip olmasıdır. Son olarak 2019 yılında güncellenen FAOSTAT verilerine göre Türkiye 3.62 milyon ton elma üretim miktarı ile dünyada üçüncü sırada iken, 950 bin ton limon üretimi ile altıncı sırada bulunmaktadır (FAOSTAT, 2019). Starking Delicious elma cinsi Türkiye’de toplam elma üretiminin %55’ini ve ihracatının %87’sini oluşturduğundan ayrıca tüm dünyada yaygın bir şekilde tüketilen uluslararası bir cins olmasından dolayı tercih edilmiştir. Limon cinsi olarak ise Türkiye’de üretim miktarında ilk sırada yer alan, yüksek verim ve meyve kalitesine sahip olan ayrıca dokuz aya kadar varan uzun süreli depolamaya bırakılan Kütdiken limon cinsi seçilmiştir. Bu limon cinsinin tercih edilmesinde ülkemize özgü olan yerel bir cins olmasının yanı sıra bu çalışmanın yapıldığı Çukurova bölgesinde yetiştiriciliğinin fazla olması etkili olmuştur. Çalışmada fungal patojen olarak elma ve limonlarda temel bozulma etkeni olan ve yaygın olarak birçok farklı meyve-sebzede de bozulmaya neden olan patulin üretici *Penicillium expansum* (mavi küf) (Liu et al, 2013) ve *Penicillium digitatum* (yeşil küf) (Pérez-Alfonso et al, 2012) patojenlerinin biyokontrolü amaçlanmıştır.

Çalışma kapsamında elma ve limon yüzeylerinden izole edilen mayalar 26S rRNA genine ait D1/D2 bölgesi sekans dizilimine göre tanımlanmıştır. Toplamda 69 adet maya izolatu tanımlanmış ve bu mayaların beş farklı cins ve altı farklı türe ait oldukları belirlenmiştir. Bu türler şu şekildedir: *Saccharomyces cerevisiae*, *Saccharomyces paradoxus*, *Aureobasidium pullulans*, *Meyerozyma guilliermondii*, *Rhodotorula mucilaginosa* ve *Wickerhamomyces anomalus*. Ön denemeler sonucunda, 69 izolat arasından *Aureobasidium pullulans* GE17 ve *Meyerozyma guilliermondii* KL3 suşları elma ve limonlarda iki önemli hasat sonrası patojenine (*Penicillium expansum* DSM62841 ve *Penicillium digitatum*

DSM2750) karşı başarılı bulunmuş ve çalışmaya bu iki suş ile devam edilmiştir. *In vitro* analizler sonucunda bu iki maya suşunun antagonistik aktivitelerinin birden fazla mekanizmanın kombinasyonu ile ortaya çıktığı belirlenmiştir. Bu mekanizmalar arasında alan ve besin için yarışma, uçucu organik bileşiklerin (VOCs) üretimi, hücre dışı litik enzim salgılanması ve fungal patojenlerin spor çimlenmesini engelleme yer almaktadır. Demir elementi için rekabet mekanizması ise her iki suşta da belirlenememiştir. *Penicillium expansum* DSM62841 ve *Penicillium digitatum* DSM2750 küflerinin misel gelişimi *Aureobasidium pullulans* GE17 suşunun tekli kültür şeklinde kullanımı sonucu sırasıyla %83.40 ve %74.70 oranında engellenmiştir. Her iki mayanın birlikte karışık kültür şeklinde 10^8 hücre/mL konsantrasyonunda kullanılması sonucu ise her iki patojenin spor çimlenmesinin %86 ile %95 arasında azaldığı tespit edilmiştir. Kullanılan maya konsantrasyonunda ki artışın mavi ve yeşil küflere karşı test edilen maya suşlarının biyokontrol aktivitelerinin artmasına yol açtığı tespit edilmiştir. *In vitro* analiz sonuçlarına göre çalışmada kontrolü hedeflenen patojenlere karşı *Meyerozyma guilliermondii* KL3 suşunun yeterince etkili olamadığı saptanmış ancak *Aureobasidium pullulans* GE17 suşunun tekli kültürü ve bu suşun *Meyerozyma guilliermondii* KL3 ile karışık kültür şeklinde kullanımı sonucu başarılı sonuçlar elde edildiği anlaşılmıştır.

Bu çalışmada kullanılan maya suşlarının (*Aureobasidium pullulans* GE17 ve *Meyerozyma guilliermondii* KL3) insan sağlığı üzerine zararlı etkileri kanıtlanmış olan ve *Penicillium* cinsi küflerinde bulunduğu çeşitli patojenler tarafından üretilen patulin mikotoksinini başarıyla degrade edebildiği belirlenmiştir. İnkübasyondan 40 saat sonra, karışık kültür kullanımı ile başlangıç (7 µg/mL) patulin miktarının %99.93'ü degrade edilirken, KL3 ve GE17 suşları kullanılan denemelerde ise bu oran %99.74 ve 96.12% olarak belirlenmiştir.

Elma yaralı bölgelerinde her iki maya suşunun oldukça başarılı şekilde kolonize olduğu belirlenmiştir. Yedi günlük inkübasyon sonucunda *Aureobasidium pullulans* GE17 tekli kültürü ve karışık kültür uygulanan denemelerde maya sayısı,

sırasıyla 10.14 log kob/yara ve 9.51 log kob/yara olarak belirlenmiştir. Limonlarda ise aynı analizde karışık kültürün *Aureobasidium pullulans* GE17 tekli kültürüne göre yaralı bölgelerde daha iyi çoğalabildiği belirlenmiştir. Bir haftalık inkübasyon sonucunda karışık kültür ve *Aureobasidium pullulans* GE17 tekli kültürün uygulandığı limonların yaralı bölgelerinde ortalama maya sayısı sırasıyla 8.31 log kob/yara ve 4.83 log kob/yara olmuştur.

Mayalar tarafından üretilen uçucu organik bileşiklerin elma ve limonlarda patojenlerin oluşturduğu lezyon çapı ayrıca patojen gelişimine karşı inhibisyon (%) ve meyvelerde bozulma şiddeti ve bozulma oranı üzerine etkisi incelenmiştir. Elde edilen verilere göre elmalarda karışık kültür kullanımı sonucu *Aureobasidium pullulans* GE17 tekli kültür uygulamasına kıyasla daha yüksek patojen misel gelişim inhibisyonu (%) ve daha düşük bozulma oranı ve şiddeti değerleri belirlenmiştir. Benzer sonuçlar limonlarda da elde edilmiş ve *Aureobasidium pullulans* GE17 suşunun tekli kültür şeklinde uygulanması sonucu düşük etki görülmüştür. Ancak karışık kültür kullanımı sonucu bozulma oranı ve şiddeti değerlerinde önemli bir azalma ve patojen misel gelişimleri inhibisyonunda önemli bir artış tespit edilmiştir. Limonlarda karışık kültür kullanımı sonucu üretilen uçucu organik bileşiklerin kontrol grubuna kıyasla *Penicillium digitatum* DSM2750 misel gelişimini %73.30 oranında, *Penicillium expansum* DSM62841 gelişimini ise %75.28 oranında inhibe ettiği belirlenmiştir.

Elma ve limon meyveleri üzerine tekli, karışık kültür ve steril saf su (kontrol) içeren solüsyonlardan sprey yöntemi ile kaplama yapılmış ve standart oda koşullarında (20 ± 2 °C ve 50 ± 5 % RH) 45 gün boyunca depolanan meyvelerin beş günde bir olmak üzere kalite özellikleri takip edilmiştir. Elde edilen verilere göre 45 günlük depolama sonunda elmaların %28.80'i enfekte olurken karışık kültür ile kaplanan elmalarda bu oran %11.11, *Aureobasidium pullulans* GE17 tekli kültürü ile muamele edilen meyvelerin ise yalnızca %8.33'ünün bozulduğu belirlenmiştir. Benzer şekilde limon yüzeylerini maya ile kaplama işlemi sonucu 45 günlük depolama sonunda bozulma oranı %8.23'ten (kontrol) %2.35 (karışık

kültür) ve %0.58'e (*Aureobasidium pullulans* GE17) düşmüştür. Bunun dışında maya uygulamasının hem elma hem de limon meyvelerinde standart oda koşullarında 45 günlük depolama boyunca pH, toplam asitlik, briks, ağırlık kaybı, esmerleşme indeksi ve tekstür gibi kalite özelliklerinin korunmasına maya ilavesiz (kontrol) örneklerle göre yardımcı olduğu elde edilen sonuçlardan açıkça anlaşılmıştır. Mayaların karışık ve tekli kültür şeklinde elma ve limon yüzeylerine inoküle edilmesi meyvelerin kalite özelliklerini olumlu yönde etkilemiştir.

Starking Delicious cinsi elmalar ve Kütdiken cinsi limonlar soğuk hava deposunda endüstriyel depolama koşullarında (elma: 4°C and 95 % RH; limon: 10°C and 90 % RH) 3 ay boyunca depolanmış ve bozulma oranı, bozulma şiddeti ve patojen lezyon çapı değerleri on beş günde bir ölçülmüştür. Elmalarda 2 aylık depolama süresi sonunda *Aureobasidium pullulans* GE17 veya karışık kültür uygulaması sonucu birbirine yakın sonuçlar elde edilmiş ve her iki uygulamanın hem mavi hem de yeşil küf gelişimini yaklaşık %50 oranında inhibe ettiği belirlenmiştir. Limonlarda ise soğuk odada depolama sonucunda *Aureobasidium pullulans* GE17 maya suşunun tekli kültür olarak uygulanmasının karışık kültüre göre test edilen her iki patojenin kontrolünde daha etkili olduğu anlaşılmıştır. Kontrol grubu limonlarda bozulma oranının %100'e ulaştığı süre içerisinde *Aureobasidium pullulans* GE17 uygulanan limonlarda *Penicillium digitatum* DSM2750 ve *Penicillium expansum* DSM62841 patojen gruplarında bozulma görülmeyen meyve oranı sırasıyla %40 ve %55 olurken, bu oran karışık kültür ile inoküle edilen limonlarda sırasıyla %35 ve %25 olarak tespit edilmiştir.

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LIST OF ABBREVIATIONS

°C	: Centigrade Degree
µg	: Microgram
µL	: Microliter
µm	: Micrometre
A.	: <i>Aureobasidium</i>
ABS	: Absorbance
ANOVA	: Analysis of Variance
BCA	: Biocontrol Agent
BEO	: Basil Essential Oil
BLAST	: Basic Local Alignment Search Tool
bp	: Base Pair
CAT	: Catalase
CFU	: Colony Forming Unit
cm	: Centimetre
CO ₂	: Carbon Dioxide
cv.	: Cultivar
d	: Day
Da	: Dalton
DMDS	: Dimethyl Disulfide
DMTS	: Dimethyl Trisulfide
DNA	: Deoxyribonucleic Acid
DSMZ	: German Collection of Microorganisms and Cell Cultures GmbH
EFSA	: European Food Safety Authority
EOs	: Essential Oils
FAO	: Food and Agriculture Organization
FB2	: Fumonisin B2
FDA	: Food and Drug Administration

FeCl ₃	: Iron (III) Chloride
FP	: Fungal Pathogen
g	: Gram
GC-MS	: Gas Chromatography-Mass Spectrometry
GPS	: Global Positioning Systemy
GRAS	: Generally Recognized as Safe
h	: Hour
H ₂ O	: Water
HCl	: Hydrochloric Acid
HPLC	: High Performance Liquid Chromatography
ITS	: Internal Transcribed Spacer
L	: Litre
LAB	: Lactic Acid Bacteria
LEO	: Lavender Essential Oil
LOD	: Limit of Detection
log	: Logarithmic
LOQ	: Limit of Quantification
M	: Molar
<i>M.</i>	: <i>Meyerozyma</i>
MAP	: Modified Atmospheric Packaging
MDA	: Malondialdehyde
MEO	: Mint Essential Oil
mg	: Milligram
min	: Minute
mL	: Millilitre
mM	: Milimolar
mm	: Millimetre
N	: Newton
NaCl	: Sodium Chloride

NaOCl	: Sodium Hypochlorite
NEB	: Non Enzymatic Browning
ng	: Nano Gram
OD	: Optical Density
OTA	: Ochratoxin A
<i>P.</i>	: <i>Penicillium</i>
PAL	: Phenylalanine Ammonia-Lyase
PAMPs	: Pathogen-Associated Molecular Patterns
PCR	: Polymerase Chain Reaction
PDA	: Potato Dextrose Agar
pmol	: Picomole
POD	: Peroxidase
ppm	: Parts Per Million
PPO	: Polyphenol Oxidase
PR	: Pathogenesis-Related
QPS	: Qualified Presumption of Safety
QS	: Quorum Sensing
RCS	: Reactive Carbonyl Species
RFLP	: Restriction Fragment Length Polymorphism
RH	: Relative Humidity
RNA	: Ribonucleic Acid
ROS	: Reactive Oxygen Species
RPM	: Rotation Per Minute
s	: Second
SA	: Salicylic Acid
SBC	: Sodium Bicarbonate
SDW	: Sterile Distilled Water
SOD	: Superoxide Dismutase
SPME	: Solid Phase Micro Extraction

TA	: Titratable Acidity
TBE	: Tris/Borate/EDTA
TSS	: Total Soluble Solids
UPGMA	: Unweighted Pair Group Method with Arithmetic Average
UPW	: Ultra Pure Water
UV	: Ultraviolet
UV-vis	: Ultraviolet-Visible
v/v	: Volume/Volume
VOCs	: Volatile Organic Compounds
w/v	: Weight/Volume
YNB	: Yeast Nitrogen Base
YPD	: Yeast Extract Peptone Dextrose

1. INTRODUCTION

Today, food losses are a primary concern worldwide, particularly with an ever-growing world population and the fact that around one-third of all food produced for human consumption is lost or wasted globally (Leyva Salas et al, 2017). Each year, an estimated 1.3 billion tons of food are lost (FAO, 2017). At the same time, current estimates indicate that approximately 28% of the world's agricultural land area is occupied to produce food that is never consumed by humans (FAO, 2013). When considering the fruits and vegetables category, these losses correspond to more than 50% of total fruit-vegetables production (Carmona-Hernandez et al, 2019). There are various reasons for this enormous global food loss, but microbial spoilage, which affects organoleptic product quality (aspect, texture, taste, and aroma), plays a critical role. Among spoilage microorganisms, fungi are considered the main cause of losses of fresh fruits and vegetables at any stage of the food chain (postharvest, distribution and consumption levels) due to their capability to grow in diverse and even harsh conditions (Pitt and Hocking, 2009a; Spadaro and Droby, 2016). Beyond the negative effect of them on food quality, some fungal genera such as *Penicillium*, *Fusarium*, *Aspergillus* and *Alternaria* are able to produce secondary metabolites named mycotoxins that can have a toxic impact on humans and animals. Furthermore, mycotoxins have the ability to withstand different food processing steps and thus can cause the food safety concerns (Sanzani et al, 2016).

In this context, it is essential to decrease food losses by controlling fungal diseases at all stages of food process chains. While several approaches were suggested for managing postharvest decay, the application of synthetic chemical fungicides is still the most widely used controlling method for fungal pathogens worldwide (Nunes, 2012; Liu et al, 2013; Usall et al, 2016). Despite their definitely positive contribution to an efficient control of postharvest diseases and pests, concerns about the negative impacts of chemical pesticides on human health,

as well as on environment involving contamination of soil and water and toxicity to beneficial organisms, are increasing (Kim et al, 2017; Raymaekers et al, 2020). Moreover, intensive utilization of chemical pesticides resulted in development of resistant biotypes of pathogens (Spadaro and Droby, 2016; Borel, 2017; Ash, 2018). There is also a strong global trend of consumers in acquiring food products that come from systems without synthetic product application that keeps growing every day. For these reasons, there is an urgent need for alternative control strategies that are more natural, safe, effective, ecologically and economically feasible (Cao et al, 2013; Carmona-Hernandez et al, 2019). Among these strategies, biological control based on the use of microbial antagonists like yeasts, bacteria, fungi, viruses and protozoans has emerged as a promising way to reduce synthetic fungicide usage (Sharma et al, 2009; Droby et al, 2016; Köhl et al, 2019). In this regard, the use of yeasts as postharvest biocontrol agents (BCA) has been especially emphasized since they possess many features which make them particularly suitable as antagonistic agents. For instance; the production of antibiotics is generally not involved in their inhibitory activity, they have high tolerance to the adverse environmental conditions, have simple nutritional requirements, colonize fruit surfaces and also there is considerable information relating to commercial scale production of yeasts including fermentation, formulation, storage and handling (Liu et al, 2013; Pantelides et al, 2015; Spadaro and Droby, 2016).

This study was conducted on two economically important fruits (apple and lemon) that are produced and consumed worldwide. Apple and lemon are among the most important fruits for Turkey because of their great amount of production and export. Turkey is one of the leading countries in the world in the production of these two fruits. According to latest data reported by FAO (2018), Turkey ranks fourth and sixth in the world for the production of apple and lemon, respectively (FAOSTAT, 2018). cv. Starking Delicious, one of the most common apple varieties used in the world, was selected as an apple genus utilized in this thesis

due to that it constitutes 55% of total apple production and 87% of total apple exportation of Turkey (Karamürsel, 2009). On the other hand, cv. Kutdiken was selected as the lemon variety since it is the most widely produced lemon cultivar in Turkey with high yield and quality, and is also capable of long-term storage of up to nine months (Uzun et al, 2009a).

Patulin producer fungi *Penicillium expansum* is the casual pathogen of postharvest blue mold decay in a variety of fruits, including apples (Droby et al, 2009; Liu et al, 2013; Leyva Salas et al, 2017). Apples are highly susceptible to blue mold decay (Mahunu et al, 2016). This main apple spoiler fungal pathogen is considered a serious threat to the safety and quality of fruits with consequent remarkable economical losses (Cerioni et al, 2013; Sanzani et al, 2013). Similarly, green mold disease caused by *Penicillium digitatum* is one of the most encountered diseases in citrus fruits (orange, lemon, lime, mandarin, grapefruit etc.). This pathogen is of main concern as it is responsible for 90% of citrus deterioration during storage period, resulting in the most economically important postharvest disease of citrus fruits worldwide (Li et al, 2016; Mohammadi et al, 2017). Therefore, biological control of blue and green mold diseases in apples and lemons based on microbial antagonists is crucial. In the past three decades, an extensive number of research activities have explored and developed the biocontrol activity of numerous yeast species. Antagonistic yeasts used in this study belonged to the species *Aureobasidium pullulans* and *Meyerozyma guilliermondii*, and these epiphytic strains were isolated from the surfaces of apple and lemon fruits.

The objectives of the present thesis are listed below:

- to isolate and identify epiphytic yeasts from three different cultivars of lemons and apples,
- to evaluate biocontrol potential of isolates as single and mix culture against both *Penicillium expansum* (blue mold) and *Penicillium digitatum* (green mold),

- to determine the optimum number of yeast cell that exhibit the highest biocontrol activity against fungal pathogens by testing selected yeasts at different concentrations,
- to investigate the *in vitro* efficacy of selected yeasts in patulin degradation,
- to determine the effect of tested yeasts on quality parameters (e.g. texture, browning, acidity, weight loss) of cv. Starking Delicious apples and cv. Kutdiken lemons under standard room conditions,
- to observe the protective effect of selected yeasts against blue and green mold diseases in apples and lemons *in vivo* during three months of storage in a cold room under commercial conditions,
- to clarify the mechanism of actions used by selected yeast strains in the control of postharvest pathogens.

2. LITERATURE OVERVIEW

In this section, it is aimed to give information about the background of the thesis by reviewing the recent developments in the current literature. This section is divided into the following titles; basic principles of the postharvest biocontrol research with description of the terms of biocontrol and antagonist, importance of yeasts in biocontrol applications and characteristics of an ideal postharvest antagonist, mechanisms of action involved in biocontrol systems, enhancing the bioefficacy of microbial antagonists, brief information about microorganisms (*P. expansum*, *P. digitatum*, *A. pullulans*, *M. guilliermondii*) employed in this study, some biocontrol studies performed on apples and lemons and commercial applications.

2.1. Basic Concepts of the Postharvest Biocontrol Research

Fresh fruits and vegetables constitute an important part of the human diet as they contain vital substances for health such as vitamins, minerals, dietary fiber and antioxidants (Hossain et al, 2017). Increasing consumer awareness on the connection between nutrition and health has led to more consumption of fresh fruits and vegetables. According to the data from Food and Agriculture Organization of the United Nations (FAO, 2017), 865.6 million tons of fresh fruit in 65.2 million hectares and 1.1 billion tons of fresh vegetables in 57 million hectares, hence approximately two billion tons of fresh fruit and vegetables, have been produced in total. However, after harvesting, fresh fruit and vegetables undergo various spoilages resulting in economic losses. The majority of spoilages are caused by fungal diseases. These postharvest losses correspond to 25% of total fruit and vegetable production in industrialized countries, whereas it is reported to be above 50% in developing countries (Spadaro and Droby, 2016; Agirman and Erten, 2020).

Due to fungal diseases, fruits and vegetables are significantly damaged, their quality decreases and shelf life is shortened. The rate of postharvest losses in fruits and vegetables also increases due to lack of transportation, processing and storage under inappropriate conditions (Liu et al, 2013). Fungal species belonging to the *Penicillium*, *Botrytis*, *Monilinia*, *Rhizopus*, *Alternaria*, *Aspergillus*, *Fusarium*, *Geotrichum*, *Gloeosporium*, *Colletotrichum* and *Mucor* genera are accounted for many of the most important postharvest diseases (Leyva Salas et al, 2017). Beyond economic considerations, infected products represent a potential health risk because several fungal genera accounted among the most important, such as *Penicillium*, *Alternaria* and *Fusarium*, produce mycotoxins that are known to be detrimental for human health. For instance, *Penicillium expansum*, known as the cause of postharvest blue mold in a variety of fruits (apple, pear, peach etc.), produces a number of toxic secondary metabolites including patulin, citrinin and chaetoglobosins, all of which are potential carcinogens (Liu et al, 2013). Green mold caused by *Penicillium digitatum* is the most frequently encountered postharvest disease in citrus, and these phytopathogenic fungi were confirmed to be a cause of fatal pneumonia (Oshikata et al, 2013; Moraes Bazioli et al, 2019). In addition, the fungal species *Aspergillus niger*, *Aspergillus tubingensis* and *Aspergillus carbonarius* have the potential to produce mycotoxins including aflatoxin, ochratoxin A (OTA) and fumonisin B2 (FB2) during their growth stages. Among these mycotoxins, OTA has been identified as the most common toxin in grape and grape-derived products (wine, grape juice, raisin, etc.) (Pantelides et al, 2015).

Application of chemical fungicides is a common strategy to control postharvest disease worldwide. However, the development of fungicide-resistant biotypes of pathogens and strong consumer demand to reduce the use of harmful chemicals in their food supply urgently led to shift global trend to seek safer, high-efficiency, low-residue, nontoxic, environmentally and economically friendly alternative biocontrol methods (Spadaro and Droby, 2016). Among various

approaches, utilization of mainly yeasts, molds, lactic acid bacteria (LAB), Gram positive and Gram negative bacteria (Table 2.1) as a microbial biocontrol agent (BCA) has emerged as a promising way to prevent postharvest diseases of fruits and vegetables ([Sharma et al, 2009](#); [Lamont et al, 2017](#)).

The utilization of natural or added microorganisms, fermentates (i.e., an ingredient produced by the fermentation of a diversity of raw materials by a microorganism) or their metabolites to extend food shelf-life or increase food safety (by means of antibiosis of pathogenic or spoilage microorganism) is defined as “biocontrol”, “biological control” or “bio-preservation” ([Leyva Salas et al, 2017](#)). The aim of biological control strategy is to control or limit the spread or proliferation of an invasive species by employing their natural enemies ([Kang et al, 2017](#)). The term natural enemy as used herein refers to antagonist microorganisms. The term antagonist originally comes from Greek word “antagonistēs” (competitor, opponent, enemy, rival) which is derived from anti (against) and agonizesthai (to contend for a prize). The antagonist microorganism known as the opposite enemy is responsible for blocking the main character. Antagonism is one of the approaches of microorganisms to establish biological balance in nature. In this relationship, one of the two microorganisms coexisting directly or indirectly damages the other and negatively affects its development and reproduction, and limits or eliminates its survival. [Sharma et al \(2009\)](#) reported that there are two main approaches in the controlling of postharvest diseases of fruits and vegetables by microbial antagonists. The first one is to use microorganisms which already exist naturally on the product itself, which could be promoted and managed. The second approach is the artificial introduction of microbial agents against postharvest pathogens.

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Table 2.1. Some microbial antagonists effective in the control of post-harvest diseases of fruits and vegetables reported in the literature

Antagonist	Pathogen	Product	Disease	Reference
<i>Acetobacter nigricans</i>	<i>Fusarium poae</i>	Maize	Fusarium infection	Nagaraja et al, 2016
<i>Aureobasidium pullulans</i>	<i>Penicillium expansum</i>	Pear	Blue mold	Spadaro and Droby, 2016
	<i>Botrytis cinerea</i>	Apple	Gray mold	Di Francesco et al, 2015
	<i>Penicillium digitatum</i>	Mandarin	Green mold	Parafati et al, 2017
	<i>Aspergillus tubingensis</i>	Grape	<i>Aspergillus</i> infection	Pantelides et al, 2015
<i>Bacillus amyloliquefaciens</i>	<i>Alternaria alternata</i>	Jujube	<i>Alternaria</i> rot	Zhang et al, 2015
	<i>Monilinia fructicola</i>	Orange	Brown rot	Calvo et al, 2017
	<i>Monilinia fructicola</i>	Cherry	Brown rot	Gotor-Vila et al, 2017
<i>Bacillus subtilis</i>	<i>Botrytis cinerea</i>	Tomato	Rot	Kilani-Feki et al, 2016
	<i>Aspergillus flavus</i>	Pomegranate	<i>Aspergillus</i> infection	Gajbhiye et al, 2013
	<i>Colletotrichum capsici</i>	Chilli	Anthraxnose	Spadaro and Droby, 2016
<i>Bacillus</i> sp.	<i>Macrophomina phaseolina</i>	Soybean	Charcoal rot	Dave et al. 2020
<i>Candida diversa</i>	<i>Botrytis cinerea</i>	Kiwi	Gray mold	Tang et al, 2015
<i>Candida intermedia</i>	<i>Colletotrichum gloeosporioides</i>	Avocado	Anthraxnose	Campos-Martínez et al, 2016
<i>Candida membranifaciens</i>	<i>Penicillium digitatum</i>	Orange	Green mold	Terao et al, 2017
<i>Candida tropicalis</i>	<i>Colletotrichum musae</i>	Banana	Anthraxnose	Zhimo et al, 2017
<i>Clavispora lusitaniae</i>	<i>Penicillium digitatum</i>	Lemon	Green mold	Pereyra et al, 2020
<i>Clonostachys rosea</i>	<i>Botrytis cinerea</i>	Tomato	Gray mold	Gong et al, 2017
<i>Cryptococcus laurentii</i>	<i>Penicillium expansum</i>	Peach	Blue mold	Zhang et al, 2017
	<i>Botrytis cinerea</i>	Strawberry	Gray mold	Wei et al, 2014
<i>Hanseniaspora uvarum</i>	<i>Botrytis cinerea</i>	Grape	Gray mold	Qin et al, 2015
	<i>Penicillium digitatum</i>	Orange	Green mold	Li et al, 2016
<i>Lactobacillus paracasei</i>	<i>Alternaria brassicicola</i>	Cherry	<i>Alternaria</i> rot	Lipinska et al, 2016
<i>Leucosporidium scottii</i>	<i>Penicillium expansum</i>	Apple	Blue mold	Vero et al, 2013

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Table 2.1. continued

	<i>Botrytis cinerea</i>	Grape	Gray mold	Parafati et al, 2015
<i>Metschnikowia pulcherrima</i>	<i>Alternaria alternata</i>	Grape	<i>Alternaria</i> rot	Prendes et al, 2018
	<i>Penicillium digitatum</i>	Orange	Green mold	Liu et al, 2017
	<i>Botrytis cinerea</i>	Grape	Gray mold	Passera et al, 2017
<i>Paenibacillus pasadenensis</i>	<i>Botryosphaeria dothidea</i>	Apple	White rot	Kim et al, 2016
<i>Paenibacillus polymyxa</i>	<i>Penicillium expansum</i>	Apple	Blue mold	Cao et al, 2013
	<i>Penicillium expansum</i>	Apple	Blue mold	Mahunu et al, 2016
<i>Pichia caribbica</i>	<i>Penicillium digitatum</i>	Lemon	Green mold	Pereyra et al, 2020
<i>Pichia fermentans</i>	<i>Penicillium digitatum</i>	Citrus	Green mold	Chen et al, 2020a
<i>Pichia galeiformis</i>	<i>Botrytis cinerea</i>	Kiwi	Gray mold	Sui ve Liu., 2014
<i>Pichia guilliermondii</i>	<i>Colletotrichum gloeosporioides</i>	Citrus fruits	Anthracnose	Zhou et al, 2016
<i>Pseudomonas</i> sp.	<i>Macrophomina phaseolina</i>	Soybean	Charcoal rot	Dave et al. 2020
<i>Rhodospiridium paludigenum</i>	<i>Penicillium expansum</i>	Pear	Blue mold	Sun et al, 2018
<i>Rhodotorula minuta</i>	<i>Geotrichum citri-aurantii</i>	Orange	Sour rot	Ferraz et al, 2016
<i>Saccharomyces cerevisiae</i>	<i>Botrytis cinerea</i>	Grape	Gray mold	Parafati et al, 2015
	<i>Penicillium digitatum</i>	Lemon	Green mold	Perez et al, 2017
<i>Starmerella bacillaris</i>	<i>Botrytis cinerea</i>	Grape	Gray mold	Lemos Junior et al, 2016
<i>Wickerhamomyces anomalus</i>	<i>Penicillium italicum</i>	Strawberry	Blue mold	Parafati et al, 2017
	<i>Botrytis cinerea</i>	Grape	Gray mold	Parafati et al, 2015
<i>Yarrowia lipolytica</i>	<i>Talaromyces rugulosus</i>	Grape	Decay	Yang et al, 2017a

2.2. Importance of Yeasts in Biocontrol and Characteristics of an Ideal Postharvest Antagonist

The control of postharvest fruit and vegetable pathogens is one of the most important challenges encountered by the fruit and vegetable industry. The proliferation of molds on the product causes significant economic losses, and it may also bring serious health problems due to the mycotoxin contamination of fruits and vegetables. The use of synthetic fungicides is the most effective tool to control fungal diseases in fruits and vegetables. However, the utilization of synthetic fungicides is under strict legal control due to acute, cumulative, and synergistic effects ([de Paiva et al, 2017](#)). In 2014, European Food Safety Authority (EFSA) stated that residues of synthetic ingredients were found in approximately 45% of the tested food samples and 1.9% of all the samples contained residue above the legal limit. Furthermore, it has been reported that pesticides not approved by the European Union are detected in tested foods ([EFSA, 2014](#)).

Consumer demand for food free of pesticide residues, harmful microorganisms and toxins, in addition to the negative effects of synthetic chemicals on the environment, concerns about the development of human pathogens resistant to these compounds and the possible development of resistance in fruit pathogens and the presence of strict legal regulations on the use of synthetic chemical fungicides led to the search on alternative control methods ([Romanazzi et al, 2017](#), [Terao et al, 2017](#)). Biological control methods, which are generally based on the use of bacteria and yeasts, protect the products while also increasing the net value of the crops. However, the main inhibition mechanism of antagonistic bacteria is the production of antibiotics, so there is increasing interest in the use of yeasts as biocontrol agents ([Liu et al, 2017](#)).

Biocontrol yeasts are beneficial microorganisms due to the fact that they can decrease the negative effects of pathogens and also can encourage positive reaction in plants ([Shores et al, 2010](#)). The mechanism of the production of antibiotics, toxins or allergenic spores that most of the mycelial fungi and bacterial

antagonists possess is generally not found among the action mechanisms of yeasts used as biocontrol agents. This attribute reinforces the safe use of biocontrol yeasts for human consumption purposes ([Aloui et al, 2015](#); [Parafati et al, 2015](#)).

Use of microbial antagonists has emerged as a promising technique in order to manage and control the postharvest pathogens and reduce the utilization of synthetic fungicides. Among various microorganisms considered for biocontrol, the selection and application of antagonists particularly focused on yeasts, since yeasts have many properties required in an ideal antagonistic agent ([Droby et al, 2009](#); [de Paiva et al, 2017](#)). Some of these characteristics of an ideal microbial antagonist are listed below:

- Genetically stable
- Effective at low concentrations
- Not fastidious in terms of nutritional requirements
- Ability to survive adverse environmental conditions
- Effective against numerous pathogens on various commodities
- Convenient to production on inexpensive growth media
- Able to provide formulation with a long shelf-life
- Easy to distribute
- Resistant to chemicals used in the postharvest environment
- Not harmful to human health
- Overcome to the commercial processing procedures

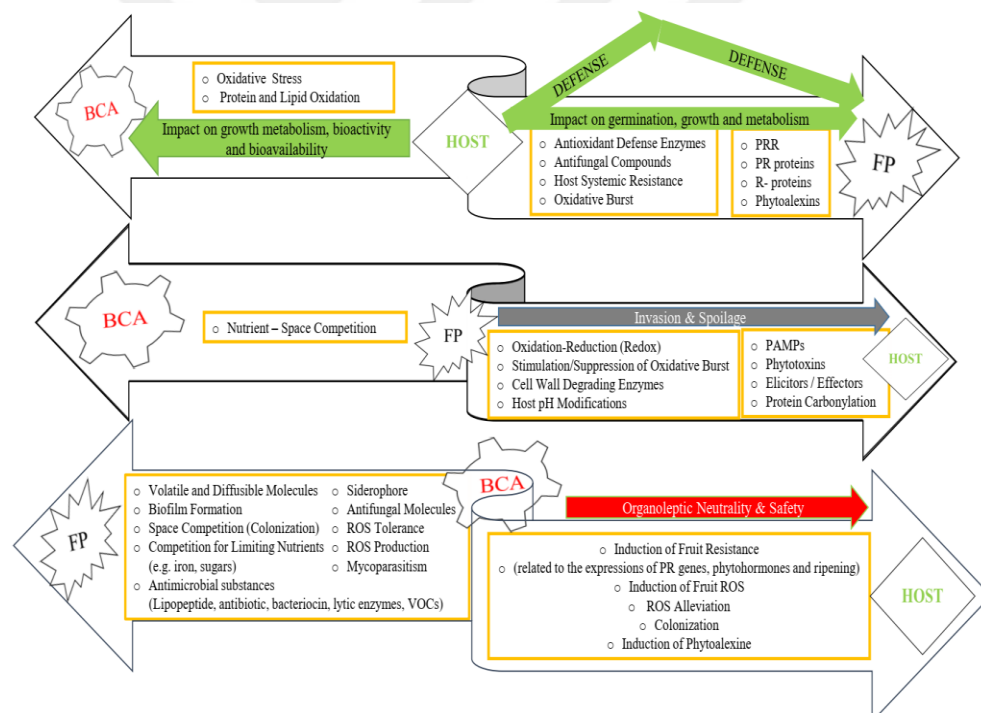
[Pantelides et al \(2015\)](#) reported that yeasts have many features which make them uniquely suitable as antagonistic agents. For instance; yeasts have simple nutritional necessity, survive a wide range of environmental circumstances; they grow quickly, could colonize rapidly on the damaged part of fruits, vegetables and plants, and are able to colonize under dry conditions for long periods. Additionally, yeasts are resistant to most agrochemicals and they do not generate anthropotoxic

compounds (Pantelides et al, 2015). Besides, yeasts have the ability to tolerate extreme environmental conditions prevailing before and after harvest, such as low and high temperatures, desiccation, a wide range of relative humidity, low oxygen levels, pH fluctuations, UV radiation. Another feature that makes yeasts superior is their unique adaptation to the fruit micro-environment (high sugar concentration, high osmotic pressure and low pH) (Spadaro et al, 2010; Spadaro and Droby, 2016). Another most important characteristics of yeast is that they could be isolated easily from various sources, including fruit and vegetable surfaces, leaves, soil, sea water and roots of plants (Liu et al, 2013). For instance; Kalogiannis et al (2006) isolated the *Rhodotorula glutinis* strain from tomato leaves, while Tang et al (2015) isolated the *Candida diversa* strain from kiwi fruit, Campos-Martínez et al (2016) isolated *Candida intermedia* yeast strain from avocado fruit. Additionally, isolation of *Saccharomyces cerevisiae* from mango fruit and *Candida tropicalis* strains from mandarin orange and bitter gourd were reported (Zhimo et al, 2017). The potential use of yeast isolated from different sources as biocontrol agents due to the positive properties mentioned above has been investigated by many researchers in the last 30 years (Liu et al, 2014; Perez et al, 2017; Sun et al, 2018). There has been a significant increase in the number of research conducted on yeasts as BCA in the last decade.

2.3. Mechanisms of Action of Microbial Antagonists

Controlling fungal spoilage using antifungal microorganisms is a complex process due to the fact that postharvest biocontrol systems are influenced by numerous parameters. The success of these systems is based on three main factors: the host which means the food itself (consisting of its natural microbiota), the fungal pathogen, and antifungal microorganism (antagonist) (Figure 2.1). Depending on the type of food and the antifungal microorganism, different mechanisms of action (single or combined) play a role in the inhibition of spoilage fungi (Liu et al, 2013; Leyva Salas et al, 2017). Understanding of the mechanism of action is a crucial subject in bio-fungicide manufacturing because it allows an increment in biocontrol performance and helps develop criteria to select biocontrol

agents (Pesce et al, 2018). Although information on the mechanisms of action for most of the antagonists investigated is still incomplete, competition for nutrients and space is clearly considered the primary mode of action for most of antagonistic yeasts in the literature (Spadaro and Droby, 2016; Mahunu et al, 2016; Oregel-Zamudio et al, 2017; Pesce et al, 2018). Other mechanisms including secretion of diffusible and volatile antifungal compounds, direct parasitism, induction of host resistance, biofilm formation, cell-wall hydrolytic enzyme production, involvement of reactive oxygen species (ROS), competition for iron, cell-to-cell contact and quorum sensing (Sharma et al, 2009; Liu et al, 2013; Ruiz-Moyano et al, 2016; Spadaro and Droby, 2016; González-Estrada et al, 2017; Agirman and Erten, 2020).



(BCA: Biocontrol agent, FP: Fungal pathogen, ROS: Reactive oxygen species, VOCs: Volatile organic compounds, PAMPs: Pathogen-associated molecular patterns, PR: Pathogenesis-related)

Figure 2.1. Several possible mechanisms operate in a tritrophic host-pathogen-antagonist interaction system

2.3.1. Competition for Nutrients and Space

Competition for nutrients (e.g. carbohydrates, nitrogen, oxygen) and space has been accepted as the main mode of action of antagonistic yeasts against postharvest spoilage fungi. Competition can be a powerful biocontrol mechanism when the antagonist is available in sufficient concentration at the right time and place, and it is able to utilize restricted resources more quickly and efficiently than the pathogen (Spadaro and Droby, 2016).

Competition for nutrients and space, considered the most important antagonism mechanism, occurs by three different ways: (i) competition for space-area, (ii) nutrient competition and (iii) attachment by the microbial antagonists to the pathogen hyphae. Competition for space depends on the growth ability of microbial antagonists more rapidly than pathogens. Thus, rapid proliferation and colonization of an antagonist on wounded parts of fruits and vegetables is a critical factor for a successful decay control (Mercier and Wilson, 1994). Attachment by microbial antagonists to the pathogen hyphae appears to be a significant factor essential for competition for nutrients as documented by the interactions between *Enterobacter cloacae* and *Rhizopus stolonifer* (Wisniewski et al, 1989), and *Pichia guilliermondii* and *Penicillium italicum* (Arras et al, 1998). In vitro research performed on such interactions showed that due to direct attachment, antagonistic microorganisms take nutrients much faster than target pathogens and thereby inhibit the spore germination and growth of the pathogens (Sharma et al, 2009).

Several studies have revealed that the hypothesis based on competition of microbial antagonists with pathogens for nutrients is one of the major mechanisms of action for antagonistic yeasts and bacteria. Studies conducted on this action of mechanism proved that competition for nutrients play a principal role in the mode of action of *Pichia guilliermondii* against *Penicillium digitatum* in citrus (Arras et al, 1998), *Pichia caribbica* against *Rhizopus stolonifer* in peach (Xu et al, 2013) and *Pichia anomala* against *Botrytis cinerea* in apple (Kwasiborski et al, 2014).

2.3.2. Iron Depletion

Competition, particularly for the element iron, is considered as a separate mechanism within the competing mechanisms of pathogen and antagonist microorganisms for nutrients. Iron (Fe^{+3}) is a biologically critical element because it is essential for the development of almost all microorganisms, and it is existing in the structure of heme-cofactored proteins (as catalase, cytochromes), iron sulphur cluster (Fe/S) including electron carriers proteins, and di-iron and mononuclear enzymes. Additionally, this element acts as a cofactor and is desired for the activity of numerous cellular enzymes involved within the various cellular processes (Nally et al, 2015; Spadaro and Droby, 2016). Besides carbohydrates and nitrogen sources, restricted iron availability due to the low solubility of Fe^{3+} ions can be a limiting factor for microbial growth (Köhl et al, 2019). Many microorganisms are able to produce a diversity of low-molecular-weight siderophores with a high affinity for ferric iron (van Loon, 2000). Microorganisms which able to produce high amounts of siderophores with high affinity to iron play a significant function in disease prevention and could be preferred for biological control via competition for iron with pathogens that produce lower amounts of siderophores with lesser affinity for iron (Whipps, 2001; Lugtenberg and Kamilova, 2009; Köhl et al, 2019).

Yeasts have the ability to perform various mechanisms, such as forming biofilms and adhering to fruit tissues or the cell walls of a pathogen, secreting hydrolytic enzymes and depleting iron (Lutz et al, 2013; Liu et al, 2017). The exhausting of iron was reported to play an important role in the biocontrol activities of *Metschnikowia* yeast strains (Saravanakumar et al., 2008). This genus is able to strongly antagonize the growth of numerous bacteria, yeasts, and filamentous fungi by producing iron-chelating ligands or siderophores (Sipiczki, 2006; Wang et al, 2009). It has been reported that pulcherrimin, a chemical complex formed between iron and pulcherriminic acid, could be produced by yeasts *Metschnikowia pulcherrima* and *Metschnikowia fructicola* (Sipiczki, 2006).

Although pulcherrimic acid is synthesized via cyclodileucine by enzymatic condensation of two leucyl-tRNAs in bacteria, the synthetic pathway of its production has still to be elucidated in yeasts (Lachance, 2016; Piombo et al, 2018). Unlike common siderophores, which facilitate cellular iron uptake, pulcherriminic acid is water-soluble until it binds iron and then precipitates as the water-insoluble, red-brown pulcherrimin complex. Accordingly, it was supposed that *Metschnikowia pulcherrima* employed this chelating agent as a competitive factor for competing fungi and bacteria by iron depletion (Türkel and Ener, 2009; Pretscher et al, 2018).

Different species belonging to the *Metschnikowia* genus have been successful in controlling the growth of different moulds on various fruits and vegetables. *Metschnikowia fructicola* was found to be successful in the control of *Botrytis cinerea* in grapes (Kurtzman and Droby, 2001), *Penicillium expansum* in apples (Spadaro et al, 2013) while *Metschnikowia pulcherrima* effectively controlled the *Monilia laxa* in nectarines (Ruiz-Moyano et al, 2016), *Botrytis cinerea*, *Alternaria alternata* and *Penicillium expansum* in apples (Saravanakumar et al, 2008), and *Penicillium expansum* in sweet cherries (de Paiva et al, 2017) through iron depletion. Liu et al (2019), have reported that iron depletion is a key action mechanism of *Metschnikowia citriensis* against *Penicillium digitatum* and *Penicillium italicum* on citrus fruit. Kantor et al (2015), hypothesized that *Metschnikowia pulcherrima* utilize the chelating agent (siderophore, pulcherrimin) as a competitive factor in order to overcome the growth of various yeasts, filamentous fungi and bacteria via iron depletion. As far as it is known, there is not any genus producing pulcherrimin with the exception of *Metschnikowia* (Agirman and Erten, 2020).

2.3.3. Mycoparasitism and Production of Lytic Enzymes

Parasitism occurs when the antagonist feeds on the pathogen and leads to the direct destruction or lysis of fungal propagules and cell structures (Spadaro and

[Droby, 2016](#)). There is limited knowledge on direct parasitism of the microbial antagonists in controlling postharvest diseases of fruits and vegetables in the literature. [Wisniewski et al \(1991\)](#) reported that a potent adhesion *in vitro* of *Pichia guilliermondii* yeast antagonist cells to *Botrytis cinerea* mycelium. After yeast cells were dislodged from the hyphae, the hyphal surface appeared to become concave in shape, and partial degradation on the cell wall of *Botrytis cinerea* at the attachment sites was determined. Conversely, cultivation of non-antagonistic yeast with *Botrytis cinerea* caused only a light attachment to the hyphae of the fungus without any pitting ([Wisniewski et al, 1991](#)). Likewise, co-culturing of *Candida saitoana* with *Botrytis cinerea* mycelium caused swelling and cytological destructions on the hyphae, such as cytoplasm degeneration as well as formation of papillae and different protuberances on the cell wall ([El- Ghaouth et al, 1998](#)).

The system of mycoparasitism shows activity by means of direct physical interaction of fungal hyphae and antagonist microorganism and secretion of cell-wall hydrolytic enzymes by antagonists ([González-Estrada et al, 2017](#)). Exhibiting chitinolytic and proteolytic activity for an antagonistic yeast is fairly important due to the potential application of them for biocontrol of fungal pathogens and insects. This is because chitin is the structural backbone of the fungal cell walls and it is arranged in regularly ordered layers. Glucan is the major structural polysaccharide of the fungal cell wall while β -1,3-glucan is accepted as chief structural component (as a filling material) to which other cell constituents are covalently attached. Moreover, β -1,3-glucan accounts for the mechanical strength and integrity of cell walls. The cell walls of filamentous fungi are composed of 20% or more chitin, 50%–60% glucan and around 20%–30% proteins by dry weight of wall ([Seidl, 2008](#), [Spadaro and Droby, 2016](#)). Proteins involved in the cell wall play important roles in many actions such as maintaining cell shape, defending the cell against foreign substances, synthesizing and modifying cell wall components, mediating adhesion for cell migration and fusion ([Spadaro and Droby, 2016](#)). Accordingly, production of different enzymes, especially proteases, chitinases and β -1,3-

glucanase, is necessary to degrade and cause lysis of pathogen cell walls. Secretion of extracellular lytic enzymes are involved directly or indirectly in the mode of actions of microbial antagonists for controlling postharvest pathogens. The inhibition activity of this mechanism starts with the production of lytic enzymes (i.e. glucanases, chitinases and proteases) after adhering to the fungal pathogen, continues with the deformation on fungus hyphae and leakage of cytoplasmic components, and ultimately result in cell lysis (Glushakova et al, 2007; Bautista-Rosales et al, 2013).

Zhao et al (2008) demonstrated that the activities of all which peroxidase, catalase, polyphenoloxidase, chitinase, superoxide dismutase, phenylalanine ammonia-lyase, and β -1,3-glucanase of tomato fruits inoculated with *Pichia guilliermondii* was changed and this situation was determined to be correlated with the initiation of induced resistance. This result indicates that tomato fruit is capable of responding to the yeast *Pichia guilliermondii*, which could activate defensive enzymes and thereby induce host disease resistance (Zhao et al, 2008). In another study showing the effect of lytic enzyme activity on biocontrol activity, *Aureobasidium pullulans* stimulated a transient increment in the activity of 1,3-gluconase, chitinase, and peroxidase enzymes in apple wounds which contributed to the wound healing processes and induced defense mechanisms against *Penicillium expansum* (Ippolito et al., 2000).

2.3.4. Role of Reactive Oxygen Species (ROS) in the Defense Response

The function of reactive oxygen species (ROS) in biocontrol systems specifically has been emphasized to play a significant role in biocontrol systems (Liu et al, 2012; Macarisin et al, 2010). When antagonistic yeasts are applied to surfaces and wounded areas of fruits and vegetables, they are encountered with ROS-generated oxidative stress that can affect the viability and/or performance of antagonist microorganisms (Castoria et al, 2003; Macarisin et al, 2010). Therefore, the relationship between the suitability of yeasts to be used as biocontrol agents in

post-harvest diseases and oxidative stress resistance was investigated, and it was stated that the capacity of yeasts to tolerate high levels of ROS is one of the essential properties for an effective biocontrol agent (Castoria et al, 2003).

The production of ROS in fruit and vegetables tissues by antagonistic yeasts is one of the earliest host responses to the pathogenic and non-pathogenic microorganisms. Thus, generation of ROS by biocontrol yeast cultures has influenced their use as a defence signal in products (Liu et al, 2013). In the case of a non-compatible host-parasite interaction, an initial moderate increase in the generation of ROS generally led to a strong oxidative burst during pathogen attacks, while in a compatible interaction no further rise is observed in the amount of reactive radicals in host tissue (Spadaro and Droby, 2016).

Oxidative burst at the wounding location after the colonization of antagonist cells was proposed to possess a role in the mechanism of action of biocontrol yeasts and can be involved in signaling pathways resulting in activation of fruit resistance system (Chan and Tian, 2006; Macarisin et al, 2010). In order to carry out this process, antagonist cells must have the capability to overcome alleviated levels of oxidative stress. In this regard, the first study to show the correlation between the postharvest biocontrol and ability of yeasts to tolerate relatively high amounts of reactive oxygen species was performed by Castoria et al (2003) by using the yeasts *Cryptococcus laurentii* and *Rhodotorula glutinis*. The findings of this study underlined the importance of oxidative stress in biocontrol systems and its probable direct and indirect impacts either on the tissue of fruits and vegetables or on the antagonist cells at intercellular and intracellular scale (Castoria et al, 2003).

After the colonization of antagonistic cells, oxidative bursts occur in the injured areas of fruits and vegetables and result in the activation of the host defense pathway, and this is considered to be one of the important action mechanisms of antagonist yeasts (Macarisin et al, 2010). Antagonistic cells must be able to tolerate high amounts of oxidative stress in order to use this mechanism. Liu et al (2012)

confirmed that applying a sub-lethal level of oxidative stress to *Candida oleophila* improved the stress tolerance of the yeast against higher lethal levels of oxidative and heat stress and also resulted in a greater biocontrol activity in comparison to non-stress-adapted yeast.

2.3.5. Biofilm Formation and Quorum Sensing

There are two significant properties affecting the success of antagonistic yeasts in their biocontrol activity; (i) to successfully reach a specific cell density on both intact and injured fruit surfaces and (ii) the adherence ability of yeast to attach themselves to plant host tissues, pathogen spores and also hyphae (Liu et al, 2013). In most cases these attributes are linked with the formation of biofilm, where micro colonies are enclosed in a hydrated matrix of microbe generated polysaccharides, proteins and nucleic acids (Bassam et al, 2009; Spadaro and Droby, 2016). Occurring a crucial change in gene expression and metabolism in the cell of antagonistic yeast often results in biofilm formation (Droby et al, 2009). In other words, biofilm formation is a developmental action in which microorganisms form morphologically distinguishable multicellular structures, altered gene expression patterns and improved resistance to stresses (Visick and Fuqua, 2005; Droby et al, 2009).

There is a strong relationship between the biofilm formation and quorum sensing (QS). In the course of surface colonization, cells of yeasts and bacteria are capable of communicating by using QS products as N-acyl homoserine lactone. Several reports elicited that cell to cell communication (QS) is involved in the formation of microbial biofilm in several yeast and bacteria species (Parsek and Greenberg, 2005; Sakuragi and Kolter, 2007). The ability to form biofilm provides some advantages in the biocontrol action of some antagonistic yeasts. For example, biofilm formation could suppress postharvest decays by interfering with pathogens to receive nutrients from the host and decreasing the physical area present for pathogen growth (Liu et al, 2013). It was reported that antagonistic yeasts supply a

protective micro-environment in the biocontrol system by using the biofilm as a coating function of exogenous compounds (Mitra et al, 2012). In addition, mixtures of biocontrol agents could form a coating layer on the fruit-vegetables surfaces that provides additional barrier (with respect to weight loss) against diffusion through stomata which is a critical pathway responsible for water loss (Ali et al, 2011). Also, biofilm formation could assist the colonisation of fruit and vegetable wounds by biocontrol cultures (Ruiz-Moyano et al, 2016).

Understanding the ecology of epiphytic microorganisms by using strategies such as biofilm formation or quorum sensing will lead to the selection of more effective antagonists and new methods of optimizing their activity in favour of biocontrol (Nunes, 2012). The role of biofilm formation and quorum sensing in biocontrol systems was documented by numerous studies. Two strains of *Metschnikowia citriensis* (FL01^T and FL02) exhibited a powerful ability to forms biofilm both *in vitro* and on the internal surface of wounds to prevent the penetration of pathogenic fungus into host tissue. These results pointed out that adhesion to mycelia and biofilm formation were significant elements for the biocontrol mechanism of *Metschnikowia citriensis* (Liu et al, 2019). Köhl et al (2019) reported that biofilm formation leads to generation of extracellular matrices by activating signalling pathways, and that it provides a protection for microorganisms against adverse environmental conditions. Lutz et al (2013) demonstrated that biofilm formation is involved among the mechanisms of actions of two strains of *Cryptococcus victoriae* (NPCC 1263 and NPCC 1259) which exhibit quite good film-forming capacity. Positive correlation between wound colonization and biofilm formation capacity was determined for *Metschnikowia pulcherrima* and *Wickerhamomyces anomalus*; on the contrary, *Aureobasidium pullulans* showed strong wound colonization ability *in vivo* but no biofilm formation *in vitro* (Parafati et al, 2015).

Environmental sensing and signal transduction pathways regulating morphogenetic transformations have been studied in detail in *Candida albicans*.

Two families of adhesin genes (HWP1 and ALS) proved to play a key role in host cell recognition, adhesion, invasion, and biofilm formation (Biswas et al, 2007). Moreover, two QS regulatory molecules, tyrosol and farnesol, organizing phenotype switching (yeast-to-hypha and vice versa), have been specified in *Candida albicans* (Chen et al, 2004; Droby et al, 2009). Gori et al (2011) identified phenylethanol, the aromatic alcohol, as a QS molecule inducing the pseudohyphal growth of *Saccharomyces cerevisiae* and *Debaryomyces hansenii*. Phenylethanol as a QS molecule also can stimulate *Kloeckera apiculata* to adhere and form biofilm on citrus fruit and embed in an extracellular matrix, by developing a mechanical barrier interfered between the injured area and the pathogen (Pu et al, 2014). The biocontrol activity of a biofilm-forming *Saccharomyces cerevisiae* strain is closely associated with the morphological phase of cell harvesting following growth in liquid culture. Only yeast cells gathered from the biofilm phase are efficient in restricting pathogen growth, clearly having the ability to colonize more effectively the inner surface of artificial wounds. In this context, the capability to form biofilms and filamentous growth are generally correlated (Ianiri et al, 2013).

2.3.6. Secretion of Diffusible and Volatile Antimicrobial Compounds (VOCs)

Treatment of BCAs for postharvest disease management has been recently oriented to the utilization of diffusible and volatile antimicrobial compounds produced by the BCAs as a promising and sustainable aspect in controlling postharvest diseases of fruits, vegetables, grains and seeds for replacement of chemicals (Parafati et al, 2017). BCAs including various species of yeasts, bacteria and mould are able to produce numerous antimicrobial compounds which include peptides and non-peptides, such as killer toxins, antibiotics and volatile organic compounds (Spadaro and Droby, 2016; Carmona-Hernandez et al, 2019).

Antibiotic production is considered as the second important mechanism after space and nutrient competition. It is known that production of antibiotics is

generally involved in the mechanisms of action of bacteria and rarely in yeasts. Large numbers of discovered antibiotics are produced by actinomycetes (8700 different antibiotics), fungi (4900) and bacteria (2900) (Köhl et al, 2019). Iturin (a strong antifungal peptide produced by *Bacillus subtilis*), natamycin (produced by *Streptomyces natalensis*) and pyrrolnitrin (produced by *Burkholderia cepacia* previously named as *Pseudomonas cepacia*) are some of the main antibiotics produced by antagonistic bacteria (Torres et al, 2014). The only microbial originated antifungal compound employed as food preservative is natamycin (E235) which belongs to the group of polyene macrolide antibiotics. The utilization of natamycin on cheese as a surface treatment is allowed by most countries and its supplementation into other foods relies on legislation (Leyva Salas et al, 2017). It is also remarkable that most of the antibiotics are produced only when a microbial population reaches a specific threshold value (Köhl et al, 2019).

Production of antimicrobial substances with broad-spectrum activity has been documented for biocontrol bacteria including *Agrobacterium*, *Bacillus*, *Pantoea*, *Pseudomonas*, *Serratia*, *Streptomyces*, *Stenotrophomonas* and many other genera. For instance; particularly lipopeptides such as iturin, surfactin, and fengycin have been studied in *Bacillus* species, (Ongena and Jacques, 2008), and many antibiotic metabolites such as pyrrolnitrin, phenazine and DAPG have been investigated in *Pseudomonas* species (Raaijmakers and Mazzola, 2012). Additionally, fungal antagonists can also produce antimicrobial metabolites. Gliovirin, gliotoxin, viridian, 6-PAP and several other compounds showing antimicrobial activity have been detected for *Trichoderma* and in the closely related genus *Clonostachys* (formerly known as *Gliocladium*) (Ghorbanpour et al., 2018).

Some LAB are able to produce many antifungal molecules including organic acids (hydrocinnamic acid, DL- β phenyllactic acid, DL- β -hydroxyphenyllactic acid, polyporic acid, azelaic acid, 2 hydroxybenzoic acid, 4-hydroxybenzoic acid, *p*-coumaric acid, vanillic acid, caffeic acid, succinic acid, 2-

pyrrolidone-5-carboxylic acid), fatty acids (decanoic acid, 3- hydroxydecanoic acid, coriolic acid, ricinoleic acid), cyclopeptides [cyclo(L-Pro-L-Pro), cyclo(L-Leu-L-Pro), cyclo(L-Tyr-L-Pro), cyclo(L-Met-L-Pro), cyclo(Phe-Pro), cyclo(Phe-OH-Pro), cyclo(L-Phe-L-Pro), cyclo(L-Phe-trans-4-OH-L-Pro), cyclo(L-His-L-Pro), and cyclo(Leu-Leu)], hydrogen peroxide, reuterin and volatile compounds such as diacetyl ([Aunsbjerg et al, 2015](#); [Valerio et al, 2016](#); [Mieszkin et al, 2017](#); [Leyva Salas et al, 2017](#)). Probiotics also apply the bacterial antagonism mechanism by producing various antimicrobial molecules, including organic acids, hydrogen peroxide, bacteriocins, and bio-surfactants which can inhibit the growth of pathogenic bacteria ([Kanmani et al, 2013](#); [Hossain et al. 2017](#)).

The main concern linked to the utilization of BCAs having the ability of antibiotic production in food products is the occurrence of human pathogens resistant to these compounds and the possible development of resistance in fruit pathogens. Special attention is required for the application of BCAs generating antimicrobials on fruit-vegetables, though these antibiotic producer cultures could have the capability to control wound infections formed before antagonist treatment.

Among possible modes of actions, production of VOCs by mainly yeasts and other microorganisms represents a significant means for biological control against a broad range of postharvest pathogens ([Di Francesco et al, 2015](#)). VOCs are low molecular weight substances (generally <300 Da), with low water solubility and high vapour pressure ([Vespermann et al, 2007](#)), which have a direct effect on the pathogen growth ([Carmona-Hernandez et al, 2019](#)). Yeasts are known to produce a wide range of VOCs belonging to several chemical classes including hydrocarbons, alcohols, thioalcohols, aldehydes, ketones, esters, thioesters, lactones, terpenes, cyclohexanes, heterocyclic compounds, phenols, sulfur compounds, benzene derivatives and many compounds at very low concentration ([Batista et al, 2011](#); [Morath et al, 2012](#); [Parafati et al, 2017](#); [Freimoser et al, 2019](#)). The volatile characteristic of these compounds provides a crucial advantage for BCAs. Since, these compounds can travel important distances

in structurally heterogeneous environments, as well as in solid, liquid, or gas compounds (Carmona-Hernandez et al, 2019). VOCs have received limited attention compared to other antagonistic mechanisms. However, recent research studies, some of which are presented below, have focused on these volatile metabolism products.

Candida intermedia and *Sporodiobolus pararoseus* were capable of suppressing conidial germination and mycelial growth of *Botrytis cinerea* and control grey mould of strawberry by releasing of several VOCs, including 2-nonanone (Huang et al, 2011) and 2-ethyl-1-hexanol (Huang et al, 2012). The production of VOCs, including 2-phenethyl alcohol, play an important role in the biocontrol activity of *Aureobasidium pullulans* against some fruit postharvest pathogens both *in vitro* and *in vivo* (Di Francesco et al, 2015). *Pichia anomala*, following the application in fruit wounds, initially may deplete a great part of the available oxygen, but later, during the stationary phase, it could use alcoholic fermentation to secrete antifungal VOCs, such as ethanol or ethyl acetate (Kwasiborski et al, 2014), and these compounds contribute to its antimicrobial activity against *Botrytis cinerea* (Fredlund et al, 2004).

VOCs produced by *Pichia anomala*, *Pichia kluyveri*, and *Hanseniaspora uvarum* suppressed the growth of *Aspergillus ochraceus* and production of ochratoxin A during coffee processing (Masoud et al, 2005). In addition, 2-phenylethanol was described as the key molecule of the *Pichia anomala* volatilome inhibiting spore germination, mycelial growth and toxin production by *Aspergillus flavus* (Hua et al, 2014). More than twenty different VOCs were determined in the volatilomes of selected biocontrol strains of *Candida friedrichii*, *Candida intermedia*, *Cyberlindnera jadinii* and *Lachancea thermotolerans*. However, 2-phenylethanol was the most abundant compound and accounts for the inhibition of both mycelial growth and ochratoxin A formation by *Aspergillus carbonarius* and *Aspergillus ochraceus* (Farbo et al, 2018; Tilocca et al, 2019).

A total of eight different VOCs belonging to the different chemical classes as ethanol (alcohol), acetic acid (acid), 3-methyl-1-butanol (alcohol), 3-methyl-1-butanol acetate (ester), benzaldehyde (aldehyde), pentyl propanoate (ester), benzeneacetaldehyde (aldehyde) and phenylethyl alcohol (alcohol) produced by *Pichia galeiformis* (BAF03) were identified through gas chromatography-mass spectrometry (GC-MS). Seven of them showed effective antimicrobial activity against *Penicillium digitatum* except benzeneacetaldehyde (Chen et al, 2020a). Volatile organic molecules produced by *Aureobasidium pullulans* were effective in lessening the growth and infection by *Botrytis cinerea*, *Colletotrichum acutatum*, *Penicillium expansum*, *Penicillium digitatum* and *Penicillium italicum* both *in vitro* and *in vivo* (Di Francesco et al, 2015). The biocontrol activity of different food yeasts such as *Aureobasidium pullulans*, *Metschnikowia pulcherrima*, *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* against *Botrytis cinerea* *in vitro* and on table grapes was mainly related with the secretion of VOCs (Parafati et al, 2015).

Many fungal species, such as *Trichoderma harzianum*, *Fusarium oxysporum*, and *Aureobasidium pullulans*, are familiar to produce low concentrations of volatile antifungal molecules (Mari et al, 2012). *Muscodor albus* and *Muscodor crispans* are endophytic fungi that produce various VOCs with antimicrobial activity (Mitchell et al, 2010). The potency and types of VOCs, which include 3-methyl-1-butanol, 2-nonanone, and phenylethyl alcohol, vary among isolates. An isolate of *Muscodor albus*, obtained from a cinnamon tree, was capable of killing a broad spectrum of plant pathogens and other microorganisms both *in vitro* and *in vivo* (Schnabel and Mercier, 2006). Another antifungal volatile-producing species, *Oxyporus latemarginatus*, was able to prevent the mycelial growth of *Alternaria alternata*, *Botrytis cinerea* and *Colletotrichum gloeosporioides*, by mycofumigation. Mycofumigation with solid cultures of this strain effectively reduced also the development of postharvest apple decay caused

by *Botrytis cinerea*, due to the production of 5-pentyl-2-furaldehyde (Lee et al, 2009).

Microorganisms producing VOCs create new possibilities to control microbial diseases in the agro-food chain, as bio-fumigation does not need physical contact with the product or commodity to be treated. VOCs are ideal antimicrobials because their spectrum of activity extends from proximal interaction through water diffusion to greater distances via air diffusion. However, the safety of using such an approach needs to be thoroughly evaluated.

2.3.7. Induction of Resistance

Induction of defense responses in the harvested fruits and vegetables by the microbial antagonists has been postulated to be one of the mechanisms of action of postharvest biocontrol agents (Droby et al, 2009; Sharma et al, 2009). Antagonistic yeasts possess the ability to interact with the host tissue, especially the wounds, raising the cicatrization processes. These biocontrol cultures were much more effective when applied before pathogen inoculation. This examination increased the assumption that yeast cells could induce resistance processes in fruit-vegetables tissue via elicitors that are either produced or constituent of their cell wall (Spadaro and Droby, 2016). Studies on the induction of resistance responses in fruit and vegetables by utilization of antagonists within a wound or on the host surface has also been significant in explaining the biology of postharvest biocontrol. Some of these reports were discussed below.

El-Ghaouth et al (1998) reported that *Cryptococcus saitoana* induced the chitinase enzyme activity and led to formation of structural barrier (papillae) on host cell walls in apple against *Penicillium expansum*. Rodov et al (1994) demonstrate that production of the phytoalexin scoparone was increased following the application of *Pichia guilliermondii* on lemon wounds. This increment evidenced the development of biochemical and molecular defence reaction of the host after the treatment of antagonistic yeast to lemon tissues (Rodov et al, 1994).

Similarly, treatment of *Candida oleophila* to surface wounds or intact grapefruits aroused systemic resistance against *Penicillium digitatum*, which is an important postharvest pathogen of many fruits and vegetables (Droby et al, 2002).

β -1,3- glucanase activity rised following the treatment of *Cryptococcus laurentii* to jujube fruit, and the expression of the related Glu-1 gene in fruit tissue was extremely induced. As a result, it was determined that the product of this gene could play a role in the defence characteristic against infection by *Alternaria alternata* and *Penicillium expansum* (Tian et al, 2007).

Chan et al. (2007) reported that induction of numerous proteins in peach tissue comprising antioxidant proteins, such as methionine sulfoxide reductase, glutathione peroxidase, peroxiredoxin, CAT and polyphenol oxidase that are associated in the repair of oxidative damage and the preservation of the tissue against oxidative damage, accounts for diseases resistance after the application of the yeast antagonist *Pichia membranifaciens* was detected through a proteomic approach. Lu et al (2013) demonstrated that preharvest employment of *Rhodospiridium paludigenum* as antagonistic yeast induced resistance against postharvest diseases in mandarins by activation of defence-connected enzymes, e.g. β -1,3-glucanase, polyphenoloxidase, POD and phenylalanine ammonia-lyase.

Even though all the results related to the induction of resistance responses in the host tissue following the application of antagonist microorganisms are correlative, direct evidence for the capability of induced substances to prevent pathogen infection and development has not yet been clearly confirmed. Whatever omic technique (genomic, transcriptomic or proteomic) has been employed, researches of postharvest BCAs have been limited and it is believed that further details about interactions in the whole biocontrol system will be forthcoming (Droby et al, 2016).

2.4. Improving the Bioefficacy of Microbial Antagonists

Once antifungal cultures have been selected and the molecules supporting the activity have been clarified, the next step that can be increased concerns the potential enhancement of their activity in terms of efficiency or action spectrum (i.e., the number of inhibited species/strains). Other important questions which need to be answered are: (i) determine the product type that antagonists could be used, (ii) detect the application method of the microbial antagonist on these products (Leyva Salas et al, 2017). Today, control of postharvest diseases in fruits and vegetables cannot be achieved 100% by using antagonist cultures alone. During large-scale fermentation and formulation of a commercial product, yeasts are exposed to various adverse environmental conditions as high temperature, oxidative stress and freeze/spray drying (desiccation). For these reasons, during the commercial production of yeasts, various approaches are used to increase their bioavailability as a result of improving their stress tolerance, vitality and performance (Sharma et al, 2009; Liu et al, 2013). Main strategies used to increase their bioefficacy and effectiveness are as follows: manipulations in the physical and chemical environment during storage, use of mixed cultures, addition of low doses of fungicides in the microbial cultures, addition of salt additives in the microbial cultures, addition of nutrients and plant products in microbial cultures, use of the microbial cultures in association with physical treatments, use of the microbial cultures with other approaches/additives (Sharma et al, 2009; Leyva Salas et al, 2017). Some studies with positive results as a result of using the mentioned approaches are summarized below.

Teixidó et al (1998) explained that raising the intracellular content of total polyols and sugars in *Candida sake* through the addition of glucose, glycerol or trehalose to the growth medium results in a development in the biocontrol efficacy and water stress tolerance of *Candida sake* when used to control *Penicillium expansum* on apple fruit. In a similar study, increasing the levels of intracellular trehalose in the growth medium (a trehalose-containing medium) of *Cryptococcus*

laurentii, led to the enhancement of viability and biocontrol efficacy of yeast after it was freeze dried and used at low temperatures or under controlled atmosphere circumstances (Li and Tian, 2006).

Salt additives also improve the bioefficacy of microbial antagonists in the control of spoilage of fruits and vegetables. Among the different salt additives used for this purpose, the use of calcium chloride, calcium propionate, sodium carbonate, sodium bicarbonate, potassium metabisulphite, ethanol and ammonium molybdate etc., together with microbial antagonists, has been reported to be highly effective and successful in controlling post-harvest diseases in fruits and vegetables (Karabulut et al, 2005; Zhang et al, 2005; Torres et al, 2007; Cao et al, 2008; Sharma et al, 2009).

Wang et al (2010) demonstrated that salt-adapted *Rhodospiridium paludigenum* exhibited higher viability in comparison to non-adapted cells under low water activity and freezing stress. In another study, it was determined that pre-heating (30 minutes at 40 ° C) increased the tolerance of *Metschnikowia fructicola* against high temperatures and oxidative stress (Liu et al, 2011). In both of the above studies, it has been observed that stress adaptation improved both subsequent high levels of stress tolerance and biocontrol activity of the yeasts. Therefore, pre-adaptation of yeast to abiotic stress may have practical implications for increasing the efficacy and reliability of yeast biocontrol products utilized to manage postharvest diseases in fruits under a broader scale of environmental conditions.

Some fungicides such as imazalil and thiobendazole are extremely effective in controlling post-harvest diseases. It is quite difficult to find a microbial antagonist that is as equally effective as these fungicides. Thus, there is an approach that provides effective biocontrol by combining microbial antagonists with fungicides. The method in which the microbial antagonist and synthetic fungicide are combined offers an advantage of strong biocontrol with reduced level of the fungicide. It is recognized that post-harvest diseases in fruits and vegetables can be effectively controlled by this strategy (Sharma et al, 2009). For instance,

some biocontrol formulations have emerged which provide almost 100% control of postharvest diseases if low doses of synthetic fungicides are also added to them (Wisniewski et al, 2001). Diseases caused by *Penicillium expansum* on pear and apples during storage period were reduced effectively by the use of *Pseudomonas syringae* with low doses of fungicide, cypronidil (Sugar and Basile, 2008). Studies have shown that the synergistic effect resulting from the combined use of yeast and low dose fungicide is higher than the effect that was exhibited by yeast and fungicide when they were used alone.

Karabulut et al (2010) evaluated the efficiency of short (30 or 60 second) immersion in water with temperature 24, 50, 55, 60, 65, or 70 °C for the control of brown rot (agent *Monilinia fructicola*) on peaches, plums and nectarines. Inoculated fruits were stored at 20 °C for 5 days or at 0 °C and 95% RH for 30 days followed by 5 days at 20 °C to provide commercial marketing conditions. It is notable that immersion in water at 55 °C for 60 seconds or at 60 °C for 30 or 60 seconds significantly reduced both decay incidence and severity. In addition, immersion fruits in water at 65 °C or higher causes severe injuries to the fruit surfaces. Immersion in water at 60 °C for 60 seconds reduced the incidence of brown rot from more than 80% among control fruit to less than 2% in plums while the same treatment decreased decay incidence from 100% to less than 5% on nectarines stored at 20 °C and from 73% to 28% on cold stored nectarines. Therefore, it has been emphasized that the short-term immersion approach in hot water can be effective in controlling *Monilinia fructicola* pathogen in stone fruits.

The association of bio-protective cultures with other molecules, such as chitosan, has been described and is currently used to protect postharvest crops by the coating technique. Chitosan protects fruits and vegetables from fungal decay, creates a barrier with the environment, and delays ripening. Zhou et al (2016) coat the citrus fruits by a mixture containing chitosan and *Pichia membranifaciens* culture to control diseases caused by *Colletotrichum gloeosporioides*. The results demonstrate that application of chitosan coating was more effective than utilizing

the yeast culture alone. Similarly, biocontrol effectiveness of the yeast *Rhodotorula glutinis* against *Penicillium expansum* and *Alternaria alternata* in cherry fruit was increased by combination with 2-hydroxybenzoic acid (salicylic acid) (Qin et al, 2003).

2.5. Commercial Applications

Increasing public interest in pesticides-free foods has strongly encouraged the scientists to use antifungal cultures as bio-preservatives. Despite all the attempts performed during the last 30 years to improve biocontrol products in order to control postharvest diseases, the low success ratio has been mentioned in the literature. Thus, a very limited number of biocontrol products are available on the market and they are applied exceptionally (Usall et al, 2016). Biocontrol systems are still in their infancy compared to fungicidal application, but advancement made in this field during the last three decades has been noticeable. There are several obstacles preventing the commercialization of candidate antifungal cultures. The first restraint trouble is the discrepancy between a detected activity in a culture medium (the *in vitro* effect) and the actual activity in the final matrix (in situ effect). Although various studies have reported that numerous microorganisms can exhibit interesting results in *in vitro* situations, the actual number drastically lessens when they tested in the target food matrices (Leyva Salas et al, 2017). For instance, solely one strain belonging to *Lactobacillus harbinensis* species among the eleven bacteria showing significant antifungal activity *in vitro* was proved to be active in yoghurt (Delavenne et al, 2013). As a result, it is clear that a product that shows lack of consistency and unreliable performance under commercial conditions cannot be successful on the market. Other difficulties encountered in commercializing a candidate organism are the inability of antagonist microorganisms to be as effective as fungicides in controlling postharvest diseases and the lack of financial resources (Droby et al, 2009). Furthermore, according to their metabolism, biocontrol microorganisms may directly influence the

organoleptic quality of the product as positive, negative or neutral. For example, CO₂ production by heterofermentative microbial candidates directly affects the product itself (bubble or air formation) or the packaging (blown packages) while other candidates which exhibit strong enzymatic activities (i.e. lipolytic, proteolytic or amylolytic) directly display effects on the product texture and overall quality. In this context, antifungal culture selection should aim towards organoleptic neutrality through using the sensory evaluation methods (i.e., triangle test, rapid methods for sensory profiling) to identify and measure a potential organoleptic impact in the final product ([Leyva Salas et al, 2017](#)).

The safety of the selected cultures is another key factor beyond the constraints mentioned above related to antagonist culture effectiveness and product quality issues. In this sense, safety assessment involving any regulatory considerations that need to be addressed, has now become an essential step during the strain selection for biotechnological application. Therefore, the European Union developed the Qualified Presumption of Safety (QPS) approach in order to evaluate the safety of a broad range of biological agents ([EFSA, 2016](#)). Premarket assessment of microorganisms utilized in food and feed production, and the employment of biological agents as a source of food and feed additives, enzymes, plant protection products are aimed by QPS approach. Similarly, there is a “Generally Recognized as Safe” (GRAS) status for food and foodstuffs in the United States ([FDA, 2021](#)).

Another important issue which should be taken into consideration in the commercialization process of a biocontrol product is the shelf life, which should be as long as possible. A biofungicide must be effective for at least 6 months and preferably up to 2 years ([Pusey, 1994](#)). [Usall et al \(2016\)](#) reported that economical and regulatory challenges are the primary limitation for successful entrance of biocontrol products in the market, even though technical shortcomings play some role in the low success of these products. The expenses related with development of biocontrol product, registration and marketing are very high. In most cases, the

companies who take in BCA commercialization are small, and generally they do not have the needed experience and economical sources to finish all the development process and marketing. Some BCAs were commercialized before the product was fully completed and then failed. Otherwise, big companies are not interested in these products due to the small size of the postharvest market (Droby et al, 2016; Usall et al, 2016).

A great number of microbial antagonists (yeast, mould and bacteria) against postharvest pathogens have been identified in laboratory, semi-commercial and commercial settings. However, few antagonists have been commercially available yet due to above mentioned troubles and difficulties. *Candida oleophila* (Aspire, Ecogen, US), *Candida sake* (Candifruit, IRTA, Spain), *Cryptococcus albidus* (YieldPlus, Lallemand, Canada), *Pseudomonas syringae* (BioSave, JET Harvest, US) are regarded as the first generation of biocontrol products approved and commercially available on the market (Droby et al, 2016; Usall et al, 2016). However, Aspire and Yieldplus were available only for a few years but discontinued. These products have been lost from the market due to reasons such as low and inconsistent productivity of product under commercial conditions, reluctance of companies to maintain registration, low profit margin, difficulties in introduction to the market, the perspective of the customers and the industry, and the presence of small-scale companies with low sources to develop and commercialize the products. On the other hand, there are some bio-protective cultures which achieved commercial success in the market (Table 2.2). For example; *Metschnikowia fructicola*, known under the trade name Shemer, was originally registered in Israel and has been successfully used in pre- and postharvest applications on many fruits and vegetables such as apricots, citrus fruits, grapes, peaches, peppers, strawberries. ShemerTM was acquired by Bayer CropScience (Germany) and is currently on the market as a sub-license of Koppert (Netherlands) (Spadaro and Droby, 2016). *Aureobasidium pullulans*, known as BoniProtectTM (BioFerm GmbH, Germany), is a successful broad-spectrum

biocontrol product and it has a suggested use as a preharvest application to control various wound pathogens such as *Penicillium expansum*, *Botrytis cinerea* and *Monilinia fructigena* that develop on pome fruits during storage (Leyva Salas et al, 2017). Additionally, the biocontrol product known under the trade name Nexy (BioNext SPRL, France) containing *Candida oleophila* strain has been approved throughout the European Union in 2013 (Massart and Jijakli, 2014).

The application of commercialized antagonistic microorganisms to the product is important in terms of product efficiency. Today, biocontrol microorganisms were applied to fruit and vegetables by spraying, dipping, drenching and coating which are usual methods for practice of fungicides. Moreover, nebulization of the biocontrol product in fruit and vegetable storage chambers could be a new postharvest method of treating. Environmental factors such as pH, oxidative stress, temperature and water activity of the harvested commodities on which commercial antagonists will be applied have an effect on the viability and efficacy of antagonist microorganisms. Therefore, companies that commercialize antagonist products should take into account the method of application while regulating the product formulation and should inform the packinghouses by giving further education on how to use this method of application (Liu et al, 2013; Droby et al, 2016).

2. LITERATURE OVERVIEW

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Table 2.2. Some commercially available bioprotective products developed to control postharvest diseases in fruits and vegetables (Spadaro and Droby, 2016; Leyva Salas et al, 2017; Agirman et al, 2019)

Product Name	Microorganism	Target Pathogens	Application Field	Manufacturer Country
Aspire	<i>Candida oleophila</i>	<i>Penicillium expansum</i> <i>Botrytis cinerea</i> <i>Rhizopus stolonifer</i>	Citrus Fruits Pome Fruits Apple, Peach	USA
Candifruit	<i>Candida sake</i>	<i>Penicillium expansum</i> <i>Botrytis cinerea</i> <i>Rhizopus stolonifer</i>	Pome Fruits	Spain
YieldPlus	<i>Cryptococcus albidus</i>	<i>Botrytis</i> spp. <i>Penicillium</i> spp. <i>Mucor</i> spp.	Pome Fruits Citrus, Pear Apple	Canada
BioSave	<i>Pseudomonas syringae</i>	<i>Penicillium expansum</i> <i>Botrytis cinerea</i> <i>Mucor piriformis</i> <i>Fusarium sambucinum</i> <i>Geotrichum candidum</i>	Pome Fruits Citrus, Pear Potatoes Cherries Apple	USA
BoniProtect	<i>Aureobasidium pullulans</i>	<i>Botrytis cinerea</i> <i>Penicillium expansum</i> <i>Monilinia fructigena</i>	Pome Fruits	Germany
Shemer	<i>Metschnikowia fructicola</i>	<i>Aspergillus niger</i> <i>Botrytis cinerea</i> <i>Penicillium digitatum</i> <i>Penicillium italicum</i> <i>Rhizopus stolonifer</i>	Grape, Carrot Strawberry Sweet potatoes, Apricot, Peach Pepper, Citrus	Netherlands
Nexy	<i>Candida oleophila</i>	<i>Botrytis cinerea</i> <i>Penicillium expansum</i>	Pome Fruits Citrus, Banana	France
Pantovital	<i>Pantoea agglomerans</i>	<i>Botrytis cinerea</i> <i>Penicillium digitatum</i> <i>Penicillium expansum</i> <i>Rhizopus stolonifer</i> <i>Monilinia</i> spp.	Citrus Pome Fruits	Spain
Serenade	<i>Bacillus subtilis</i>	<i>Monilinia fructicola</i> <i>Erwinia amylovora</i> <i>Phytophthora infestans</i>	Grape, Apple, Pear, Legumes, Peanuts	USA
Avogreen	<i>Bacillus subtilis</i>	<i>Cercospora</i> spp. <i>Colletotrichum</i> spp.	Avocado	South Africa

2.6. Some Biocontrol Studies on Apples

Apple (*Malus domestica*) is a significant nutritional crop commonly consumed as fresh fruit worldwide, and a small part of it is processed into cooked slices, juices and jellies. Fresh apples are stored in cold rooms after harvest in order to use them throughout the year. However, fungal diseases associated with wounds that occur during the transportation and packaging processes, especially during or after harvest, have a limiting effect on the storage life of apples (Li et al, 2011). The apple is an important fruit type in botany with its good adaptation ability to different ecological conditions, growing capacity in a broad range of geographic areas and its rich gene resources. Apple cultivation has been carried out in almost each region of Turkey for a very long time due to suitable ecological conditions and being the gene center of this fruit (Karamürsel, 2009). According to the data, last updated in 2018, from the Food and Agriculture Organization (FAO), Turkey is in the fourth rank for apple production in the world after China, the United States and Poland (FAOSTAT, 2018). As can be understood from the production capacity, Turkey is one of the leading countries in the world for the production of apples with a reported amount of 3.625.960 tonnes in 2018. In this section, information will be given about the postharvest diseases of apples that cause significant economic losses and the biocontrol studies performed on apples.

Fresh apples are attacked by pathogens directly after harvest due to their plentiful water and nutrient composition. Grey mould decay caused by *Botrytis cinerea* and blue mould caused by *Penicillium expansum* are two of the most destructive post-harvest diseases of apples. Both pathogens germinate in a suitable environment parallel to fruit maturity and cause diseases (Tolaini et al, 2010; Zhao et al, 2013). Blue mould decay caused by species of *Penicillium* is the most important disease of stored apples (Morales et al, 2008). In addition, growth of *Penicillium expansum* led to accumulation of patulin in apples. Patulin (4-hydroxy-4H-furo [3, 2c] pyran, 2[6H]-one) is an unsaturated heterocyclic lactone, a mycotoxin produced mainly by *Penicillium expansum* and other species like

Penicillium griseofulvum, *Penicillium roqueforti*, *Alternaria alternata* and *Bysochlamis nivea* (Ritieni, 2003; Morales et al, 2007). Patulin is a toxic compound for animals, causing intestinal injuries, involving epithelial cell degeneration, inflammation, ulceration, and hemorrhages; it has also been pointed out that it is a potential genotoxic, which stimulates oxidative DNA damages in human cells, resulting in mutagenesis which can lead to cancer (Liu et al, 2003; Morales et al, 2008). In this context, there are some effective BCAs against common fungal pathogens present in apples. For instance, mycelial growth of *Botrytis cinerea* and *Penicillium expansum* was successfully controlled by *Cryptococcus laurentii* (Castoria et al, 1997) in apples by the mechanism competition for nutrients while *Metschnikowia pulcherrima* (Saravanakumar et al, 2008) suppressed the same pathogens and provided an efficient biocontrol through iron consumption in apples.

Cao et al (2013) conducted a study on the efficiency of *Pichia caribbica* in controlling postharvest blue mold and natural decay development of apples and degrading the patulin produced by *Penicillium expansum*. The decay incidence of the blue mold was importantly decreased in apples through *Pichia caribbica* compared to the control samples. Additionally, increasing the concentration of *Pichia caribbica* provided higher efficacy for biocontrol. Otherwise, *Pichia caribbica* significantly controlled the natural decay development of apples following storage at 20 °C for 35 days or 4 °C for 45 days followed by 20 °C for 15 days. Germination of spores and growth of *Penicillium expansum* were remarkably reduced by *Pichia caribbica* in *in vitro* tests. Apple wounds were quickly colonized by the yeast in fruit stored at 20 °C or 4 °C. After incubation with *Pichia caribbica* at 20 °C for 15 days, patulin production by *Penicillium expansum* in apples was significantly reduced compared with the control. The initial patulin concentration was diminished over 58% in the presence of *Pichia caribbica* cells when incubated at 28 °C for 12 h, about 78% after 30 h incubation

and finally patulin was not detected after 36 h. In vitro testing demonstrated that *Pichia caribbica* can degrade patulin directly.

Recent study of [Madbouly et al \(2020\)](#) aimed to control *Monilinia fructigena*, causal agent of brown rot in apples, by using five different endophytic yeasts (*Schwanniomyces vanriijiae*, *Galactomyces geotrichum*, *Pichia kudriavzevii*, *Debaryomyces hansenii*, and *Rhodotorula glutinis*) isolated from healthy apples. In vitro plate assays showed that yeast BCAs have the ability to secrete fungal cell wall hydrolyzing enzymes (i.e. chitinases, pectinases, β -1,3-glucanase, and protease) and also produce killer toxins. In in vivo assays, inoculation of fruits with each of the 5 BCAs and *Monilinia fructigena* simultaneously, 24 h before, and 24 h after pathogen inoculation resulted in a noticeable decrease of disease incidence by 84.02–89.5%, 80.1–86.9%, 56.3–86.9% respectively, compared to the controls. In another study conducted on apples, apple decay caused by *Penicillium expansum* was controlled effectively by combining low doses of cypronidil with *Pseudomonas syringae* as a BCA ([Errampalli and Brubacher, 2006](#)).

[Sun et al \(2021\)](#) examined the biocontrol efficacy of *Meyerozyma guilliermondii* Y-1 with and without its combination with melatonin (MLT) against *Botrytis cinerea* in the postharvest apple fruit and underlying molecular mechanisms. Results demonstrated that the combination of *Meyerozyma guilliermondii* Y-1 (1×10^8 cells/mL) and MLT (100 μ mol/L) synergistically and significantly decreased the decay incidence and lesion diameter. MLT improved the ability of strain Y-1 to colonize in apple fruit wounds and surface whether at 22 or 4 °C. Furthermore, BCA and MLT treatment considerably elevated the defense-related enzyme activities comprising catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL), as well as the total antioxidant capacity, total phenolics and lignin contents. With regard to molecular mechanism, expression levels of four pathogenesis-related (PR) genes (MdPR1, MdPR5, MdGLU, and MdCHI) were significantly increased in response to strain Y-1 and MLT treatment.

Mahunu et al (2016) integrated the use of phytic acid with *Pichia caribbica* for the controlling of wound infection in apples, and this combination result in rises of peroxidase (POD), polyphenoloxidase (PPO), catalase (CAT) and low malondialdehyde (MDA) activities. As a result, utilization of phytic acid as a potential elicitor against blue mold decay was recommended. Zhang et al (2016) suggest that *Streptomyces rochei* A-1 treatment induces resistance against *Botryosphaeria dothidea* in apple fruit by stimulating a series of defense responses including generation of H₂O₂, increasing the activity of the defense-related-enzymes (β-1,3-glucanase, chitinase, PAL, SOD, CAT, POD) and suppressing the lipid peroxidation.

2.7. Some Biocontrol Studies on Lemons

Lemon (*Citrus limon* L.) is one of the most significant fruit crops in the world with an annual production amount of nearly 20 million tonnes (Uzun et al, 2009b; FAOSTAT, 2018). This fruit includes numerous valuable constituents such as high content of phenolic substances, mainly flavonoids (e.g. diosmin, limocitrin, hesperidin) and phenolic acids (e.g. synapic, ferulic and p-hydroxybenzoic acids), folic acid, vitamin C, pectin, minerals, dietary fiber, essential oils (D-limonene, β-pinene, γ-terpinene) and carotenoids. Lemons are evaluated for various industrial purposes such as concentrated juice, essential oil, dried peel and frozen pulp (Palavecino-Ruiz et al, 2016; Klimek-Szczykutowicz et al, 2020). According to the latest data reported by FAO, India is the first country in the world in terms of lemon production with the amount of 3.148.000 tonnes in 2018. It was followed by Mexico, China, Argentina and Brazil. Turkey is among the most important lemon producer countries and placed in the sixth rank worldwide with the 1.1 million tonnes volume of lemon production in 2018 (FAOSTAT, 2018). Turkey has favourable climatic conditions for citrus fruits, in particular the Mediterranean region, supplying the 92% of total production, and stands out in the production of high quality lemons due to its altitude (0-700 meters) above sea level and having

ideal micro climates (e.g. appropriate temperature, wind and rains) (Uzun et al, 2004; TAGEM, 2017).

As with other fruits and vegetables, lemons are subject to various postharvest diseases due to fungal infections, causing significant economic losses for the citrus industry during storage, transport and marketing. The crop losses in lemons were reported to reach 65.4% after a four-month cold storage process (Kotan et al, 2009). Fungi are the main organisms responsible for lemon decays due to its low pH level. There are two main fungal infections in lemon fruit caused by two *Penicillium* species, blue and green rot diseases caused by *Penicillium italicum* and *Penicillium digitatum* respectively (Pérez-Alfonso et al, 2012). Also, citrus sour rot disease caused by *Geotrichum citri-aurantii* is one of the most frequently encountered diseases in lemons (Palavecino-Ruiz et al, 2016). It was observed that the number of studies conducted on the subject of biocontrol in lemons is smaller in comparison to those of apples. Some of them are presented below.

Pérez-Alfonso et al (2012) used the single and mixture of two essential oils (thymol and carvacrol) against *Penicillium italicum* and *Penicillium digitatum* by using *in vitro* and *in vivo* (lemon) tests. Results demonstrated that both essential oils displayed high levels of inhibition against fungal growth while the highest efficacy was exhibited by thymol. Additionally, the utilization of wax with thymol and carvacrol to lemons inoculated with *Penicillium digitatum* led to decrease of infection on fruit surface, as well as reduced ethylene production, respiration ratio and total acidity losses. As a result, application of thymol and carvacrol in combination with wax in the lemon packing lines was recommended as a good alternative for reducing the utilization of chemical fungicides.

Çorbacı and Uçar (2018) evaluated the *in vivo* biocontrol efficiency of killer toxins produced by *Debaryomyces hansenii* (strains TEM8 and TEM17) against some pathogenic fungi such as *Alternaria brassicicola*, *Alternaria citri*, *Aspergillus niger* and *Rhizopus stolonifer* in fruits. Results demonstrated that this

yeast and its killer toxins are effective BCAs against those post-harvest fungi in apples, tomatoes and lemons.

[Perez et al \(2019\)](#) investigated the colonization ability of *Clavispora lusitaniae* 146 in lemon and its impact on *Penicillium digitatum* spore germination. In addition, the protection capacity of the yeast strain against an imizalil-resistant *Penicillium digitatum* and its survival potential on commercial waxes were determined. According to the results, *Clavispora lusitaniae* 146 showed an evident capability to colonize in lemon wounds and proceed to grow at both low and room temperatures. *Clavispora lusitaniae* 146 also showed high efficacy for inhibition of spore germination of both sensitive and fungicide-resistant fungi. The yeast was able to survive in natural waxes for 7 days at 25 °C and 40 days at 8 °C. Therefore, potential industrial application of *Clavispora lusitaniae* 146 strain as an efficient BCA against postharvest green rot infections caused by *Penicillium digitatum* in lemons was confirmed.

In another study using the same yeast strain, *Clavispora lusitaniae* 146, exhibited an excellent potential to overcome *Penicillium digitatum* under different stress factors. Strain 146 displayed exceptional behaviour over the other yeast strains through showing a remarkable ability to survive saline and oxidative stress, to resist disinfectants and to continue viability when exposed to high levels of UV-B irradiation or against thermal shocks. Thus, this organism is suggested as a promising candidate particularly for fungal diseases in lemons, and possibly in other citrus fruits as well ([Pereyra et al, 2020](#)).

[Chen et al \(2020b\)](#) demonstrated that the combined utilization of *Bacillus* antagonists with organic fertilizers have the possibility to be a good procedure for controlling citrus diseases and facilitating the emergence of commercial bio-organic fertilizers.

2.8. Evaluation of *Penicillium expansum* and *Penicillium digitatum* as Postharvest Pathogens

Fungal pathogens are a significant threat to human health and food security (Meyer et al, 2016). Fungi are currently a major cause of human morbidity and mortality in immunocompromised patients. In addition, they influence major crops globally and contaminate foods, resulting in unacceptable food spoilage (Garrigues et al, 2016). Particularly, infections by various pathogenic fungi, such as *Penicillium digitatum*, *Penicillium italicum*, *Penicillium expansum*, *Geotrichum citri-aurantii*, *Aspergillus niger*, and *Aspergillus flavus* cause crucial reductions in quality and marketable yield (Moraes Bazioli et al, 2019; Wang et al, 2020a). The genus *Penicillium*, one of the most important postharvest pathogens, comprises a group of anamorphic fungi in the division Ascomycota. This genus is large and includes 150 identified species (Pitt and Hocking, 2009b). Penicillia are habitually soil fungi and are ubiquitous microorganisms present in warm and moderate climates. Some species of this genus possess economic importance in the food industry due to their utilization in cheese and meat products while some of them are postharvest pathogens that cause spoilage (Ballester et al, 2015).

Penicillium expansum and *Penicillium digitatum* are considered widespread fungal pathogens of postharvest rot in pome fruits and citrus, respectively, causing remarkable fruit losses during postharvest handling (Yang et al, 2017b). *Penicillium expansum* has a wide range of hosts including apples (Buron-Moles et al, 2015), pears (Paster et al, 2009), peaches (Yang et al, 2011), hazel (Yang et al, 2014) and others (Judet-Correia et al, 2010; Cao et al, 2012), while *Penicillium digitatum* infects citrus fruits exclusively under natural conditions (Julca et al, 2015; Vilanova et al, 2017). *Penicillium expansum*, the type species of the genus, causes blue mold disease in a broad range of hosts including apples and is responsible for almost 50% of stored fruit losses (Vilanova et al, 2014a; Ballester et al, 2015). On the other hand, green mold, the most devastating disease in postharvest citrus crops, is caused by *Penicillium digitatum* (Vilanova et

al, 2014b; Julca et al, 2015) which accounts for 90% of the total loss of citrus together with *Penicillium italicum* (cause of blue mold) (Zhang et al, 2009). *Penicillium digitatum* is a necrotrophic filamentous fungus that is highly specific for citrus fruits including orange (Liu et al, 2017; Ahima et al, 2019), lemon (Mohammadi et al, 2017; Perez et al, 2019) and mandarin (Habiba et al, 2019) and produces significant economic losses in one of the main fruit tree crops worldwide (Garrigues et al, 2016).

Conidia of *Penicillium* species are ubiquitous in the atmosphere of packinghouses (Vilanova et al, 2014a) and also in manufacturing areas where the most developed storage technologies are utilized (Conway et al., 2004). Thus, mechanical injury caused during harvest and postharvest handling provides an optimal locus for infection (Cantu et al, 2008). Both pathogens can quickly infect wounds on the fruit surface. The spores of *Penicillium expansum* and *Penicillium digitatum* present in the wounds become active, germinate under nutrients, stimulating and colonizing rapidly in wound tissues. Then a visible maceration can be observed within a short time due to plentiful production of cell wall degrading enzymes and change in pH of the host. Finally, completion of the growth cycle of the pathogens within two days results in fruit diseases (Julca et al, 2015; Zhou et al, 2018; Yang et al, 2019).

Penicillium expansum causes great concern to fruit processing industries (e.g. juicing, baby food, ready-to-eat salads) due to its capacity to generate secondary metabolic toxins such as patulin, citrinin, ochratoxin A, penitrem A, rubratoxin B and penicillic acid which pose serious health hazards to consumers (Ballester et al, 2015; Zhou et al, 2018). Among these mycotoxins, patulin is the most common one produced by *Penicillium expansum* and consumption or contamination of foods with patulin may cause severe toxicosis including mutagenic, teratogenic, hepatotoxic, nephrotoxic, neurotoxic, and genotoxic effects (Hopkins 1993; Ballester et al, 2015).

Exposure to patulin at high doses results in immunosuppressive properties (Sharma, 1993). On the other hand, it was confirmed that the genes required for patulin biosynthesis were not found in the genome sequence of *Penicillium digitatum* (Marcet-Houben et al, 2012). However, *Penicillium digitatum* is well-known for the production of another mycotoxin citrinin which has nephrotoxicity, embryotoxicity, teratogenicity, and carcinogenicity effects on humans and animals (Yang et al, 2019).

The infection of fruits by the fungi *Penicillium expansum* and *Penicillium digitatum*, specifically apples, lemons and derived products, is a serious global trouble. Development of more environmentally friendly and safe postharvest *Penicillium expansum* and *Penicillium digitatum* infections control is receiving increasing attention from researchers worldwide. The combination of biological fungal control with physical (e.g. use of hot water, steam, microwave, refrigeration, UV-C) or chemical control (food additives such as ascorbic acid, sodium bicarbonate, phytic acid; plant hormones such as salicylic acid, ethylene) excluding the use of fungicides method is highlighted during the last decade due to achievement of more stable and synergistic antifungal effects in practical applications (Yu et al, 2020). However, further research will be required to explore and use beneficial microbial consortia and novel approaches like CRISPR/Cas technology for management of postharvest diseases (Wang et al, 2020a).



3. MATERIALS AND METHODS

3.1. Fungal Strains

Penicillium expansum and *Penicillium digitatum*, the dominant postharvest pathogens in apples and lemons, which cause blue mold and green mold diseases respectively, were selected as fungal species. The fungi strains used in this study, *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750, were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH) and routinely cultivated on Potato Dextrose Agar (PDA) at 24 °C.

3.2. Raw Materials Used for Isolation of Yeasts

Different varieties of apple and lemon fruits were selected as isolation sources of potential antagonistic yeasts. Two international (cv. Golden Delicious and cv. Starking Delicious) and one Turkish local (cv. Amasya) apple cultivars were used as a raw material, similarly international cultivars (cv. Meyer, cv. Interdonato) and local Turkish cultivar cv. Kutdiken were selected for lemon fruit. All apples and lemons used in this study were obtained from the orchard of the Agriculture Faculty of Cukurova University and also from local farmers (Adana Province, Turkey).

3.3. Raw Materials Used in the Experiments

In this study, cv. Starking Delicious apples and cv. Kutdiken lemons were used for *in vivo* analyses and also for storage in a cold room adapted to industrial conditions.

The apples' (cv. Starking Delicious) final pesticide application was done in September (2019) and harvested in October (2019), was obtained from Bademdere village (GPS Coordinates: 37° 55' 13.5444" North; 35° 4' 4.9944" East) in Çamardı (Niğde) district. They were purchased in November (2019) from a local farmer and

directly transported to a cold room present in the Department of Food Engineering (Faculty of Agriculture, Cukurova University, Turkey).

The lemons' (cv. Kutdiken) final pesticide application was done in the beginning of January (2020) and harvested in February (2020), was obtained from Arpaçbahşiş village (GPS Coordinates: 36° 38' 54.3048" North; 34° 20' 38.8608" East) in Erdemli (Mersin) district. They were purchased at the end of February (2020) from a local farmer and quickly transferred to the cold room as well as apples.

3.4. Molecular Characterization of Yeasts

3.4.1. Isolation of Yeast Strains

Apples and lemons were firstly chopped, then 10 g pieces of fruits were transferred into a sterile stomacher bag with a sterile 90 ml 0.85% NaCl physiological solution. Samples were homogenized for 4 min through Bag Mixer (Interscience, Model 400P, St Nom, France) followed by 2 h maceration for transmission of microorganisms to the dilution solution. Macerated samples were 10-fold serially diluted then streaked on Yeast Extract Peptone Dextrose (YPD) Agar (Sigma-Aldrich, Y1500, USA) and L-Lysine Agar (Sigma-Aldrich, USA) supplemented with 0.1 g/L chloramphenicol and 1 g/L sodium propionate (Campbell, 1988). Petri dishes were incubated at 28°C for 24 - 72 h until colony development. Each colony having morphological different characteristics was re-streaked onto YPD and L-lysine agar medium to isolate single colonies. Yeasts were differentiated according to features of textures (mucoid, fluid or viscous, butyrous); elevation (flat or raised); color (yellow, orange and red); surface (glistening or dull, smooth, rough and sectoried) and margin (entire, undulating, lobed and filaments) (Kurtzman et al, 2011). Finally, obtained yeast isolates were preserved in 40% glycerol at -80°C until identification.

3.4.2. Genomic DNA Extraction from Yeast Isolates

Genomic DNA extraction from isolates was carried out by using the Insta Gene Matrix (Bio-Rad, 732-630, Hercules, CA, USA) Kit according to the manufacturer's instructions with some modifications. Firstly, stored yeast isolates were activated in YPD Broth (Sigma-Aldrich, Y1375, USA) media for 24 h at 28°C and then genomic DNA was prepared as follows:

1. Each isolate was grown for 24 h at 28°C in a sterile thermo-stabil Eppendorf tube containing YPD Broth with a volume 1.5 mL.
2. Multiplied cultures in a micro-centrifuge tube were centrifuged (Thermo Scientific, Micro CL17, Germany) for 3 min at 13,300 rpm to pellet the cells, and supernatants were removed.
3. The pellets were then re-suspended in 1 mL of sterile ultra-distilled water to remove medium residues. Afterwards, tubes were vortexed (DragonLab, MX-S, China) at high speed for 10 s and centrifuged for 3 min at 13,300 rpm. Then, ultra-pure water (UPW) was removed.
4. If needed, second or third washing was applied as explained step 3.
5. 150 µL Insta Gene Matrix was added onto the pellet and vortexed at maximum speed for 10 s.
6. Eppendorf tubes were incubated at 56°C for 30 min. After incubation, high speed vortex was applied for 10 s.
7. Tubes were placed in a 100°C boiling water-bath for 8 min and then vortexed at maximum speed for 10 s.
8. The tubes were centrifuged at 13,300 rpm for 3 min.
9. The resulting supernatant including DNA was transferred in a new sterile Eppendorf tube and stored at - 80°C.
10. The stored DNA extract was used for further Polymerase Chain Reaction (PCR) analyzes.

3.4.3. Quantitation of DNA

The amounts of extracted DNAs were determined in order to standardize the PCR method. A fluorescence-based Qubit[®] 3 Fluorometer (Invitrogen–Life Technologies, Q33216, Malaysia) device and Qubit[®] dsDNA BR Assay Kit (Invitrogen-Life Technologies, Q32853, Oregon, USA) were used to verify the amount of DNA. Firstly, the Qubit[®] working solution was prepared by diluting the Qubit[®] reagent in the Qubit[®] buffer at the ratio 1:200. Two μL from extracted DNA were placed into the assay tubes (0.5 mL PCR tubes) and it was completed to the total volume of 200 μL with previously prepared working solution. All tubes were vortexed (Fisherbrand, ZX4 IR, Italy) 4-5 seconds. The tubes were incubated 2 min at room temperature and then tubes were inserted in the Qubit[®] Fluorometer for reading. After reading, the DNA amount of all isolates were adjusted to the 50 ng/ μL for direct use in PCR mixture.

3.4.4. Identification of Yeasts

Identification of potential yeast isolates was carried out according to method described previously by [Settanni et al \(2011\)](#) with slight modifications. Molecular identification of isolates was achieved by a combination of PCR-RFLP of the 5.8S internal transcribed spacer (ITS) rRNA region and sequencing of the D1/D2 domain of the 26S rRNA gene. Hae III, Hinf I and Cfo I restriction endonucleases were selected to differentiate the 5.8S rRNA PCR product (Table 3.1). ITS1/ITS4 and NL1/NL4 primer pairs were used for amplification of the 5.8S ITS rRNA gene and 26S rRNA gene, respectively ([Esteve-Zarzoso et al, 1999](#)). Sequences of universal primers used in this study are presented in Table 3.2.

Table 3.1. The mixture used for restriction endonucleases

Hae III		Hinf I		Cfo I (Hha I)	
Component	Amount	Component	Amount	Component	Amount
Enzyme	1 µL	Enzyme	1 µL	Enzyme	1 µL
Buffer R	2.5 µL	Buffer R	2.5 µL	Buffer Tango	2.5 µL
H ₂ O	11.5 µL	H ₂ O	11.5 µL	H ₂ O	11.5 µL
ITS Product	10 µL	ITS Product	10 µL	ITS Product	10 µL
Total	25 µL	Total	25 µL	Total	25 µL

Table 3.2. Primer sequences used for amplification of the 5.8S ITS rRNA gene and 26S rRNA gene

Primer	Sequence Alignment	Brand
ITS 1	5'-TCCGTAGGTGAACCTGCGG-3'	Thermo Fisher
ITS 4	5'-TCCTCCGCTTATTGATATGC-3'	Thermo Fisher
NL 1	5'-GCATATCAATAAGCGGAGGAAAAG-3'	Thermo Fisher
NL 4	5'-GGTCCGTGTTTCAAGACGG-3'	Thermo Fisher

Amplification was performed in thermal cycler (Eppendorf, AG 22331, Germany and Techne, TC-Plus, UK) and the protocols reported by [Leventdurur et al \(2016\)](#) were used with small modifications for PCR mixture and programme (Table 3.3 and Table 3.4).

Table 3.3. PCR mixture used for the amplification of 5.8S rRNA and 26 S rRNA genes

5.8 S rRNA	Components / Concentrations	26 S rRNA
1x	Sample Number	1x
5 µL	DNA (75-250 ng)	1.5 µL
5 µL	Dream Taq Buffer (10x + 20 mM MgCl ₂)	5 µL
2.5 µL	MgCl ₂ (25 mM)	2.5 µL
5 µL	dNTP (2.5 mM)	0.5 µL
0.126 µL (ITS1)	Forward Primer (100 pmol / µL)	0.1 µL (NL1)
0.126 µL (ITS4)	Reverse Primer (100 pmol / µL)	0.1 µL (NL4)
0.15 µL	Dream Taq DNA Polymerase (5 U / µL)	0.5 µL
32.098 µL	Sterile Ultra-Pure Water	39.8 µL
50 µL	Total Volume	50 µL

Table 3.4. PCR conditions used for amplification of 5.8S rRNA and 26 S rRNA genes

5.8 S rRNA	PCR Steps	26 S rRNA
105 °C	Pre-Heating	105 °C
95 °C – 5 min	Initial Denaturation	95 °C – 5 min
94 °C – 1 min (35 cycle)	Denaturation	94 °C – 1 min (30 cycle)
55 °C – 2 min (35 cycle)	Annealing	52 °C – 45 s (30 cycle)
72 °C – 2 min (35 cycle)	Extension	72 °C – 1 min (30 cycle)
72 °C – 10 min	Final Extension	72 °C – 7 min
4 °C	Cooling	4 °C

PCR products and their corresponding restriction fragments were separated in 1.5 and 3% w/v agarose gels stained with the SYBR[®] Safe DNA Gel Stain (Invitrogen, S33102, Carlsbad, CA, USA), respectively. The electrophoresis was run in 1 x TBE at 120 V. The sizes of the fragments were determined using a standard 100 bp DNA ladder (Thermo Fisher Scientific, SM0241, Waltham, MA,

USA) and gels were visualized under UV light by a gel imaging system (Vilber Lourmat, Infinity VX2, France). PCR-RFLP profiles were analysed with the pattern analysis software MEGA-X. Unweighted pair group method with an arithmetic average (UPGMA) clustering algorithm was used to obtain dendograms. Bootstrap method was used as a test of phylogeny.

PCR amplicons of D1/D2 domain were sent to Macrogen (Netherlands) for sequencing. Sequencing data was edited in BioEdit 7.2 sequence alignment editor software, obtained in FASTA format and compared using the sequence database on Nucleotide Basic Local Alignment Search Tool with BLAST search in Genbank/EMBL/DDBJ database. Nucleotide sequences obtained in this study were deposited in the GenBank database and accession numbers were obtained for identification of each different yeast species.

3.5. Determination of Number of Yeast Cell and Fungal Spores

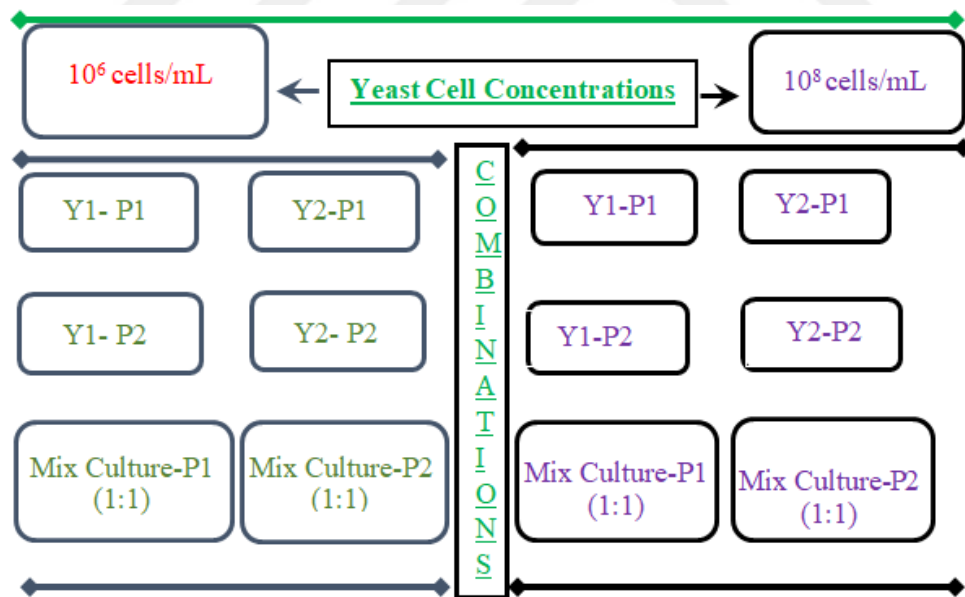
The concentrations of yeasts used in this study (*Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3) were determined by UV–Vis spectrophotometer (Agilent Technologies, Cary 60 UV–Vis, Australia) at OD 600 in order to use the advantage of quick checking of absorbance method ([Mahunu et al, 2016](#)). Yeasts were cultivated in YPD Broth (Sigma-Aldrich, Y1375, USA) separately and absorbance of liquid medium was measured after different incubation time intervals: 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48 h. Samples taken at these times were also counted by plating method to determine the correlation between OD 600 and counting by plating. Based on absorbance (OD600) and corresponding cell number values, a linear calibration line with a high R square value was drawn and an equation was obtained. The number of yeast cell was determined according to this equation for subsequent analyses.

Fungal cultures were maintained on Potato Dextrose Agar (Merck KGaA, Darmstadt, Germany) at 4 °C and fresh cultures were grown on PDA plates before use. Spore suspensions were prepared by removing the spores from the sporulating edges of a 7-day old culture with a bacteriological loop, and suspending them in

sterile distilled water (SDW). Suspensions of fungal spores (*Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750) were counted using a hemocytometer (Marienfeld Superior, Germany) under the microscope (Olympus, CX 22, Hamburg, Germany) and then adjusted to desired concentrations by SDW.

3.6. Experimental Design

Biocontrol activity of each *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 yeasts against postharvest pathogens, *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841, were investigated by using single yeast culture and mixed culture in order to determine the synergistic effect between these two yeast strains. In addition, two different cell concentrations were used as 10^6 and 10^8 cells/mL for each yeast, to understand the effect of differences in cell density on biocontrol capability. In this way, totally 12 different experiments were established and they were summarized in Figure 3.1.



Y1: *Meyerozyma guilliermondii* KL3; Y2: *Aureobasidium pullulans* GE17; P1: *Penicillium digitatum* DSM2750; P2: *Penicillium expansum* DSM62841; mixed culture (X): composed of both yeasts

Figure 3.1. Treatments applied to determine antagonistic capability of studied yeasts against pathogens.

In this study, analyses were performed as both *in vitro* and *in vivo*. Firstly, *in vitro* analyses were conducted to test the biocontrol efficiency of isolated various yeasts against both pathogens. Different yeast combinations and concentrations were also investigated in order to determine the successful trials to be used for *in vivo* analyses. Later, promising trials were applied directly on apples and lemons both in laboratory and cold room conditions. Commercial circumstances (e.g. temperature, humidity, storage duration) were provided during cold room storage. The effect of yeast application on postharvest losses and quality parameters (e.g. texture, weight loss, browning index, acidity etc.) of fruits was assessed in the course of storage. These assays are presented in detail below.

3.7. *in vitro* Analyses

3.7.1. Dual Culture Assay

All yeast strains isolated from apples and lemons were tested for *in vitro* preliminary screening to select isolates showing the highest antagonism against patulin producing *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750 strains. For this purpose, a loop of yeast cells from 2-day-old cultures was streaked on a YPD plate (9 cm diameter) at 2 cm from the rim of the plate (Pantelides et al, 2015). After 24 h, a conidial suspension of *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750 from a 5-day-old PDA culture was inoculated in the form of drop at the distance of 5 cm from the yeast line. Plates with only pathogens were used as control. Petri dishes were incubated at 25°C and mycelial growth of moulds in each plate was measured daily with digital caliper for 7 days. Experiments were carried out in triplicate. The two yeast strains that exhibited the highest inhibition on fungal growth were determined by dual culture test and the following analyses were conducted only with those successful strains.

3.7.2. Effects of VOCs on Biocontrol Activity

The method reported by Parafati et al (2015) was used with small modifications to evaluate the effect of VOCs produced by yeasts on biocontrol activity. For this purpose, aliquots of yeast suspensions with 10^6 and 10^8 cells/mL were seeded on petri dishes containing YPD Agar with pH value 4.5 and 6.0. These plates were incubated at 28°C for 24 h. Additionally, 20 µl aliquots of conidial suspensions of *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750 at 10^6 conidia/mL were inoculated on YPD Agar then dried at room temperature for 30 min. The plates with pathogens were individually covered face to face under dishes containing 24 h old yeast strains. These two plates were enwrapped together with 3 cycles of parafilm in order to avoid air leakage and incubated at 28°C for 9 days. The control trials were conducted with only pathogens.

Radial growth reduction of two fungal pathogens were calculated using formula $I\% = [(DC-DA)/DC \times 100]$ where I% represented the inhibition of radial mycelial growth, DC as the diameter of the growth zone on YPD plates without antagonistic yeasts (mm) and DA as the radial growth of the pathogen in the presence of yeast strains (mm). The experiments were conducted three times.

3.7.3. Extracellular Lytic Enzymes Activity

Ability of selected yeasts to produce and secrete five different cell wall lytic enzymes, chitinase (Souza et al, 2009), pectinase (Oskay and Yalçın, 2015), β -1,3 glucanase (Lutz et al, 2013), protease and gelatinase (Pickett et al, 1991) were characterized. Aliquots (20 µL) of 24 h yeast culture suspensions were superficially spotted on solid media containing the corresponding substrates according to previously reported techniques. All experiments were carried out twice. Analyses were carried out as explained below.

3.7.3.1. Chitinase Activity

The presence of chitinase was determined on solid medium (agar 15 g/L) containing colloidal chitin (Sigma-Aldrich, C7170, St. Louis, USA). Development of the culture medium for chitinolytic bacteria was carried out in two stages: (1) preparation of colloidal chitin; and (2) preparation of a medium containing the colloidal chitin and emended with mineral salts and agar. For this purpose, firstly colloidal chitin was prepared as described below.

Five grams of chitin powder from crab shells were added slowly to 60 ml concentrated hydrochloric acid (HCl) (Merck, Darmstadt, Germany) and kept at room temperature, with vigorous stirring, for 1 h. The mixture was filtered through glass wool and the filtrate was added to a 200 mL of 50% ethanol that was stirred vigorously during the process. The precipitate was transferred to a glass funnel with filter paper (80 g/m²) and washed with SDW until the colloidal chitin became neutral (pH 7.0). The colloidal chitin that was retained on a filter paper was removed with a spatula, weighted and stored in the dark at 4°C (Souza et al, 2009).

Secondly, minimal medium was prepared according to Furukawa et al (1978) and contained (NH₄)₂SO₄ (1 g/L), KH₂PO₄ (0.2 g/L), K₂HPO₄ (1.6 g/L), MgSO₄·7H₂O (0.2 g/L), NaCl (0.1 g/L), FeSO₄·7H₂O (0.01 g/L) and CaCl₂·2H₂O (0.02 g/L). A duplicate set of minimal medium containing 1.5% (w/v) of Bacto Agar (Becton Dickinson and Company, 214010, Sparks, USA) was made. Each set was supplemented, separately, with 1.2% (w/v) of colloidal chitin. The final pH was adjusted to 7.0 and sterilized by autoclaving at 121 °C for 15 min.

Extracellular chitinase activity was detected by the presence of clear zones around the colonies after incubation at 25°C for 7 days.

3.7.3.2. Pectinase Activity

Pectinase activity was tested on solid medium (agar 15 g/L) containing 6.7 g/L Yeast Nitrogen Base (YNB) (Sigma-Aldrich, 51483, India) and 10 g/L (Sigma-Aldrich, P9135, Denmark) citrus pectin. The pH of the medium was adjusted to

7.0. After cell growth, plates containing pectin were flooded with hexadecyltrimethylammonium bromide (10 g/L) (Sigma-Aldrich, H5882, China) and a clear halo around a colony in an otherwise opaque medium indicated degradation of the pectin (Buzzini and Martini, 2002).

3.7.3.3. β -1,3 Glucanase Activity

β -1,3 glucanase activity was tested on solid medium (agar 15 g/L) containing 5 g/L laminarin (Sigma-Aldrich, L9634, St. Louis, USA), 6.7 g/L YNB, K_2HPO_4 (0.65 g/L), KH_2PO_4 (2.5 g/L), $(NH_4)_2SO_4$ (0.5 g/L), NaCl (2.50 g/L), $MgSO_4 \cdot 7H_2O$ (0.12 g/L) and yeast extract (1.5 g/L) (Dewi et al, 2016). The pH of the medium was adjusted to between 6-7 and medium was sterilized at 121°C for 15 min by autoclaving. Plates were incubated at 25 °C for 72 h. After cell growth, plates containing laminarin were stained with congo red (0.6 g/L) (Sigma-Aldrich, C6277, St. Louis, USA) for 90 min with gentle shaking, and excessive congo red was removed, then plates were washed with 1M NaCl for 30 min. Strains were evaluated as positive for endoglucanase activity by the presence of a yellow-orange zone around the yeast colonies (Lutz et al, 2013).

3.7.3.4. Protease Activity

The medium, composed of 10% skim milk powder (Sigma-Aldrich, 70166, Switzerland) and 2% agar, was sterilized by autoclaving at 110 °C for 5 min and then poured into petri dishes. Inoculated plates were incubated at 25°C and examined daily for 1 week. Yeasts strains were evaluated as protease positive when a light halo surrounded the inoculum zone (Gardini et al, 2006).

3.7.3.5. Gelatinase Activity

Gelatinase activity was checked on sterilized tubes including gelatin as a substrate. The medium was prepared by dissolving 100 g of gelatine, 5 g of glucose (Sigma-Aldrich, 16301, France), 15 g of agar (Merck, Darmstadt, Germany) and 1

g of yeast extract (Merck, Darmstadt, Germany) in 1 L of demineralized water, pouring 9 ml of the medium into tubes sterilized by autoclaving at 121 °C for 15 min. Tubes were cooled in vertical position and then inoculated with 10 µl of an overnight culture at 25 °C of each yeast. Gelatinase activity was examined regularly during three weeks checking for signs of gelatine liquefaction ([Gardini et al, 2006](#)). All experiments were carried out triplicate.

3.7.4. Effect of Iron Concentration on Antagonistic Activity of Yeasts

The effect of ferric ions on antagonistic activity was determined on YPD plates unsupplemented and with 5, 10, and 20 mg/L of FeCl₃ (Merck, Darmstadt, Germany) before autoclaving. *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM270 were inoculated on solidified plates with 20 µl of a conidial suspension (3×10^5 spores/mL). After drying, cells of *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondi* KL3 were streaked onto YPD Agar plates. The width of reddish halos developing around the yeast colonies was measured after 6 days of incubation at 25°C in presence and absence of fungal strains ([Saravanakumar et al, 2008](#)). Unsupplemented YPD plates were used as control.

3.7.5. Effect of Antagonistic Yeasts on Spore Germination of Fungal Pathogen

Aliquots of 100 µl of a suspension of 10^8 spores/mL of *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM270 were separately inoculated in glass tubes containing 5 ml of YPD Broth. Then, one hundred microliters (100 µl) from each selected potential antagonist yeasts were inoculated in different tubes at different concentrations: 10^6 and 10^8 cells/mL. Tubes without yeast cells were used as control. The tubes were incubated at 25°C for 12 h in an orbital shaker with speed set to 100 rpm. A spore was considered germinated when the length of the germ tube was greater than the diameter of the spore, and at least 100 spores per replicate were examined microscopically to detect germination rate

(Cao et al, 2013; Ruiz-Moyano et al, 2016). All experiments were performed in triplicate and repeated twice.

3.7.6. Determination of Percent Inhibition of Mycelial Growth

Six millilitres of YPD medium with a final concentration of 10^6 and 10^8 cells/mL of selected antagonistic yeasts were poured over the previously prepared and solidified YPD plates. Once the top agar had solidified, 20 µl of the spore suspension (10^5 spores/mL) was transferred in the middle of the plate using a 6 mm diameter Whatman® disc with 5-day-old spores of *Penicillium expansum* DSM62841 or *Penicillium digitatum* DSM2750 (Ruiz-Moyano et al, 2016). Plates without yeasts were used as controls and incubation was carried out at 25°C for 10 days. The mycelial diameter of each colony was measured daily by digital caliper and results were expressed as the percent inhibition of radial growth calculated according to the formula $I\% = [(DC-DA)/DC \times 100]$ as previously described. All experiments were repeated three times.

3.8. *in vivo* Analyses

3.8.1. Wound Size Colonization

The colonization ability of *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 strains in artificial wounds on the surfaces of cv. Starking Delicious apples and cv. Kutdiken lemons were evaluated following a previously described procedure (Cirvilleri et al, 2005) partially modified. Before inoculation and treatment, selected apples and lemons were washed with tap water, surface disinfected by dipping for 1 min in 1% (w/v) of sodium hypochlorite (NaOCl) solution, rinsed with SDW and then allowed to air dry at room temperature. Uniform artificial wounds (4 mm diameter and 10 mm deep) were made along the fruit's equatorial areas (eight wounds/fruit) using the tip of a sterile cork borer. Each wound on fruits was inoculated with 20 µL of yeast suspensions (1×10^8 cells/mL) which contain single or mix culture according to trial type. Sterile

distilled water (20 µL) pipetted fruits were used as control. Fruits were incubated at 25 °C for 7 d. *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 were recovered from the wounds after incubation at 25 °C for 0 (2 h after treatment), 1, 2, 3, 4, 5, 6 and 7 days. The wounded whole tissue was extracted with sterile bistoury and homogenised for 1 min using Bag Mixer (Interscience, Model 400P, St Nom, France) in 50 mL of sterile 0.85% sodium chloride solution in a sterile Stomacher bag (Interscience, Bag Light-400, France) with volume of 400 mL, then macerated for 1 h. 10-fold serial dilutions were made and 0.1 mL of each dilution was spread onto YPD Agar added with chloramphenicol (100 mg/L) (Sigma-Aldrich, C0378, St. Louis, USA). Yeast colonies were counted after 48 h at 25 °C and population densities were expressed as log₁₀ CFU per wound. There were three single fruit replicates per treatment and the experiments were repeated twice.

3.8.2. Efficacy of the VOCs in Controlling Blue and Green Mold Rots in Apples and Lemons

The procedure reported by Parafati et al (2015) was partially modified and used in order to determine the effect of VOCs secreted by antagonists in controlling blue and green mold rots in apples and lemons. The experiments were done in food type sterile polypropylene packages (Whirl-Pak, Stand-Up Bag B01451, USA) that can stand upright completely by itself. Fruits (cv. Starking Delicious apples and cv. Kutdiken lemons) were surface disinfected with 1% NaOCl for 1 min, washed with SDW and then air-dried. Artificial wounds were performed using a sterile cork borer to make 10 mm deep and 4 mm wide wounds (one wound for each apple or lemon) along the fruit equatorial areas. Two different pathogen concentrations (1×10^6 conidia/mL and 1×10^8 conidia/mL) were used in this analysis for each treatment and each wound was inoculated separately with 20 µL of pathogen suspensions of *Penicillium expansum* DSM62841 or *Penicillium digitatum* DSM2750. A total of 24 trials were established, twelve trials for each of

the apples and lemons as presented in Table 3.5. The selected antagonists were inoculated separately on YPD Agar on the day before experimentation and incubated 24 h at 25 °C. Thereafter, the plates were transferred into the polypropylene packages (four plates per packaging) containing six inoculated fruits and sealed on both sides and on the cover to prevent air leakage. The inoculated apples and lemons were held at 25 °C for 5 days. At the end of the incubation period, disease incidence (DI), disease severity (DS) and lesion diameter (LD) were measured for each treatment as described below (Galanihe et al, 2010; Mamta et al, 2017). The same packaging with inoculated fruits and with agar plates without the antagonists served as controls. The experiments were repeated twice.

LD: Lesion diameter was assessed by measuring the average diameter (mm) of the damaged area five days after pathogen inoculation.

DI: Disease incidence was determined according to formula below:

$$DI (\%) = \frac{\text{Number of infected fruits}}{\text{Total number of fruits assessed}} \times 100$$

DS: The DS was evaluated by using an empirical 0-to-6 rating scale given in Table 3.6. Average DS was calculated by the following formula:

$$DS (\%) = \frac{\sum(C_i)}{M \times N} \times 100$$

Where DS is the average severity index, C the number of fruits in each class, i (0-to-6) the numerical values of classes, M the maximum rating scale number and N the total number of fruits examined.

Table 3.5. Treatments applied for *in vivo* VOCs analysis

Code of Treatment	Fruits Inoculated with	Pathogen Concentrations (conidia/mL)	Plates containing YPD Agar
1	P1(control)	20 µL from 1×10^6	Without yeast
2	P1 (control)	20 µL from 1×10^8	Without yeast
3	P2 (control)	20 µL from 1×10^6	Without yeast
4	P2 (control)	20 µL from 1×10^8	Without yeast
5	P1	20 µL from 1×10^6	With Y2
6	P1	20 µL from 1×10^8	With Y2
7	P2	20 µL from 1×10^6	With Y2
8	P2	20 µL from 1×10^8	With Y2
9	P1	20 µL from 1×10^6	With X
10	P1	20 µL from 1×10^8	With X
11	P2	20 µL from 1×10^6	With X
12	P2	20 µL from 1×10^8	With X

Y1: *Meyerozyma guilliermondii* KL3; Y2: *Aureobasidium pullulans* GE17; P1: *Penicillium digitatum* DSM2750; P2: *Penicillium expansum* DSM62841; X: mixed culture of two yeasts

Table 3.6. Damage scoring system used to measure disease severity

Rating Scale	Description - Severity of Damage
0	No visible symptom (0 % of fruits damaged)
1	Slight rot (1-15 % of fruits damaged)
2	Medium rot (15-30 % of fruits damaged)
3	High rot (30-45 % of fruits damaged)
4	Mycelium (45-60 % of fruits damaged)
5	Sporulation (60-75 % of fruits damaged)
6	Decay (75-100 % of fruits damaged)

3.8.3. Efficacy of *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 for Reducing Natural Decay Development and Their Effect on Quality Parameters of Apples and Lemons

A total of 540 healthful apple and 510 lemon fruits having close characteristics such as size, colour and maturity were selected within the scope of this analysis. Intact fruits were firstly washed with tap water in order to remove dust, leaf and other foreign substances, then disinfected with 1% (w/v) of sodium hypochlorite solution, rinsed with SDW and then allowed to air dry at room temperature. Apples and lemons were inoculated by spraying them with a suspension of single culture of *Aureobasidium pullulans* GE17 (1×10^8 cells/mL) (1st treatment) and its combination with *Meyerozyma guilliermondii* KL3 (1×10^8 cells/mL) (2nd treatment) (Cao et al, 2013). The spraying of SDW was used as a control treatment (3rd treatment). After absorbing yeast suspensions, dried fruits were stored at ambient condition (20 ± 2 °C and 50 ± 5 % relative humidity (RH)) for 45 days. Infection rate was measured in order to determine natural decay development under normal storage conditions. Fruits showing any symptoms of microbial incidence were marked as infected. Furthermore, pH, titratable acidity (TA), total soluble solids (brix), weight loss, browning index and texture properties, which are among the most important quality parameters of fruits, were determined at the beginning and then per five days during storage. The number of fruits required in each analysis was predetermined and the fruits were divided into different groups according to the analyses to be applied. The testing methods are described below.

Weight loss (g): For both apple and lemon experiments, 10 fruits were selected for each treatment (10 fruits x 3 treatment = a total of 30 apples and 30 lemon fruits) and codes were given to them. Then, their mass (g) was measured by balance (± 0.0001 g) (Precisa, XB 220A SCS, Switzerland) before treatment (A) and every five days (B) during storage. The weight loss was calculated according to formula $(A-B)/A$ as a percentage (%) at the end of the storage.

pH: The juice of the apples and lemons was obtained by fruit juicer and the pH of the liquid samples was directly measured with a digital pH meter (Mettler Toledo, SevenCompact™ S220, Leicester, United Kingdom).

Titrateable acidity (%): The acidity level of the apple and lemon juices was measured by titration to pH 8.2 with 0.1 N and 1 N NaOH, respectively. The analysis was repeated twice and 20 mL of fruit juices were used for each replicate. TA value was calculated as percent malic acid for apples and as percent citric acid for lemons (Wright and Kader, 1997). The following formula was used for determination of TA.

$$\text{TA (\%)} = \frac{\text{Volume of titrate} \times \text{N of titrate} \times \text{Eqv. Wt. of corresponding acid}}{\text{Volume of sample} \times 1000} \times 100$$

Brix: Total soluble solids (TSS) were determined by measuring the refractive index by using a hand refractometer (KEM Kyoto, RA-130, Tokyo, Japan) in the apple and lemon juices. Results were expressed as percentages (g per 100 g fruit weight) (Larrigaudière et al, 2002).

Fruit firmness (N): Firmness values of each individual apple and lemon were measured by using Texture Analyser Device (Stable Micro Systems, TA.XTPlus, Surrey, United Kingdom) with a 2 mm stainless steel cylinder probe (Stable Micro Systems, P/2, Surrey, United Kingdom). As analysis parameters, pre-test speed was determined as 1 mm/s, and the probe descended toward the sample at speed 10 mm/s. Additionally, the penetration distance of the probe inside the fruit was determined as 15 mm, trigger type as auto (force) and trigger force as 5.0 g. Apples and lemons were divided into two equal parts before analysis and the firmness of fruits was measured at three different points of the equatorial region of each part. The maximum force applied was defined as fruit firmness (N). A total of 90 of each of the apple and lemon fruits were used for texture analysis due to utilization of three apple and lemon fruits per treatment for each analysis day (10 analyses days' x 3 fruits x 3 treatment = 90 fruits).

Browning Index: The method described by Lee et al (1990) was used with some modifications. 10 mL of fresh pressed fruit juices (apple and lemon) were mixed with 10 mL of distilled water and were centrifuged at 14.000 rpm for 10 min at 4 °C. Then supernatants were mixed with 95% ethanol at a ratio of 1:1 and transferred into new eppendorf tubes. The mixture was vortexed vigorously and then centrifuged again at 14.000 rpm for 10 min at 4 °C. Finally, the absorbance of supernatants was recorded at A_{420} against blank (95% ethanol) by UV–Vis spectrophotometer (Agilent Technologies, Cary 60 UV–Vis, Australia).

3.8.4. Efficacy of the Yeast Cells in Controlling Blue and Green Mould Decay on Apples and Lemons During Cold Room Storage

Healthy and homogenous apple and lemon fruits were selected and randomly assigned to different treatments. Before inoculation and treatment, fruits were washed with tap water and the surface disinfected by dipping for 1 min 1% (w/v) of NaOCl solution, rinsed with SDW and then air dried. One artificial lesion for each fruit was made with a sterilised cork borer (4 mm wide and 5 mm deep) along the fruit equatorial areas. Each wound was inoculated with 30 µL drop of 1×10^8 cells/mL of yeasts and sterile distilled water was used as the control. After 2 h, the same lesions were inoculated with 30 µL of suspension of *Penicillium expansum* DSM62841 (1×10^5 spores/mL) or *Penicillium digitatum* DSM2750 (1×10^5 spores/mL). After air drying, fruits were stored for 3 months under the conditions as used in the industry. For this purpose, apples were stored in a cold room with a high RH (95%) at 4 °C while lemons were stored in an air conditioning cabinet (Nüve, TK-252, Ankara, Turkey) arranged to 90% RH and 10 °C temperature. To create a humid environment, a humidifier device (Faran, Cezio, Korea) that had the capacity to spray 3 to 5 liter moisture per hour with particle size between 5 to 20 micron was used in the cold room. There were 50 fruits for each treatment (50 fruits x 6 treatments = 300 apple and 300 lemon fruits). All treatments were presented in Table 3.7. Infection rate, disease severity and lesion

diameter of stored fruits were recorded (as described previously in subtitle 3.8.2) per 15 days during 3 months of storage.

Table 3.7. Description of the treatments applied in cold storage

Code of Treatment	Inoculated Pathogens in Lesion	Pathogen Concentration (spores/mL)	Inoculated Yeasts in Lesion	Yeast Concentration (cells/mL)
1	P1	30 µL from 1×10^5	SDW (control)	Without yeast
2	P2	30 µL from 1×10^5	SDW (control)	Without yeast
3	P1	30 µL from 1×10^5	Y2	30 µL from 1×10^8
4	P2	30 µL from 1×10^5	Y2	30 µL from 1×10^8
5	P1	30 µL from 1×10^5	X	30 µL from 1×10^8
6	P2	30 µL from 1×10^5	X	30 µL from 1×10^8

SDW: Sterile distilled water; Y1: *Meyerozyma guilliermondii* KL3; Y2: *Aureobasidium pullulans* GE17; P1: *Penicillium digitatum* DSM2750; P2: *Penicillium expansum* DSM62841; X: mixed culture of two yeasts

3.9. The Degradation of Patulin by Yeasts *in vitro*

The method of sample preparation was described by Coelho et al (2007) with some modifications. Suspensions of *Aureobasidium pullulans* GE17 (1 ml; 1×10^7 cells/mL), *Meyerozyma guilliermondii* KL3 (1 ml; 1×10^7 cells/mL) and their mixture (1 ml; 1×10^7 cells/mL) were separately added to aliquots of 50 ml YPD Broth with patulin (Sigma-Aldrich, P1639, St. Louis, USA) at a concentration of 7.0 µg/mL in 250 mL erlenmeyer flasks. Sterile distilled water used as control treatment. All erlenmeyer flasks were placed on a rotary shaker (IKA, KS-501, Germany) and incubated at 150 rpm at 28 °C.

3.9.1. Extraction and Purification of Patulin

Samples were taken at 8, 16, 24, 32 and 40 h, and were centrifuged (Hettich, Universal 320R, Germany) at 13.000 rpm for 10 min. The supernatant was filtered through a sterile 0.22 µm filter (Millipore, Millex-GS, Ireland). In the extraction of patulin from samples, the method suggested by [Gökmen and Acar \(1998\)](#) was used with small modifications.

A 2-ml volume of centrifuged and filtered sample was extracted twice with 10 mL of ethyl acetate (Merck, Darmstadt, Germany) by shaking vigorously for 1 min using a vortex mixer. Organic phases were combined and extracted with 5 mL of 3% sodium carbonate (Na₂CO₃) solution by shaking for 1 min. Then, the phases were allowed to separate and the aqueous phase was immediately extracted with 10 mL of ethyl acetate by shaking for 1 min. Combined organic phases were dried over 2.5 g of anhydrous sodium sulfate. Subsequently, the dried extract was filtered through a black band filter paper to remove the remaining particles of anhydrous sodium sulfate. A 2-ml excess of ethyl acetate was added to wash the filter cake layer and the filtrate obtained was combined with the filtered extract. Then the extract was evaporated just to dryness using rotary evaporator (Heidolph, Hei-VAP-HL, Germany) at 35°C. The residue was immediately dissolved in 4 mL of UPW that had been acidified with acetic acid (pH 4.0) and filtered by 0.45 µm PTFE membrane filter. 20 µl of this solution were injected into the column. Final solutions were kept in a deep freezer until the chromatographic measurements.

All treatments consisted of three replicates, and the experiments were repeated twice.

3.9.2. HPLC Conditions for Patulin Detection

High performance liquid chromatography (HPLC) analyses of patulin were performed according to the method described by [Ağçam and Akyıldız \(2014\)](#) with some modifications. HPLC analyses were carried out by means of a Shimadzu LC-20AT (Japan) system, consisting of a quaternary pump, a column temperature

control oven (CTO-10AS VP), an autosampler unit (SIL-20A), a degasser module (DGU-20A_{5R}), and a photodiode array detector (SPD-M20A). 20 µL of supernatant was injected into the C18 ACE (4.6 x 250 mm) column. The column was maintained at 30 °C with a flow rate of 0.5 mL/min. The photodiode array detector was set at 275 nm. Methanol/water/acetic acid (20/79/1, v/v/v) was used as mobile phases.

Patulin was identified comparing its UV-visible spectra and retention time to that of the corresponding standards based on the quantification of an external standard method. Calibration curve was obtained using the commercial standards of the concentrations at 1-100 ppm for patulin, consequently with the coefficient of determination (R^2) above 0.995. The method's limit of detection (LOD) and limit of quantification (LOQ) were determined as 4.6 ppb and 5.1 ppb, respectively.

All the analyses were conducted in triplicate.

3.10. Statistical Analysis

A one-way analysis of variance (ANOVA) testing was used to compare the *in vitro* and *in vivo* results of different treatments. Tukey multiple comparisons were applied as a post-hoc test to determine significant differences using the software SPSS.23 (SPSS Inc., Version 23.0, Chicago, USA) at the 5% ($p < 0.05$) level of significance between treatments.



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4.1. Biodiversity of Yeasts in Different Varieties of Apples and Lemons

The molecular identification of epiphytic yeasts from three different apple and lemon cultivars was carried out by sequencing the D1/D2 region of the 26S rRNA gene to confirm the preliminary identification obtained by RFLP analysis. The first analysis consisted in molecular weight determination of the ribosomal RNA region 5.8S ITS which generated six products. The electrophoretic separation of DNA fragments obtained after enzymatic digestion produced six different profiles with endonuclease CfoI, seven with HaeIII and seven with HinfI. In order to confirm the identification of species' level of isolates, sequencing of the D1/D2 domain of the 26S rRNA gene was performed for at least three representative strains of each of the RFLP groups. A total of 69 isolates were identified, belonging to 5 different genera and 6 different species (*Saccharomyces cerevisiae*, *Saccharomyces paradoxus*, *Aureobasidium pullulans*, *Meyerozyma guilliermondii*, *Rhodotorula mucilaginosa* and *Wickerhamomyces anomalus*) (Table 4.1). As far as we know, this is the first document that screened the epiphytic yeasts of apple and lemon varieties used in this study.

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Table 4.1. GenBank accession numbers and some information about identified yeast strains

Isolation Source	Isolate Code	Strain Name	B. N.	Q. C. (%)	P. I. (%)	A.N.
Apple (cv. Amasya)	AE2	<i>Aureobasidium pullulans</i>	489	99	100	MK907969
Apple (cv. Amasya)	AE3	<i>Aureobasidium pullulans</i>	505	100	100	MK907970
Apple (cv. Amasya)	AE4	<i>Saccharomyces cerevisiae</i>	560	99	100	MK907971
Apple (cv. Amasya)	AE8	<i>Aureobasidium pullulans</i>	583	99	100	MK907972
Apple (cv. Amasya)	AE13	<i>Saccharomyces cerevisiae</i>	590	99	100	MK907973
Apple (cv. Amasya)	AE14	<i>Aureobasidium pullulans</i>	558	100	100	MK907974
Apple (cv. Amasya)	AE19	<i>Saccharomyces cerevisiae</i>	581	99	100	MK907975
Apple (cv. Amasya)	AE19 (2)	<i>Saccharomyces cerevisiae</i>	486	99	99.79	MK907976
Lemon (cv. Interdonato)	IL9	<i>Saccharomyces cerevisiae</i>	571	99	99.82	MK907977
Lemon (cv. Meyer)	ML11	<i>Saccharomyces cerevisiae</i>	567	99	100	MK907978
Lemon (cv. Interdonato)	IL33	<i>Saccharomyces paradoxus</i>	495	99	100	MK907979
Lemon (cv. Kutdiken)	KL5	<i>Meyerozyma guilliermondii</i>	580	99	100	MK907980
Lemon (cv. Interdonato)	IL11	<i>Meyerozyma guilliermondii</i>	484	99	99.59	MK907981
Lemon (cv. Kutdiken)	KL3	<i>Meyerozyma guilliermondii</i>	564	99	100	MK907982
Lemon (cv. Meyer)	ML4	<i>Meyerozyma guilliermondii</i>	583	99	100	MK907983
Apple (cv. Golden)	GE1	<i>Saccharomyces cerevisiae</i>	578	99	100	MK907984
Apple (cv. Golden)	GE11	<i>Aureobasidium pullulans</i>	548	99	100	MK907985
Apple (cv. Golden)	GE16	<i>Aureobasidium pullulans</i>	569	99	100	MK907986
Apple (cv. Golden)	GE17	<i>Aureobasidium pullulans</i>	562	99	100	MK907987
Apple (cv. Golden)	GE18	<i>Saccharomyces cerevisiae</i>	565	100	100	MK907988
Lemon (cv. Interdonato)	IL1	<i>Rhodotorula mucilaginosa</i>	564	99	100	MK907989

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Lemon (cv. Interdonato)	IL7	<i>Saccharomyces paradoxus</i>	573	99	100	MK907990
Lemon (cv. Kutdiken)	KL4	<i>Saccharomyces cerevisiae</i>	590	99	100	MK907991
Lemon (cv. Kutdiken)	KL6	<i>Saccharomyces paradoxus</i>	555	99	100	MK907992
Lemon (cv. Meyer)	ML3	<i>Saccharomyces cerevisiae</i>	592	99	100	MK907993
Lemon (cv. Kutdiken)	KL22	<i>Meyerozyma guilliermondii</i>	580	100	100	MK907994
Apple (cv. Starking)	SE1	<i>Rhodotorula mucilaginosa</i>	556	99	100	MK907995
Apple (cv. Starking)	SE4	<i>Wickerhamomyces anomalus</i>	572	99	100	MK907996
Apple (cv. Starking)	SE5	<i>Aureobasidium pullulans</i>	558	99	100	MK907997
Apple (cv. Starking)	SE11	<i>Saccharomyces cerevisiae</i>	582	99	100	MK907998
Apple (cv. Starking)	SE18	<i>Saccharomyces cerevisiae</i>	563	100	99.82	MK907999
Apple (cv. Starking)	SE18 (2)	<i>Aureobasidium pullulans</i>	565	100	100	MK908000
Apple (cv. Starking)	SE19	<i>Saccharomyces paradoxus</i>	559	99	100	MK908001
Apple (cv. Starking)	SE19 (2)	<i>Saccharomyces cerevisiae</i>	585	99	100	MK908002
Apple (cv. Starking)	SE23	<i>Saccharomyces cerevisiae</i>	587	99	100	MK908003
Lemon (cv. Meyer)	ML5	<i>Saccharomyces cerevisiae</i>	583	99	100	MK908004
Lemon (cv. Kutdiken)	KL25	<i>Saccharomyces cerevisiae</i>	570	99	100	MK908005
Lemon (cv. Kutdiken)	KL28	<i>Saccharomyces cerevisiae</i>	546	99	100	MK908006

B. N: Base number, Q. C: Query cover, P. I.: Percent identification, A. N.: Accession number, GE: cv. Golden delicious apple, SE: cv. Starking delicious apple, AE: cv. Amasya apple, IL: cv. Interdonato lemon, KL: cv. Kutdiken lemon, MY: cv. Meyer lemon.

Saccharomyces cerevisiae was found as the predominant species with a frequency of 40% (28 isolates), and it was the only species present in all apple and lemon varieties (Figure 4.1). *Aureobasidium pullulans* was isolated as the second dominant yeast species with the ratio of 30% (21 isolates), and this yeast-like fungus was found in all apple varieties whereas not in any of the lemon cultivars. On the contrary, *Meyerozyma guilliermondii* (formerly known as *Pichia guilliermondii*) was successfully isolated from all lemon varieties although it was not detected in any of the apple varieties examined in this study, and this species constituted 12% (8 isolates) of the total yeasts isolated. *Rhodotorula mucilaginosa*, *Saccharomyces paradoxus*, and *Wickerhamomyces anomalus* yeast species represented 9% (6 isolates), 6% (4 isolates) and 3% (2 isolates) respectively. *Wickerhamomyces anomalus* was not detected in any lemon variety similar to *Aureobasidium pullulans*. *Saccharomyces paradoxus*, the closest known relative of model yeast *Saccharomyces cerevisiae*, was isolated solely from cv. Starking Delicious among apple varieties besides from cv. Kutdiken and cv. Interdonato lemons.

The Starking Delicious apple variety had the highest yeast biodiversity with availability of five out of six different yeast species identified in this study, with the exception of *Meyerozyma guilliermondii*. Moreover, only two yeast strains belonging to the same species (*Aureobasidium pullulans* and *Saccharomyces cerevisiae*) could be isolated from the surface of both Amasya and Golden delicious apples. When the lemon varieties were examined, it was determined that cv. Interdonato, cv. Kutdiken and cv. Meyer hosted four, three and two different yeast species, respectively.

Some of the yeast species isolated from different apple varieties have been reported as *Candida krusei*, *Aureobasidium pullulans*, *Rhodotorula minuta*, *Cryptococcus victoriae*, *Saccharomyces cerevisiae* (Erten et al, 2019), *Cryptococcus laurentii*, *Metschnikowia pulcherrima* (Conway et al, 2007), *Rhodotorula mucilaginosa*, *Rhodotorula glutinis* and *Pichia guilliermondii*

(Mokhtari et al, 2012). Yeast microbiota of lemons belonging to the different varieties was composed of *Rhodotorula glutinis*, *Hanseniaspora opuntiae*, *Hanseniaspora occidentalis*, *Cryptococcus flavescens* and *Cryptococcus magnus* (Mokhtari et al, 2012), *Pichia guilliermondii*, *Candida tropicalis*, *Candida lusitanae* (Erten et al, 2019). To our knowledge, this is the first report of *Saccharomyces paradoxus* and *Wickerhamomyces anomalus* in apple and lemon.

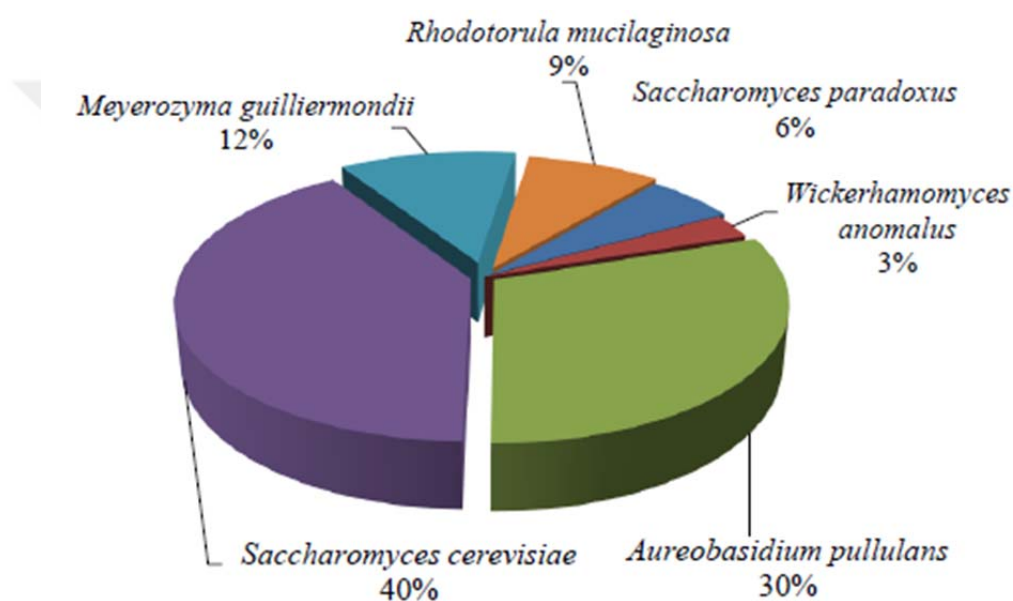


Figure 4.1. Distribution of six yeast species isolated from apple and lemon fruits

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree is shown (Figure 4.2). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al, 2004), and are in the units of the number of base substitutions per site. This analysis involved 38 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion

option). There were a total of 627 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al, 2018).

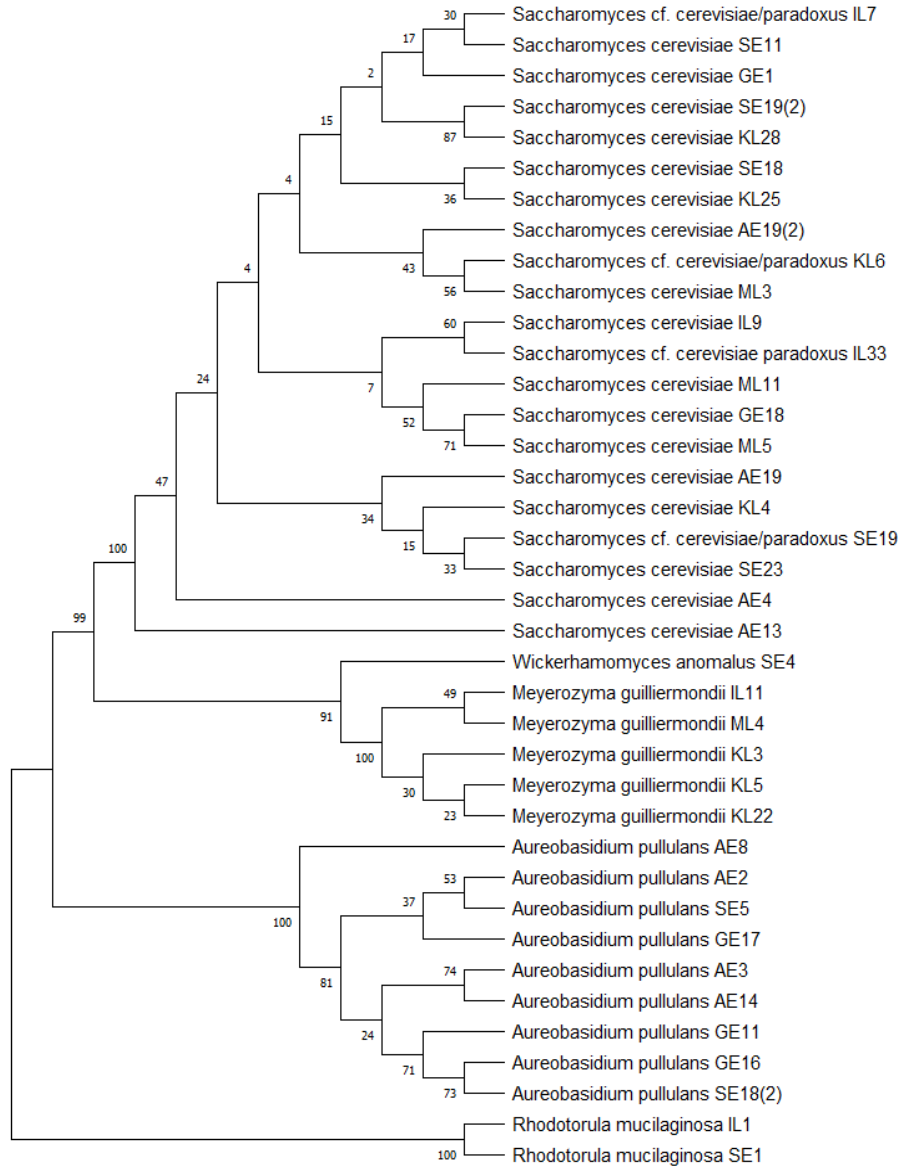


Figure 4.2. Dendrogram obtained from UPGMA cluster analysis based on combined RFLP and D1/D2 domain fingerprints

4.2. Calibration Curve for Determination of Yeasts Cell Number

Cells of the two selected yeasts, *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3, were counted by spectrophotometer due to the ease and speed of this method. The number of yeast cells were determined according to the growth curve of yeasts which were drawn based on the correlation between the results of agar counting and OD600 readings. A linear calibration line was drawn with a high R square including equation (Figure 4.3). Linear regression as $y = 7.695x + 0.1849$ with $R^2 = 0.98$ value was obtained for *Aureobasidium pullulans* GE17 and $y = 10.314x + 0.4378$ with $R^2 = 0.94$ value for *Meyerozyma guilliermondii* KL3.

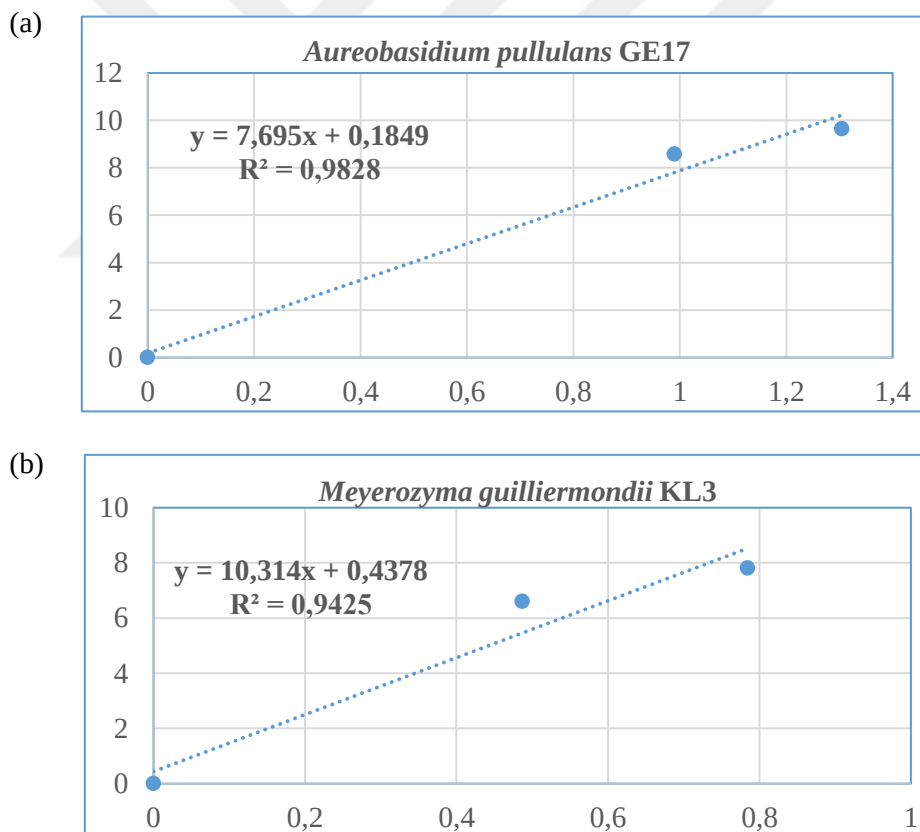


Figure 4.3. The linear part of the growth curve of strains *Aureobasidium pullulans* GE17 (a) and *Meyerozyma guilliermondii* KL3 (b)

4.3. *in vitro* Analyses

4.3.1. Preliminary Screening of Isolated Yeasts by Dual Culture Test

The antagonistic capability of all 69 isolated yeast strains against target molds, *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750, was pre-screened by dual culture *in vitro* method, and isolates showing high levels of inhibitory effect against blue and green molds were selected for subsequent analyses. Although the fungal growth was not fully inhibited by any yeast, mycelium growth of both fungi was confined over 50% by only two yeast species (*Aureobasidium pullulans* GE17, *Meyerozyma guilliermondii* KL3) compared to the control treatment after 7-day incubation. Inhibition rates of the remaining 67 isolates ranged from 4% to 33%. Therefore, those strains were not considered for latter trials due to their low antagonistic effect. Furthermore, both pathogens colonized the entire surface of YPD plates without yeasts after 6-day incubation. However, *Penicillium digitatum* DSM2750 covered the surface of the whole plate after 12 days when this pathogen was inoculated separately with *Aureobasidium pullulans* GE17 or *Meyerozyma guilliermondii* KL3. Other mold, *Penicillium expansum* DSM62841, grew over the yeasts and colonized the entire surface of the plates after 9 and 10 days when it was plated with *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3, respectively.

[Pantelides et al \(2015\)](#), tested the antagonism capacity of all yeasts isolated from grapes against an ochratoxin producing isolate of *Aspergillus tubingensis* by dual culture assay. Seven taxonomically distinct groups at species level was identified and results showed that none of those isolates (*Aureobasidium pullulans*, *Rhodotorula mucilaginosa*, *Candida zeylanoides*, *Candida sake*, *Cryptococcus magnus*, *Pseudozyma aphidis*, *Hanseniaspora uvarum*) were able to completely inhibit mycelial growth of tested mold. However, fungal sporulation of *Aspergillus tubingensis* was inhibited by 27 out of the 37 identified *Aureobasidium pullulans* isolates.

4.3.2. Efficacy of VOCs in Controlling Blue and Green Moulds

The inhibitory effect of volatile antifungal metabolites produced by antagonists was evaluated at two different pH values and also yeast cell concentrations (Table 4.2). Results clearly proved that an increase in the concentration of antagonistic yeasts led to a decrease in radial growth of both *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 in all treatments ($p < 0.05$). Additionally, the greatest inhibition values of both pathogens by VOCs was observed as a result of using mixed culture rather than their usage alone. Data presented in Table 4.2 indicated that the highest level of inhibition for *Penicillium expansum* DSM62841 was achieved at the ratio 56.73% at pH 4.5 whereas it was 50.12% at pH 6.0 for *Penicillium digitatum* DSM2750 by volatile substances produced via combination of two yeasts. Likewise, the combined usage of the two yeasts was inhibited by the mycelial growth of *Penicillium digitatum* DSM2750 by 47.61% at pH 4.5 while *Penicillium expansum* DSM62841 by 47.96% at pH 6.0.

The inhibition rate was below 40% in all trials using *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 yeasts alone. One of the remarkable results is that radial growth (mm) of *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750 was lower at acidic pH (4.5) than pH 6.0 on the whole treatments including control (Figure 4.4). Similarly, the maximum inhibition of *Botrytis cinerea* was observed at pH 4.5 by VOCs produced from four different yeast species as *Metschnikowia pulcherrima*, *Saccharomyces cerevisiae*, *Aureobasidium pullulans* and *Wickerhamomyces anomalus* (Parafati et al, 2015).

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Table 4.2. Effect of volatile metabolites of antagonistic isolates on mycelial growth of *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750 under different pH and yeast cell concentrations

Treatments	Cell numbers of yeasts/plate	pH 4.5		pH 6.0	
		Mean radial growth (mm)	Inhibition (%)	Mean radial growth (mm)	Inhibition (%)
P1 (control)	None	48,07±0,69 ^A	-	50,97±1,45 ^{AB}	-
P2 (control)	None	47,52±0,78 ^{AB}	-	58,47±0,84 ^A	-
Y1P1	10 ⁶	41,50±2,58 ^{ABC}	13,67	49,19±1,59 ^{AB}	3,50
Y1P1	10 ⁸	38,98±1,26 ^{ABC}	18,90	39,92±1,14 ^{BCD}	21,68
Y2P1	10 ⁶	31,38±2,97 ^{BCD}	34,72	43,93±0,55 ^{ABC}	13,82
Y2P1	10 ⁸	30,94±2,92 ^{CD}	35,63	35,10±2,87 ^{BCD}	31,13
Y1P2	10 ⁶	40,20±1,81 ^{ABC}	15,40	49,68±2,22 ^{AB}	15,03
Y1P2	10 ⁸	32,95±2,64 ^{ABCD}	30,66	44,40±1,58 ^{ABC}	24,07
Y2P2	10 ⁶	38,95±1,06 ^{ABC}	18,03	39,71±3,75 ^{BCD}	32,08
Y2P2	10 ⁸	37,35±3,80 ^{ABC}	21,41	38,47±2,82 ^{BCD}	34,21
XP1	10 ⁶	26,22±1,13 ^D	45,45	27,19±0,49 ^D	46,66
XP1	10 ⁸	25,18±1,85 ^D	47,61	25,42±2,07 ^D	50,12
XP2	10 ⁶	29,69±3,08 ^{CD}	37,52	31,84±3,60 ^{CD}	45,55
XP2	10 ⁸	20,56±2,36 ^E	56,73	30,43±2,32 ^{CD}	47,96

Y1: *Meyerozyma guilliermondii* KL3, Y2: *Aureobasidium pullulans* GE17, P1: *Penicillium digitatum* DSM2750, P2: *Penicillium expansum* DSM62841, X: Mixed culture, Mean values with different letters in each column have significant differences according to Duncan's multiple range test at $p < 0.05$.

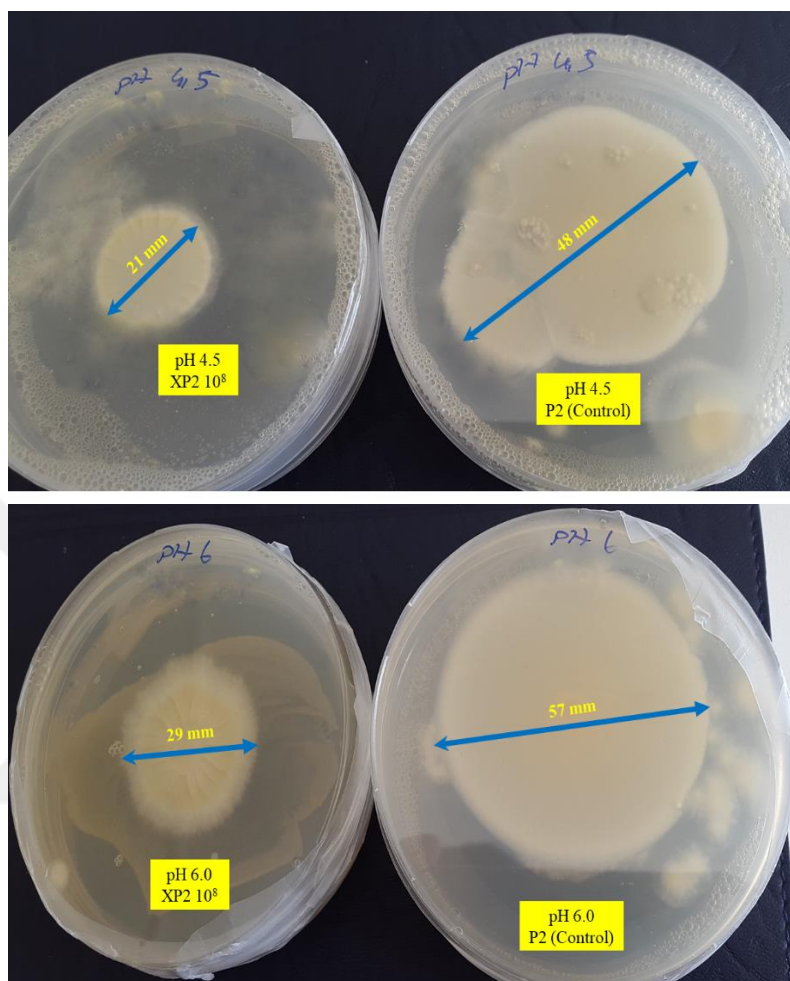


Figure 4.4. Changes between radial growth of *Penicillium expansum* DSM62841 in control and mixed culture experiment at pH 4.5 and 6.0

It is very clear that in recent years, the interest in production of VOCs by biocontrol agents, among the various modes of action, has received considerable attention (Mari et al, 2016; Contarino et al, 2019). Several studies have reported some antifungal compounds, belonging to different chemical classes (alcohols, aldehydes, hydrocarbons and terpene), produced by yeast strains such as 2-phenylethanol, 2-ethylhexanol, D-limonene, benzaldehyde, 2-methylbutanol, 3-methylbutyl hexanoate, 2-methylpropyl hexanoate, and pentylhexanoate which

demonstrate efficiency in controlling some postharvest pathogens such as *Penicillium digitatum*, *Penicillium expansum*, *Botrytis cinerea*, *Alternaria alternata*, *Aspergillus carbonarius* and *Aspergillus ochraceus* (Arrarte et al, 2017; Farbo et al, 2018).

Di Francesco et al (2015) identified some volatile compounds with regard to two strains (L1-L8) of *Aureobasidium pullulans* by SPME-GC technique and reported as 2-phenyl, 1-butanol-3-methyl, 1-butanol-2-methyl and 1-propanol-2-methyl belonging to the group of alcohols. It was also stated that those compounds were primarily produced in the first 4 days of growth for both strains and production of VOCs could play a key role for antagonistic activity of *Aureobasidium pullulans* L1 and L8 against five fruit postharvest pathogens (*Penicillium expansum*, *Penicillium digitatum*, *Penicillium italicum*, *Botrytis cinerea*, *Colletotrichum acutatum*).

4.3.3. Secretion of Extracellular Lytic Enzymes

Aureobasidium pullulans GE17 and *Meyerozyma guilliermondii* KL3 strains demonstrate the same characteristics for tested β -1,3 glucanase, protease, gelatinase, pectinase and chitinase enzymes. In this context, both yeast strains are capable of producing extracellular β -1,3 glucanase, protease and gelatinase enzymes, whereas they are incapable of producing the remaining two enzymes (Table 4.3). Our findings are compatible with some previous researchers as Liu et al (2013) who reported the secretion of β -1,3 glucanase by *Meyerozyma guilliermondii* for degrading the cell walls of *Botrytis cinerea* after strongly attaching to its hyphae. Parafati et al (2015) also determined the activities of protease, gelatinase and β -1,3 glucanase enzymes for *Aureobasidium pullulans* and documented their role in the mode of action of this biocontrol agent. Unlike our findings, *Aureobasidium pullulans* (Spadaro and Droby, 2016) and *Meyerozyma guilliermondii* (Zhang et al, 2011) strains showing pectinase and chitinase activity have also been reported in the literature.

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Table 4.3. *In vitro* enzymatic activity assays of two selected yeasts

Yeast Strains	Isolation Source	Enzymatic Activity				
		Protease	Chitinase	β -1,3 glucanase	Pectinase	Gelatinase
<i>Aureobasidium pullulans</i> GE17	cv. Golden delicious apple	+	-	+	-	+
<i>Meyerozyma guilliermondii</i> KL3	cv. Kutdiken lemon	+	-	+	-	+

Having chitinolytic and proteolytic activity for an antagonistic yeast is noticeably important due to potential application of them for biocontrol of fungal pathogens and insects. This is because chitin is the structural backbone of the fungal cell walls and is arranged in regularly ordered layers. Glucan is the main structural polysaccharide of the fungal cell wall while β -1,3-glucan is regarded as the chief structural component to which other cell constituents are covalently attached. Moreover, β -1,3-glucan accounts for the mechanical strength and integrity of cell walls. The cell walls of filamentous fungi are composed of 20% or more chitin, 50% – 60% glucan and around 20%–30% proteins by dry weight of wall (Seidl, 2008). Proteins in the cell wall are involved in many actions such as maintaining cell shape, protecting the cell against foreign materials, synthesizing and adjusting cell wall components, mediating adhesion for cell migration and fusion (Spadaro and Droby, 2016). Therefore, secretion of different enzymes in particular β -1,3-glucanase, chitinases and proteases is essential to degrade and cause lysis of pathogen cell walls. It seems that production ability of β -1,3 glucanase, protease and gelatinase by two yeasts utilized in this study can directly or indirectly contribute to their biocontrol activity (Figure 4.5).

Pectinase enzyme led to the softening of fruits tissue as a result of the destruction of pectin molecules, which are high molecular weight acid polysaccharides present in plant cell walls primarily made up of α -(1→4) linked D-galacturonic acid residues (Oskay and Yalçın, 2015). The lack of pectinase activity in the yeasts *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 used in this study was considered as an advantage, because this characteristic could contribute to maintaining fruit firmness and preventing softening when these yeasts are applied to the surfaces of fruits for biocontrol intention.

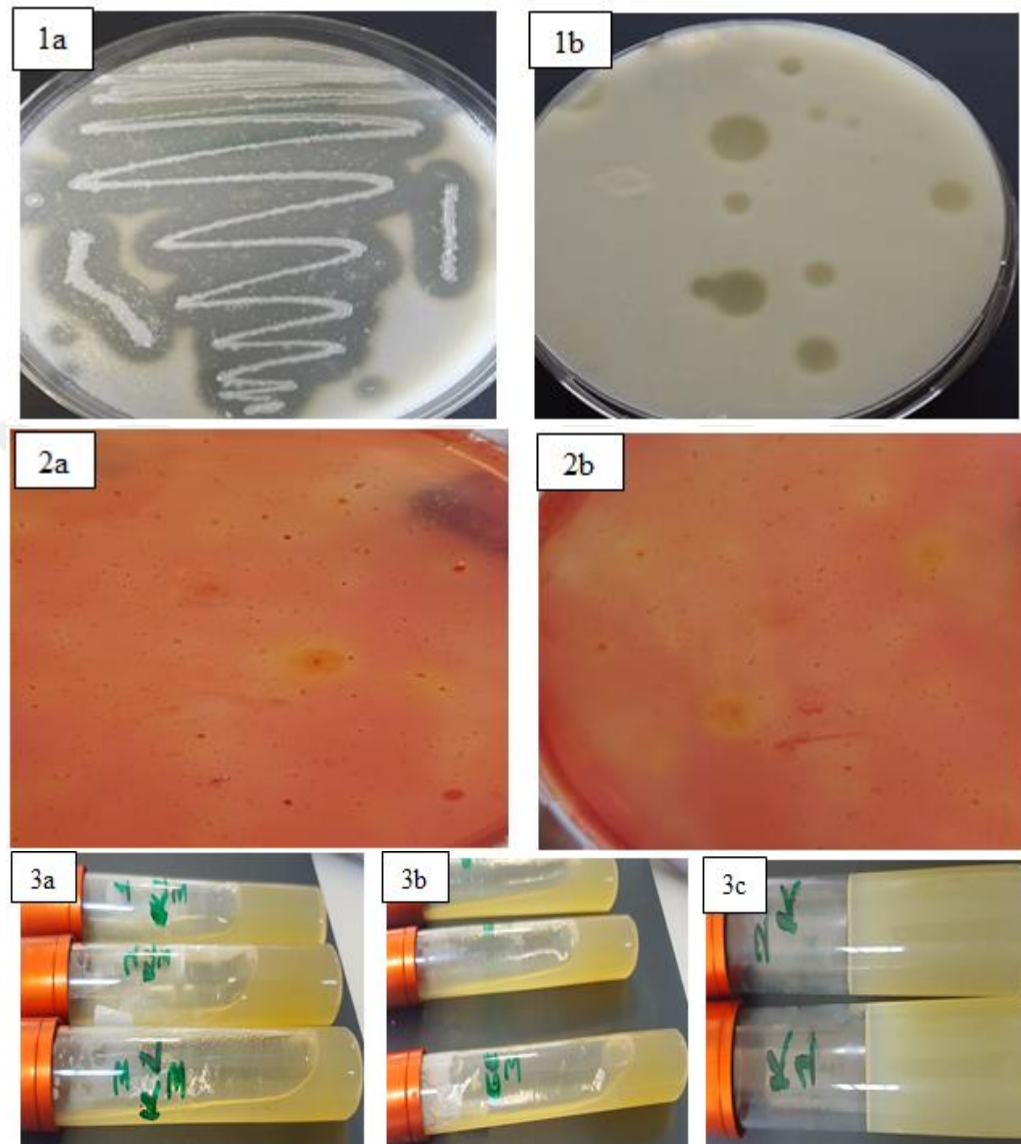


Figure 4.5. View of positive plates from enzyme assays. (1) protease activity; (2) β -1,3 glucanase activity; (3) gelatinase activity; (a) plate including *Meyerozyma guilliermondii* KL3, (b) plate including *Aureobasidium pullulans* GE17, (c) control plates (without yeasts)

4.3.4. Study of Iron Depletion Assay

Iron (Fe³⁺) is a biologically significant element because it is essential for the growth of almost all microorganisms, and it is present in the structure of heme-cofactored proteins (as catalase, cytochromes), iron sulphur cluster (Fe/S) including electron carriers' proteins, and di-iron and mononuclear enzymes. Additionally, this element acts as a cofactor and is desired for the activity of many cellular enzymes involved within the numerous cellular processes (Nally et al, 2015; Spadaro and Droby, 2016). Therefore, it is believed that iron competition could be an important mechanism affecting biocontrol activity in some biocontrol systems. Siderophore producing yeast species are able to chelate iron, and this reduces the growth chances of pathogenic fungi due to non-availability of iron, which is a crucial element of survival for them.

Results of iron assay performed with different iron concentrations (5, 10, and 20 mg/L of FeCl₃) proved that antagonistic activity of yeast strains *Aureobasidium pullulans* GE17 and *M. guilliermondii* KL3 is not dependant on the iron competition mechanism. The reddish halo was not visible after 6 days of incubation for two yeasts tested under the study. Similarly, iron depletion mechanism was not confirmed for *Aureobasidium pullulans*, *Wickerhamomyces anomalus*, *Saccharomyces cerevisiae* (Parafati et al, 2015) and *Meyerozyma guilliermondii* but confirmed for *Metschnikowia pulcherrima* strains whose main mode of action against postharvest pathogens was extensively reported as iron depletion (Saravanakumar et al, 2008). Several studies have reported that yeast species in genus *Metschnikowia* such as *Metschnikowia pulcherrima* (Kantor et al, 2015), *Metschnikowia citriensis* (Liu et al, 2019) and *Metschnikowia fructicola* (Saravanakumar et al, 2008) use chelating agent (siderophore, pulcherrimin) as a competitive factor in order to overcome the growth of various filamentous fungi and bacteria through iron depletion. As far as it is known, there is not any genus producing pulcherrimin, a chemical complex formed between iron and pulcherriminic acid, with the exception of *Metschnikowia*.

4.3.5. Effect of Antagonistic Yeasts on Spore Germination of Pathogenic

Moulds

Yeasts were assayed for their capability to suppress germination of *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 conidia at 25 °C for 12 h in an orbital shaker. The results showed that reduction in germination rate for *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 reached up to 88.13% and 94.69%, respectively. Application of 100 µl from 10⁸ cells/mL *Aureobasidium pullulans* GE17 showed the best control efficacy in inhibiting the germination of *Penicillium digitatum* DSM2750 in YPD broth. Similarly, utilization of the same volume from 10⁸ cells/mL mixed culture led to the highest decrease of conidial germination of *Penicillium expansum* DSM62841 of around 95%. The results distinctly indicate that an increase in the concentration of antagonist yeasts led to higher inhibition of fungal spore germination, thereby lower concentration of yeast cells (10⁶ cells/mL) increased the mean germination rate (Table 4.4). As can be seen from Figure 4.6, eight from the whole applied 12 treatments significantly prevented the spore germination of *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 by at least 77.43% ($p < 0.05$).

Previous studies have shown that competition for nutrients have a direct effect on the spore germination (Liu et al, 2013), because conidia requires nutrients (carbon sources, nitrogen, glucose, fructose, iron) for germination (Gramisci et al, 2018; Adnan et al, 2019). Production of VOCs, proteins, enzymes and antibiotic secondary metabolites also helps to delay the germination of fungal spores (Moraes Bazioli et al, 2019). For instance; the inhibition of spore germination, growth and also aflatoxin production in *Aspergillus flavus* was achieved by an antifungal compound 2-phenylethanol which is produced by *Wickerhamomyces anomalus* as a volatile compound (Hua et al, 2014).

Table 4.4. Effect of yeasts on spore germination of *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841

Treatments	Concentration of Yeasts (cells/mL)	Count of Germinated Spore	Inhibition of Germination (%)
P1 (Control)	Without yeast	46,33±4,70 ^A	-
P2 (Control)	Without yeast	37,66±0,47 ^B	-
Y1P1	10 ⁶	35,50±2,12 ^B	23,38
Y1P1	10 ⁸	9,00±1,41 ^{DE}	80,58
Y2P1	10 ⁶	6,50±0,70 ^E	85,97
Y2P1	10 ⁸	5,50±4,36 ^E	88,13
Y1P2	10 ⁶	35,00±0,00 ^B	7,08
Y1P2	10 ⁸	17,00±2,82 ^C	54,87
Y2P2	10 ⁶	16,00±4,65 ^{CD}	57,52
Y2P2	10 ⁸	8,50±3,53 ^{DE}	77,43
XP1	10 ⁶	8,50±5,36 ^{DE}	81,65
XP1	10 ⁸	6,50±0,70 ^E	85,97
XP2	10 ⁶	5,50±0,70 ^E	85,40
XP2	10 ⁸	2,00±1,41 ^E	94,69

Y1: *Meyerozyma guilliermondii* KL3, Y2: *Aureobasidium pullulans* GE17, P1: *Penicillium digitatum* DSM2750, P2: *Penicillium expansum* DSM62841, X: Mixed culture, Mean values with different letters in each column indicate significant differences among treatments according to Duncan's multiple range test at $p < 0.05$.

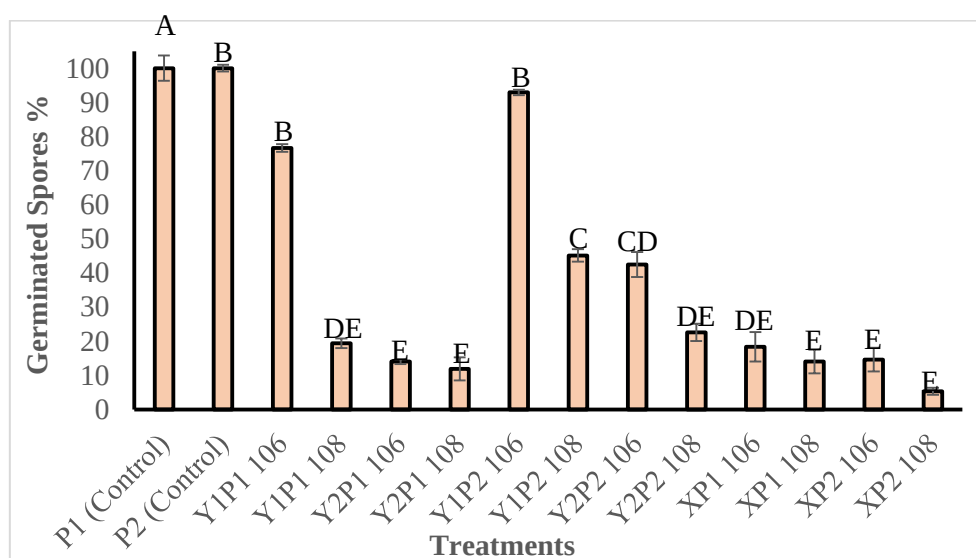


Figure 4.6. The percentage of germinated spore

4.3.6. Percent Inhibition of Radial Growth by Selected Yeasts

The colony diameter and percentage inhibition of pathogens were calculated in comparison to the control experiment by the formula detailed in materials and methods. The pathogenic moulds were challenged with two selected yeasts on YPD agar *in vitro* at two different yeast concentrations (10^6 and 10^8 cells/mL), and the data from these assays showed that all tested treatments inhibited the mycelial growth of both pathogens with various efficacy. Diameter values determined by measuring radial growth of both pathogens during 10-day incubation were presented in Table 4.5. Percent inhibition of *Penicillium digitatum* DSM2750 ranged from 45.42% to 74.66%, whereas it was 45.77% to 83.37% for *Penicillium expansum* DSM62841 after 10 days of incubation (Figure 4.7). The highest level of percentage inhibition of both *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 was obtained by using the yeast *Aureobasidium pullulans* GE17 at 10^8 cells/mL concentration. Other treatments which used single culture of *Meyerozyma guilliermondii* KL3 and mixed culture exhibited inhibition between 45.42% and 58.19 % for tested pathogens which is lower than that of *Aureobasidium pullulans* GE17.

As the concentrations of yeast cells increased, radial diameters of moulds decreased significantly in all trials as shown in Figure 4.7 and Table 4.5 ($p < 0.05$). Mycelium diameter (mm) values were 69.29 ± 3.27 and 72.02 ± 3.19 mm in the control plates of *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 at the end of the incubation, respectively (Figure 4.8). The radial diameter of *Penicillium digitatum* DSM2750 was reduced by 31.47–51.74 mm, whereas it was between 32.96 mm and 60.05 mm for *Penicillium expansum* DSM62841 when they were plated with yeasts (Table 4.5).

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Table 4.5. Radial growth diameter of *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 during ten days incubation

Experiments	1.day (mm)	2.day (mm)	3.day (mm)	4.day (mm)	5.day (mm)	6.day (mm)	7.day (mm)	8.day (mm)	9.day (mm)	10.day (mm)
P1	17,34±1,51 ^{hA}	26,40±1,75 ^{gA}	32,65±2,12 ^{gA}	37,53±2,08 ^{efA}	42,00±1,17 ^{eA}	50,07±1,28 ^{dA}	55,07±0,89 ^{cdA}	60,43±2,25 ^{bcA}	66,00±4,58 ^{abA}	69,29±3,27 ^{aA}
P2	14,62±0,12 ^{IA}	23,76±0,07 ^{hA}	31,37±0,21 ^{gA}	38,11±0,63 ^{IA}	43,41±1,01 ^{eA}	50,25±1,61 ^{dA}	56,53±2,91 ^{cA}	60,24±3,65 ^{bcA}	64,65±4,76 ^{bA}	72,02±3,19 ^{aA}
Y1P1 10 ⁶	9,47±0,44 ^{bBC}	12,54±0,86 ^{ghCD}	17,09±0,34 ^{gD}	19,29±1,38 ^{fC}	21,90±1,30 ^{efD}	25,32±1,63 ^{deCD}	28,99±2,72 ^{cdBCD}	31,05±2,85 ^{bcBCD}	35,16±3,06 ^{abB}	37,82±2,91 ^{aB}
Y1P1 10 ⁸	7,94±0,57 ^{BCDE}	11,10±0,08 ^{efDE}	12,23±0,79 ^{deE}	12,59±0,89 ^{deD}	14,80±1,73 ^{deE}	15,78±1,09 ^{cdFG}	19,04±1,50 ^{cEFG}	24,37±0,14 ^{bDE}	30,00±0,75 ^{aB}	31,83±1,83 ^{aBCD}
Y2P1 10 ⁶	5,06±0,83 ^{bEF}	9,06±0,56 ^{abEF}	9,24±0,26 ^{abFG}	9,88±0,61 ^{abDE}	11,25±1,84 ^{abEF}	13,58±1,15 ^{abG}	16,94±1,80 ^{abFGH}	18,34±1,53 ^{abEF}	19,29±1,70 ^{aC}	20,19±1,75 ^{aCDE}
Y2P1 10 ⁸	5,94±0,78 ^{aDEF}	7,42±0,96 ^{aFG}	9,07±0,67 ^{aFG}	12,11±1,22 ^{aD}	13,61±1,54 ^{aEF}	14,40±1,63 ^{aG}	15,27±2,94 ^{aFGH}	15,65±2,75 ^{aF}	17,29±2,87 ^{aC}	17,55±2,04 ^{aDE}
Y1P2 10 ⁶	9,68±0,34 ^{gBC}	13,00±0,48 ^{fCD}	15,00±1,32 ^{fD}	19,33±0,14 ^{eC}	21,16±1,71 ^{eD}	26,46±1,58 ^{dCD}	27,78±1,68 ^{dBCD}	32,25±1,51 ^{cBC}	35,82±0,88 ^{bB}	39,06±2,75 ^{aB}
Y1P2 10 ⁸	8,57±0,51 ^{hBCD}	11,15±0,52 ^{gDE}	12,36±0,58 ^{fgE}	13,15±0,39 ^{fD}	13,70±0,54 ^{efE}	19,63±1,44 ^{eEF}	21,97±1,36 ^{dDEF}	26,01±0,93 ^{cCD}	29,69±0,98 ^{bB}	32,10±0,81 ^{aBC}
Y2P2 10 ⁶	5,55±0,37 ^{eDEF}	8,41±0,14 ^{def}	9,46±1,22 ^{cdF}	10,37±1,26 ^{bcdDE}	11,34±1,36 ^{abDEF}	11,64±1,24 ^{abG}	12,02±1,11 ^{abGH}	12,30±1,32 ^{abF}	12,56±1,34 ^{abC}	13,31±0,72 ^{aE}
Y2P2 10 ⁸	3,02±0,14 ^{dF}	5,33±0,18 ^{cdG}	6,56±0,42 ^{bcG}	8,68±0,88 ^{abE}	10,10±1,63 ^{aF}	10,88±1,40 ^{aG}	11,02±1,79 ^{aH}	11,35±1,09 ^{aF}	11,66±1,93 ^{aC}	11,97±1,41 ^{aE}
XP1 10 ⁶	9,79±0,38 ^{fBC}	15,19±0,69 ^{eBC}	20,78±1,17 ^{dC}	23,72±1,20 ^{dB}	27,32±1,04 ^{BC}	28,68±1,86 ^{bcBC}	29,84±1,77 ^{bcBC}	31,12±1,66 ^{abBCD}	33,86±1,41 ^{aB}	34,05±1,39 ^{aBC}
XP1 10 ⁸	7,15±0,02 ^{gCDE}	11,17±0,17 ^{fDE}	16,17±1,67 ^{eD}	18,96±1,95 ^{deC}	20,12±1,99 ^{dD}	21,47±1,49 ^{cdDE}	24,36±1,92 ^{bcCDE}	27,40±1,45 ^{bBCD}	31,05±0,73 ^{aB}	32,98±2,04 ^{aBC}
XP2 10 ⁶	11,06±0,88 ^{gB}	17,38±0,40 ^{fB}	23,99±0,81 ^{eB}	25,68±0,96 ^{eB}	29,42±0,79 ^{dB}	32,00±1,30 ^{CB}	34,00±1,38 ^{bcB}	34,94±1,21 ^{abB}	36,71±1,14 ^{aB}	37,16±0,97 ^{aB}
XP2 10 ⁸	7,01±0,19 ^{fCDE}	11,44±0,35 ^{eDE}	16,70±0,12 ^{dD}	17,83±0,43 ^{dC}	23,68±1,56 ^{cD}	24,22±1,10 ^{cCDE}	24,37±0,98 ^{cCDE}	27,35±0,79 ^{bBCD}	29,02±0,61 ^{aB}	30,11±0,99 ^{aBCD}

Y1: *Meyerozyma guilliermondii* KL3, Y2: *Aureobasidium pullulans* GE17, P1: *Penicillium digitatum* DSM2750, P2: *Penicillium expansum* DSM62841, X: Mixed culture, ^{A-H}: Uppercase superscripts indicate significant differences within the same column ($p<0.05$), ^{a-i}: Lowercase superscripts indicate significant differences within the same row ($p<0.05$).

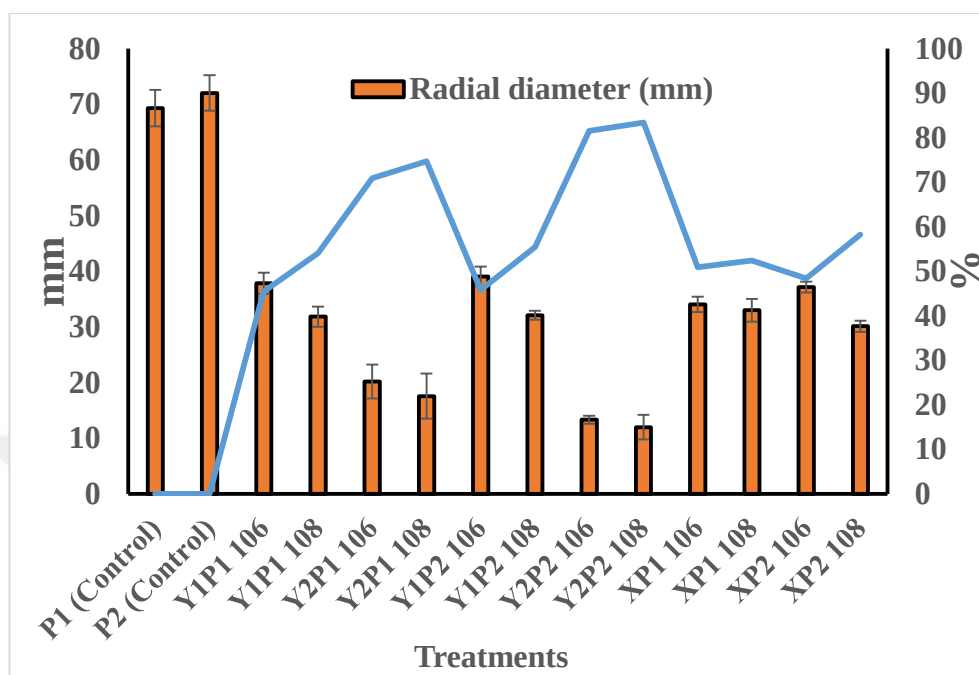


Figure 4.7. The mycelial diameter (mm) and percent radial growth inhibition of pathogens after 10 days incubation at 25 °C

Recent studies reported the mycelium growth inhibition (%) of several important postharvest pathogens such as *Monilinia fructicola* by yeast strains *Debaryomyces hansenii* MI1a and *Wickerhamomyces anomalus* BS91 as 22.41–69.53 (Czarnecka et al, 2019), *Botrytis cinerea* by yeast strains *Metschnikowia pulcherrima*, *Aureobasidium pullulans*, *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus* as 47.67–81.50 (Parafati et al, 2015), *Penicillium expansum* by yeast strains *Hanseniaspora opuntiae* and *Metschnikowia pulcherrima* as 45.20–63.70 (Ruiz-Moyano et al, 2016) and *Rhizoctonia solani* by bacterial strain *Bacillus velezensis* 8-4 as 48.96 (Cui et al, 2020). Similarly, the inhibition zone of 45 different yeast isolates belonging to eight different genera against *Penicillium digitatum* was determined to be between 2.03 mm to 15.73 mm (Liu et al, 2017).

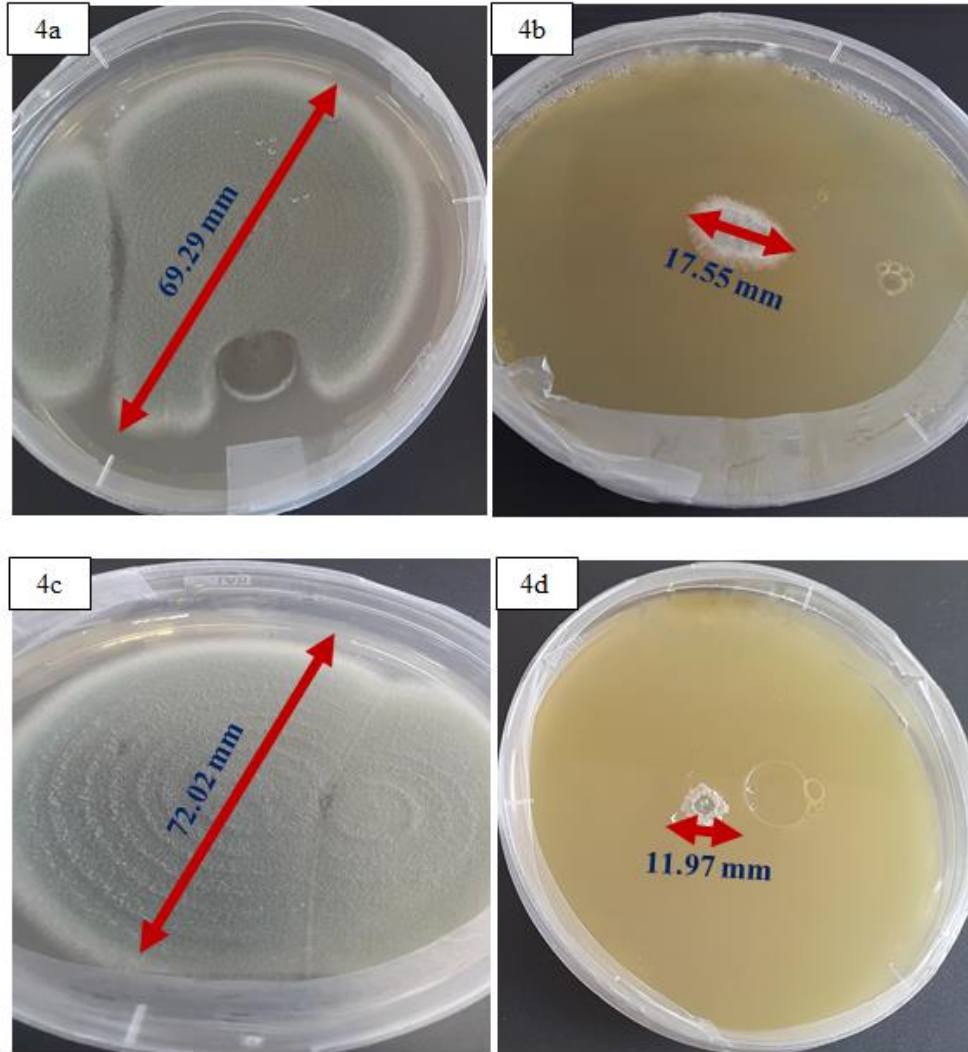


Figure 4.8. Radial diameter (mm) of pathogens in plates with or without yeast after 10 days' incubation (a) *Penicillium digitatum* DSM2750 without yeast (control), (b) *Penicillium digitatum* DSM2750 with *Aureobasidium pullulans* GE17 (10^8 cells/mL), (c) *Penicillium expansum* DSM62841 without yeast (control), (d) *Penicillium expansum* DSM62841 with *Aureobasidium pullulans* GE17 (10^8 cells/mL)

4.3.7. The Degradation of Patulin by Yeasts *in vitro*

Retention time of patulin was determined as 10.07 min. Figure 4.9. illustrates the HPLC chromatograms of a standart solution and sample containing patulin.

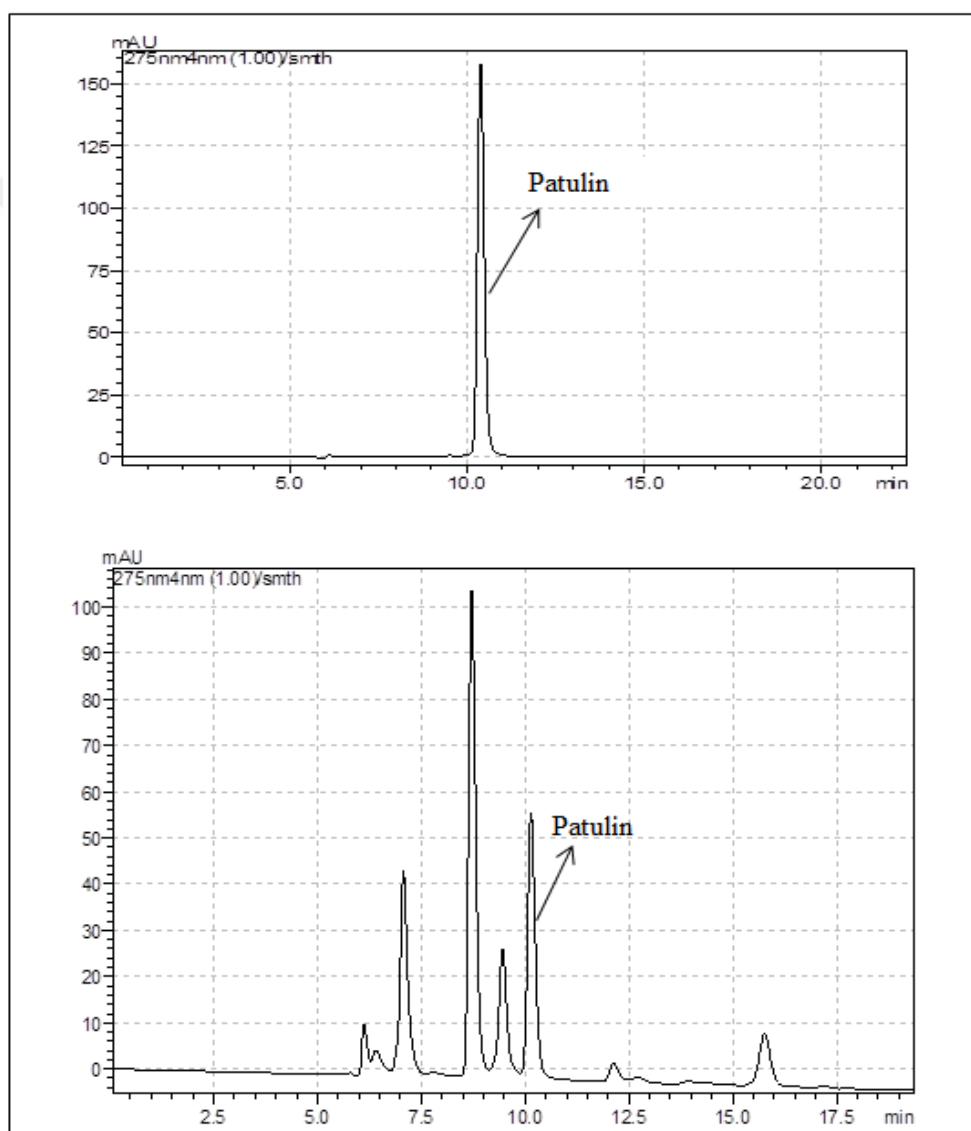


Figure 4.9. HPLC chromatograms of patulin a) from standard solution, b) from sample

The efficacy of yeast strains to degrade patulin *in vitro* was quantified. Although there was a partial degradation of patulin in the control system at all the tested times, the degradation of patulin in the system treated with *Aureobasidium pullulans* GE17 (single culture), *Meyerozyma guilliermondii* KL3 (single culture) and their combination (mixed culture) was greater than that of control at all the tested times (Table 4.6). The initial patulin concentration of 7.0 µg/mL decreased over 31%, of the original concentration when incubated at 28 °C for 8 h, and 57% after 40 h incubation time. Patulin concentration was significantly reduced in all cases in presence of strain GE17, KL3 and mixed culture. After 8 h of incubation at 28 °C, initial patulin degradation reduced by 38.71 %, 40.39 % and 42.45 % by strain KL3, GE17 and mixed culture, respectively. Only 16 h after incubation, high biodegradation level of patulin determined and the degradation rate increased to about 72 % and 73 % with the activity of the strain KL3 and mixed culture, respectively. Over the same time, the degradation of patulin was observed to be slower (55.37 %) in the case of application the strain of *Aureobasidium pullulans* GE17 compared to the strains KL3. The difference between this two strains may be due to the longer adaptation time of the strains GE17 to the patulin including medium and also role of certain intracellular enzymes which are present in the strain KL3 and GE17. Since it was reported that intracellular enzymes (Ianiri et al, 2017) play a key role in the degradation of patulin.

After 32 h incubation period, almost all initial patulin concentration was degraded by strain KL3 (98.50 %) and mixed culture (99.44 %), while quite good ratio was also determined in sample employed with GE17 (89.57 %). At the end of the incubation (after 40 h), patulin amount was determined to be below the LOD in sample treated with mixed culture. In the last tested time, biodegradation of initial patulin at the ratio of 99.74 % and 96.21 % was achieved by strain KL3 and GE17, respectively. Conversely, initial patulin concentration was reduced by 57.33 % without yeast application (control) at the end of the incubation.

To sum up, both yeast strain (KL3 and GE17) have ability for successful degradation of patulin which is the toxic compound for human health produced by several fungal genera including *Penicillium*. Biodegradation of patulin was found to be faster by strain KL3 than GE17. Biodegradation of patulin increased slightly with utilization of mixed culture compared to single use of strain KL3 and the highest degradation rate of patulin was determined in the case that used combination of two yeast at all tested times.

Patulin degradation activity was determined in the same yeast genus that studied in this study and it was reported that patulin degradation occurred mainly inside the cells of a *Candida guilliermondii* strain (Chen et al, 2017). In another comparative study, partial degradation (58.32 %) of the initial patulin concentration (6.67 µg/mL) was reported in the control group (Cao et al, 2013). In the same study, the original concentration of patulin decreased about 78% by *Pichia caribbica* after 30 h incubation at 28 °C.

Penicillium expansum is one of the most important postharvest pathogens of apple worldwide. This mold causes blue mold which can lead to huge losses during storage of apples. In addition, it produces patulin, a secondary metabolite with toxic effects such as immunosuppression, cytotoxicity, neurotoxicity and damage to liver and kidneys in mammals (Pal et al, 2017). The most effective way to avoid patulin contamination in apple and other fruits and vegetables and their derived products is to minimize *Penicillium expansum* rot in them. Therefore, the interest on biological control methods is increasing due to the consumer reluctance to accept chemical residues in food and public concern for environmental safety (Alvarez et al, 2019).

Table 4.6. The degradation of patulin by yeasts *in vitro*

Treatments		Initial Concentration ($\mu\text{g/mL}$)	Final Concentration ($\mu\text{g/mL}$)	Degradation Rate (%)
Control	8 h	7.0	4.7642	31.94
	16 h		4.1499	40.71
	24 h		3.6571	47.75
	32 h		3.4266	51.04
	40 h		2.9866	57.33
GE17	8 h	7.0	4.1721	40.39
	16 h		3.1238	55.37
	24 h		2.7696	60.43
	32 h		0.7300	89.57
	40 h		0.2651	96.12
KL3	8 h	7.0	4.2900	38.71
	16 h		1.9627	71.96
	24 h		0.8105	88.42
	32 h		0.1046	98.50
	40 h		0.0178	99.74
Mixed	8 h	7.0	4.0284	42.45
	16 h		1.8815	73.12
	24 h		0.5978	91.46
	32 h		0.0386	99.44
	40 h		0.0046	99.93

4.4. *in vivo* Analyses

Twelve different experiments (as previously shown in Figure 3.1) including single and mixed culture of selected yeasts with different cell concentrations were tested against *Penicillium expansum* DSM62841 (blue mold) and *Penicillium digitatum* DSM2750 (green mold) under *in vitro* conditions. According to the obtained results of *in vitro* analyses, utilization of single culture of the yeast strain *Aureobasidium pullulans* GE17 and its combination with *Meyerozyma guilliermondii* KL3 exhibited the highest antagonistic activity against both blue and green molds. However, it was determined that application of *Meyerozyma guilliermondii* KL3 yeast strain as a single culture resulted in the restriction of its antagonistic capability. When the cell concentrations examined in this study were evaluated, it can be clearly understood from the results that application of 10^8 cells/mL was more effective with regard to controlling blue and green moulds in comparison to 10^6 cells/mL.

Based on these findings, it was decided to continue *in vivo* analyses on apples and lemons using single culture (*Aureobasidium pullulans* GE17) and mixed culture (*Aureobasidium pullulans* GE17 + *Meyerozyma guilliermondii* KL3) of yeasts at the concentration of 10^8 cells/mL.

4.4.1. Apple Treatment

4.4.1.1. Population Studies of Yeasts in Apple Wounds

The population dynamics of single culture of *Aureobasidium pullulans* GE17 and its mixture with *Meyerozyma guilliermondii* KL3 on artificially wounded cv. Starking Delicious apples are demonstrated in Figure 4.10 and Table 4.7. A photo taken during the population studies analysis of yeasts is presented in Figure 4.11.

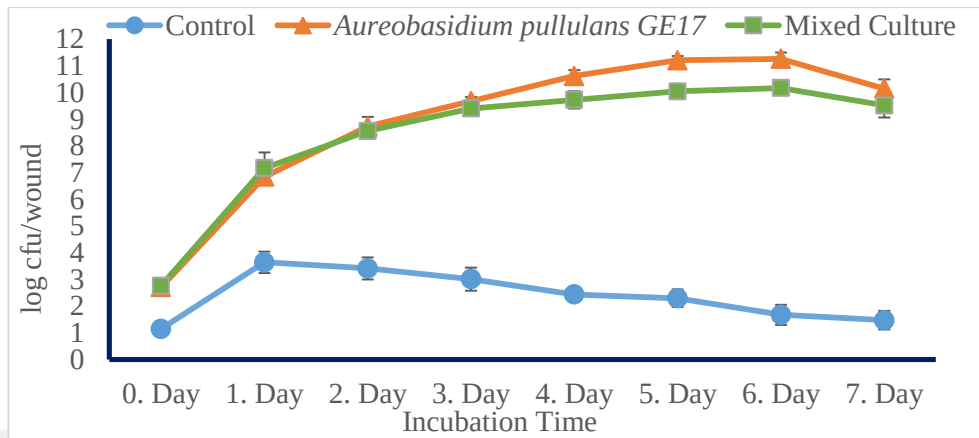


Figure 4.10. Population dynamic of control and two selected yeasts in apple wounds. (Bars represent the standard deviations).

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Table 4.7. Population density of yeasts in artificially wounded apples as log cfu/wound

Treatments	0. Day	1. Day	2. Day	3. Day	4. Day	5. Day	6. Day	7. Day
Control	1,14±0,175 ^{dB}	3,63±0,400 ^{aB}	3,40±0,410 ^{abB}	3,01±0,432 ^{abB}	2,43±0,153 ^{abcC}	2,28±0,332 ^{bcdC}	1,67±0,378 ^{cdC}	1,46±0,352 ^{cdB}
<i>Aureobasidium</i>								
<i>pullulans</i>	2,70±0,027 ^{gA}	6,84±0,050 ^{fA}	8,72±0,358 ^{eA}	9,68±0,148 ^{dA}	10,62±0,213 ^{bcA}	11,20±0,163 ^{abA}	11,26±0,230 ^{aA}	10,14±0,341 ^{cdA}
GE17								
Mixed Culture	2,76±0,023 ^{dA}	7,17±0,590 ^{cA}	8,55±0,081 ^{bA}	9,40±0,223 ^{abA}	9,72±0,324 ^{aB}	10,04±0,173 ^{aB}	10,16±0,141 ^{aB}	9,51±0,451 ^{aA}

^{A-C} : Uppercase superscripts indicate significant differences within the same column ($p<0.05$), ^{a-g} : Lowercase superscripts indicate significant differences within the same row ($p<0.05$).

As it can be seen from the results, both yeasts uniquely adapted to the apple environment and they rapidly colonized the wounded parts of apples, even reaching an average 2.73 log cfu/wound in day 0 (after 2-hour inoculation). After 24-hour incubation at 25 °C, the population density of single culture of *Aureobasidium pullulans* GE17 and mixed culture exceeded their starting inoculum concentration (6.30 log cfu/mL) by reaching 6.84 log cfu/wound and 7.17 log cfu/wound, respectively. The concentration of yeasts in apple wounds continuously increased during the first six days and then a slight decrease was observed on the last day of incubation. Conversely, yeast population in the control treatment (inoculated with SDW) increased only on the first day (3.63 log cfu/wound) and then decreased continually. Single culture of *Aureobasidium pullulans* GE17 and mixed culture (*Aureobasidium pullulans* GE17 + *Meyerozyma guilliermondii* KL3) reached the maximum population peak at 6 days as 11.26 log cfu/wound and 10.16 log cfu/wound, respectively. After one-week of incubation, *Aureobasidium pullulans* GE17 (10.14 log cfu/wound) and its mixture with *Meyerozyma guilliermondii* KL3 (9.51 log cfu/wound) were found at quite significant numbers in apple wounds (Table 4.7). On the other hand, yeast population in control wounds was found to be very low (1.46 log cfu/wound) at the end of the incubation period. According to the results, the highest increment in the density of *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 antagonist yeast strains was determined during the first 24 h and then population density increased slower and stabilized between 5th and 6th days. The differences between separate experiments on the same day and also between the same experiments on the different incubation days were found statistically significant ($p<0.05$).

It can be concluded that both yeast strains tested in this thesis exhibited great colonization ability in wounds of cv. Starking Delicious apples during 7 days of incubation at 25 °C. However, the number of yeast population was found higher in the treatment which used a single culture of *Aureobasidium pullulans* GE17 than its combination with *Meyerozyma guilliermondii* KL3 strain during all the

incubation period except the first day. This result means that if both yeasts are present together, they also compete for space and nutrition with each other.

Colonization ability of candidate yeast strains in fruit wounds play a crucial role in their biocontrol activity due to it reflecting strong space and nutrient competition mechanism against postharvest fungal pathogens. Successful colonization of antagonistic yeasts competitively inhibits the adhesion of pathogenic microorganisms to host and lower the risk of pathogen colonization. Moreover, rapid growth and colonization of fresh wounds by biocontrol agents is necessary for limiting nutritional resources at the wound site and this characteristic results in inhibiting pathogen growth.

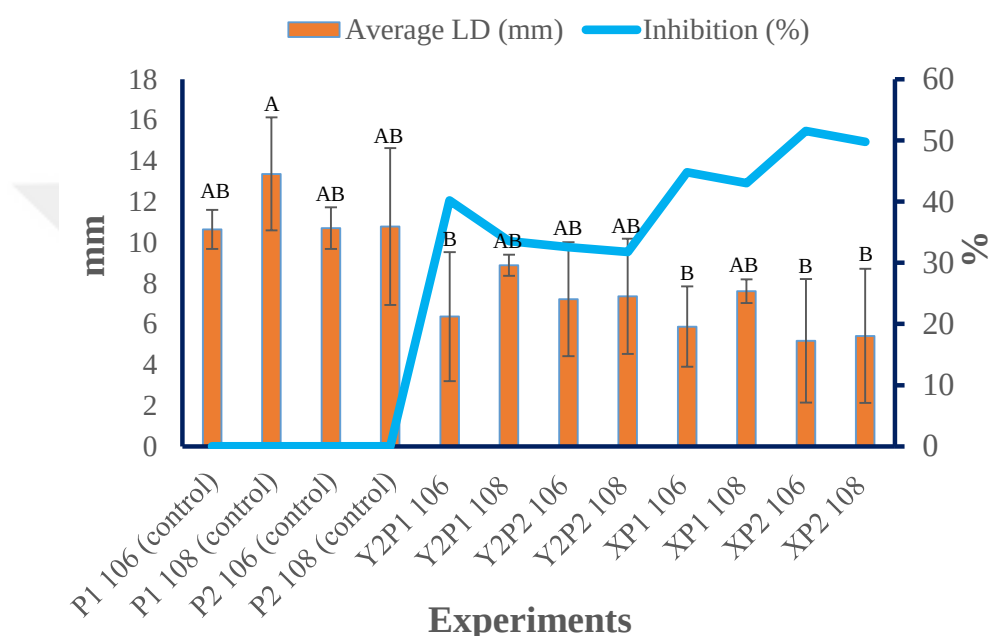
Cao et al (2013) studied the population dynamics of *Pichia caribbica* in apple wounds for 8 days at 20°C and reported that yeast grew quickly in apple wounds specifically in the first days. The population of *Pichia caribbica* yeast reached the maximum level after 2 days of incubation (1.3×10^8 cfu/mL) from an initial inoculum of 3.1×10^7 cfu/mL, and after that it declined and stabilized with a minimum level of 3.6×10^7 cfu/mL. In another comparative study (Parafati et al, 2015), population dynamics of *Aureobasidium pullulans* PI1 strain was determined on artificially wounded grape berries for 96 h at 25 °C. The population dynamics rapidly increased within the first 24 h and appeared stable enough after 48 h incubation. Population density of *Aureobasidium pullulans* PI1 reached a level of 7.9 log cfu/g of tissue from an inoculum 4.27 log cfu/g of tissue.



Figure 4.11. An image from wound size colonization assay of apples

4.4.1.2. *In Vivo* Efficacy of the VOCs in Controlling Blue and Green Mold Rot of Apples

The efficacy of VOCs produced by testing two yeast strains on the growth of blue and green moulds in apples is demonstrated in Figure 4.12.



(LD: Lesion diameter, Y2: *Aureobasidium pullulans* GE17, P1: *Penicillium digitatum* DSM2750, P2: *Penicillium expansum* DSM62841, X: Mixed culture, mean values with different letters indicate significant differences among the experiments according to Duncan's multiple range test at $p < 0.05$.)
Figure 4.12. Effect of VOCs on the mycelial diameter (mm) and percent radial growth inhibition of pathogens.

As can be understood from Figure 4.12, the least radial growth and the highest percent inhibition values were achieved in the treatment which used a combination of two yeasts. The results indicate that utilization of mixed culture of two antagonistic yeast isolates apparently resulted in the production of volatile substances that suppressed the growth of *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 at level of 44.83% and 51.58%, respectively. Conversely, VOCs produced by utilization of single culture of *Aureobasidium*

pullulans GE17 strain showed lesser inhibition activity on both pathogens in comparison to mixed culture. This result is due to the fact that different yeasts produce different antifungal volatile metabolites. Therefore, utilization of two different yeasts in combination led to the release of a high amount and broad variety of VOCs to the environment and results in more effective biocontrol activity against fungal pathogens in comparison to the usage of those yeasts as a single culture. Yeasts are known to secrete a wide range of VOCs, generally identified as alcohols, aldehydes, esters and many compounds at very low concentration (Fialho et al, 2010). Therefore, efficacy of VOCs is not based on the activity of the single component, but seems to be correlated to the synergic effect of a large number of compounds (Mercier and Jimenez, 2004; Strobel et al, 2001). For instance, Choińska et al (2020) identified the VOCs in the headspace of *Meyerozyma guilliermondii* by combination of SPME / GC-MS and reported a broad range of volatile, potentially anti-mold compounds, including aldehydes (tetradecanal), alcohols (2-decen-1-ol, phenylethyl alcohol, isogeraniol, 9-octadecanol), esters (isoamyl butanoate, phenethyl acetate, citronellyl formate, ethyl dodecanoate, ethyl nonanolate, methyl dihydrojasmonate), ketones (acetophenone, 2-nonanone), terpenes (*o*-cymene, α -limonene, α -bisabolol, α -himachalene, β -farnesene) and others (benzene, 1,3-bis (1,1-dimethylethyl)). Similarly, Di Francesco et al (2020) identified 3-methyl-1-butanol, 2-methyl-1-butanol, 2-methyl-1-propanol and phenethyl alcohol through HS-SPME-GCMS as the main volatile compounds produced by two strains of *Aureobasidium pullulans*.

Effects of VOCs produced by single culture of *Aureobasidium pullulans* GE17 and its combination with the *Meyerozyma guilliermondii* KL3 yeast strain on disease severity (DS), disease incidence (DI), percent inhibition (%I) and lesion diameter (LD) caused by *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 was presented in Table 4.8.

Table 4.8. Effects of VOCs produced by tested yeasts on LD, DI and DS

Experiments	Average LD (mm)	Inhibition (%)	DI (%)	DS (%)
P1 (10 ⁶)	10,64±0,961 ^{AB}	control	100	14,28
P1 (10 ⁸)	13,35±2,770 ^A	control	100	14,28
P2 (10 ⁶)	10,69±1,023 ^{AB}	control	83,33	11,9
P2 (10 ⁸)	10,78±3,854 ^{AB}	control	100	14,28
Y2 - P1 (10 ⁶)	6,36±3,167 ^B	40,21	83,33	11,9
Y2 - P1 (10 ⁸)	8,87±0,528 ^{AB}	33,53	100	14,28
Y2 - P2 (10 ⁶)	7,21±2,802 ^{AB}	32,55	83,33	11,9
Y2 - P2 (10 ⁸)	7,36±2,831 ^{AB}	31,75	83,33	11,9
X - P1 (10 ⁶)	5,87±1,975 ^B	44,83	83,33	11,9
X - P1 (10 ⁸)	7,60±0,580 ^{AB}	43,04	100	14,28
X - P2 (10 ⁶)	5,18±3,032 ^B	51,58	50	7,14
X - P2 (10 ⁸)	5,41±3,296 ^B	49,78	66,66	9,52

(LD: Lesion diameter, DI: Disease incidence, DS: Disease severity, Y2: *Aureobasidium pullulans* GE17, P1: *Penicillium digitatum* DSM2750, P2: *Penicillium expansum* DSM62841, X: Mixed culture, mean values with different letters in each column indicate significant differences among the experiments according to the least significant difference test $p<0.05$.)

Data presented in Table 4.8 clearly indicated that increasing the concentration of pathogens from 10⁶ conidia/mL to 10⁸ conidia/mL led to an increment in LD, DI and DS values. Regarding the VOCs effects against *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 (Table 4.8), experiments of X-P1(10⁶) and X-P2 (10⁶) displayed the highest *in vivo* efficacy in reducing infections (DI, DS and LD values) on wounded apple fruits followed by treatments of X-P1 (10⁸) and X-P2 (10⁸). Single culture of *Aureobasidium pullulans* GE17 strain provided lesser efficacy in controlling blue and green mold decay on apple fruits. It is noteworthy that the percent inhibition values of the mixed culture against both pathogens which are inoculated at higher concentrations (10⁸ conidia/mL) was even higher than that of single culture of the *Aureobasidium pullulans* GE17 against the same pathogens inoculated with lower numbers (10⁶ conidia/mL). Based on the results of higher %I and lower DS and DI

values, it is proved that VOCs produced by utilization of mixed culture were more effective in controlling blue mold than the green mold. Some photos taken during the VOCs assay were presented in Figure 4.13.

Studies on the modes of action of BCAs reported as the secretion of antifungal VOCs might be a critical mechanism for the suppression of the infections in harvested fruits under airtight conditions (Mari et al, 2016). *Wickerhamomyces anomalus* BS91 exhibited the best performances among all tested yeasts (*Wickerhamomyces anomalus* BS91, *Metschnikowia pulcherrima* MPR3, *Aureobasidium pullulans* PI1 and *Saccharomyces cerevisiae* BCA61) in reducing LD, DI and DS parameters caused by *Penicillium digitatum* on mandarin fruits. However, *Aureobasidium pullulans* PI1 displayed the highest *in vivo* efficacy against *Penicillium italicum* in reducing infections (LD, DI and DS) on mandarin fruits (Parafati et al, 2017).

In another comparative study on VOCs, volatile metabolites of strain *Aureobasidium pullulans* L1 inhibited (*in vitro*) *Monilinia laxa* and *Monilinia fructigena* mycelia more than strain L8 (40% and 75% for L1 and 20% and 50% for L8, respectively) (Di Francesco et al, 2020). The inhibitory effect of the VOCs emitted by the yeast strain *Meyerozyma guilliermondii* against five target molds (*Penicillium chrysogenum* M24, *Fusarium poae* M28, *Aspergillus fumigatus*, *Mucor* sp., *Botrytis cinerea*) was evaluated after 5 days of incubation at 25 °C and the growth of molds tested was distinctly inhibited (above 60%), except for *Botrytis cinerea*, where the percentage of its growth reduction was the lowest (11%) (Choińska et al, 2020).



Figure 4.13. An image from *in vivo* VOCs assays of cv. Starking Delicious apples

4.4.1.3. The Effect of Yeast Coating on the Quality Parameters of Apples During Storage

The surfaces of pre-selected healthful cv. Starking Delicious apples were inoculated by spraying them with a suspension of single culture of *Aureobasidium pullulans* GE17 (1×10^8 cells/mL) or mixed culture (1×10^8 cells/mL), using sterile distilled water as the control. Yeast-coated apples were stored at ambient condition (20 ± 2 °C and 50 ± 5 % RH) for 45 days. Natural decay development (infection rate) and quality characteristics (weight loss, pH, brix, titratable acidity, fruit firmness and browning) of apples were determined at the beginning and every five days during storage period. All results obtained during 45 days of storage are presented in Table 4.9.

Table 4.9. Changes of quality characteristics of apples during 45 days of storage

Storage Time	Analyses	Control	<i>Aureobasidium pullulans</i> GE17	Mixed Culture
0. Day	Weight (g)	128,76±10,811 ^{aB}	135,08±10,610 ^{aAB}	142,69±6,268 ^{aA}
	Brix (%)	13,06±0,152 ^{gA}	13,30±0,100 ^{fA}	12,70±0,000 ^{eB}
	pH	3,95±0,014 ^{eA}	3,90±0,007 ^{fB}	3,87±0,007 ^{gB}
	T. Acidity (%) [*]	0,24±0,018 ^{aAB}	0,21±0,004 ^{aB}	0,29±0,000 ^{aA}
	Firmness (N)	9,31±1,097 ^{aA}	10,02±1,399 ^{aA}	10,24±2,001 ^{aA}
	Browning (OD ₄₂₀)	0,16±0,009 ^{gA}	0,12±0,003 ^{fB}	0,13±0,002 ^{hB}
5. Day	Weight (g)	127,27±10,782 ^{aB}	133,58±10,440 ^{aAB}	141,14±7,705 ^{aA}
	Brix (%)	13,90±0,000 ^{fB}	14,40±0,000 ^{eA}	13,33±0,152 ^{dC}
	pH	3,97±0,007 ^{deA}	3,96±0,007 ^{eA}	3,91±0,000 ^{fB}
	T. Acidity (%) [*]	0,19±0,004 ^{bcdeB}	0,20±0,000 ^{abB}	0,22±0,004 ^{bA}
	Firmness (N)	8,82±2,202 ^{abA}	9,90±1,403 ^{abA}	9,64±2,241 ^{abA}
	Browning (OD ₄₂₀)	0,18±0,007 ^{fgB}	0,12±0,003 ^{fC}	0,21±0,009 ^{gA}
10. Day	Weight (g)	125,75±10,756 ^{aB}	132,02±10,296 ^{aAB}	139,56±7,637 ^{aA}
	Brix (%)	14,03±0,057 ^{efB}	14,60±0,000 ^{dA}	13,63±0,057 ^{dC}
	pH	3,97±0,007 ^{deB}	3,99±0,007 ^{dA}	3,96±0,000 ^{eB}
	T. Acidity (%) [*]	0,21±0,000 ^{bA}	0,20±0,004 ^{abA}	0,19±0,009 ^{dA}
	Firmness (N)	7,78±1,326 ^{abcB}	9,20±1,176 ^{abcA}	9,46±1,300 ^{abA}
	Browning (OD ₄₂₀)	0,23±0,006 ^{efA}	0,18±0,003 ^{eC}	0,20±0,007 ^{gB}
15. Day	Weight (g)	124,55±10,737 ^{aB}	130,83±10,204 ^{aAB}	138,32±7,601 ^{aA}
	Brix (%)	14,10±0,000 ^{eB}	14,76±0,057 ^{cdA}	14,03±0,115 ^{cB}
	pH	4,02±0,000 ^{cA}	4,02±0,014 ^{dA}	4,04±0,007 ^{cA}
	T. Acidity (%) [*]	0,18±0,004 ^{cdefB}	0,19±0,004 ^{bcAB}	0,20±0,004 ^{cA}
	Firmness (N)	6,45±1,572 ^{cdB}	9,11±1,588 ^{abcdA}	9,40±1,004 ^{abCA}
	Browning (OD ₄₂₀)	0,22±0,003 ^{efgA}	0,17±0,002 ^{eB}	0,21±0,016 ^{gA}

Table 4.9. Continued

Storage Time	Analyses	Control	<i>Aureobasidium pullulans</i> GE17	Mixed Culture
20. Day	Weight (g)	123,64±10,726 ^{aB}	129,94±10,143 ^{aAB}	137,39±7,581 ^{aA}
	Brix (%)	14,33±0,057 ^{dB}	14,80±0,000 ^{cA}	14,10±0,000 ^{cC}
	pH	3,97±0,000 ^{deA}	3,99±0,007 ^{dA}	3,97±0,014 ^{deA}
	T. Acidity (%) [*]	0,19±0,000 ^{bcB}	0,19±0,000 ^{bcC}	0,22±0,002 ^{bA}
	Firmness (N)	6,24±1,213 ^{cdB}	8,43±1,382 ^{bcdA}	8,83±0,853 ^{abcdA}
	Browning (OD ₄₂₀)	0,23±0,008 ^{efA}	0,20±0,004 ^{eB}	0,23±0,009 ^{fgA}
25. Day	Weight (g)	122,64±10,715 ^{aB}	128,96±10,082 ^{aAB}	136,38±7,559 ^{aA}
	Brix (%)	14,43±0,057 ^{dB}	14,73±0,057 ^{cdA}	14,16±0,057 ^{cC}
	pH	3,99±0,007 ^{dB}	4,08±0,000 ^{cA}	3,99±0,007 ^{dB}
	T. Acidity (%) [*]	0,19±0,004 ^{bcdA}	0,19±0,006 ^{bcA}	0,18±0,000 ^{dA}
	Firmness (N)	7,35±2,253 ^{bcdB}	9,04±1,027 ^{abcdA}	8,84±0,962 ^{abcdA}
	Browning (OD ₄₂₀)	0,25±0,006 ^{deA}	0,20±0,005 ^{eB}	0,26±0,026 ^{efgA}
30. Day	Weight (g)	121,84±10,706 ^{aB}	128,20±10,032 ^{aAB}	135,55±7,539 ^{aA}
	Brix (%)	14,50±0,000 ^{dAB}	14,90±0,000 ^{bcA}	14,33±0,321 ^{bcB}
	pH	4,07±0,000 ^{bC}	4,16±0,007 ^{abA}	4,13±0,000 ^{abB}
	T. Acidity (%) [*]	0,16±0,004 ^{efgB}	0,19±0,004 ^{bcA}	0,15±0,002 ^{eB}
	Firmness (N)	8,35±2,226 ^{abA}	9,06±1,275 ^{abcdA}	9,01±1,190 ^{abcA}
	Browning (OD ₄₂₀)	0,26±0,006 ^{deAB}	0,25±0,013 ^{dB}	0,29±0,020 ^{defA}
35. Day	Weight (g)	120,90±10,700 ^{aB}	127,30±9,979 ^{aAB}	134,61±7,533 ^{aA}
	Brix (%)	14,90±0,000 ^{cA}	15,06±0,057 ^{abA}	14,60±0,173 ^{bB}
	pH	4,05±0,007 ^{bcC}	4,14±0,000 ^{bA}	4,10±0,014 ^{bB}
	T. Acidity (%) [*]	0,17±0,004 ^{defB}	0,18±0,000 ^{cdA}	0,14±0,004 ^{efC}
	Firmness (N)	7,41±1,479 ^{bcdA}	8,29±1,506 ^{cdA}	8,23±1,268 ^{bcdA}
	Browning (OD ₄₂₀)	0,27±0,006 ^{deAB}	0,26±0,015 ^{dB}	0,30±0,013 ^{cdeA}

Table 4.9 Continued

Storage Time	Analyses	Control	<i>Aureobasidium pullulans</i> GE17	Mixed Culture
40. Day	Weight (g)	119,99±10,699 ^{aB}	126,42±9,930 ^{aAB}	133,72±7,546 ^{aA}
	Brix (%)	15,20±0,000 ^{bA}	15,10±0,100 ^{aA}	15,06±0,057 ^{aA}
	pH	4,11±0,014 ^{aB}	4,16±0,007 ^{abA}	4,12±0,000 ^{bB}
	T. Acidity (%) [*]	0,15±0,004 ^{fgB}	0,18±0,004 ^{cdA}	0,14±0,000 ^{fc}
	Firmness (N)	6,47±1,211 ^{cdB}	8,26±2,181 ^{cdA}	7,97±1,139 ^{cdA}
	Browning (OD ₄₂₀)	0,27±0,013 ^{deA}	0,30±0,009 ^{cA}	0,30±0,024 ^{cdeA}
45. Day	Weight (g)	118,61±10,663 ^{aB}	125,66±9,886 ^{aAB}	132,84±7,480 ^{aA}
	Brix (%)	15,93±0,057 ^{aA}	15,20±0,100 ^{aB}	15,20±0,000 ^{aB}
	pH	4,13±0,000 ^{aB}	4,17±0,007 ^{aA}	4,15±0,007 ^{aA}
	T. Acidity (%) [*]	0,13±0,004 ^{gB}	0,17±0,000 ^{dA}	0,14±0,000 ^{fB}
	Firmness (N)	5,90±1,597 ^{dB}	7,58±1,086 ^{dA}	7,50±1,143 ^{dA}
	Browning (OD ₄₂₀)	0,27±0,006 ^{deC}	0,31±0,010 ^{cB}	0,34±0,004 ^{bcdA}
75. Day	Browning (OD ₄₂₀)	0,31±0,019 ^{cdA}	0,33±0,025 ^{cA}	0,35±0,012 ^{bcA}
105. Day	Browning (OD ₄₂₀)	0,35±0,008 ^{cB}	0,38±0,006 ^{bA}	0,36±0,007 ^{bB}
135. Day	Browning (OD ₄₂₀)	0,50±0,012 ^{bA}	0,42±0,025 ^{aB}	0,38±0,011 ^{bB}
165. Day	Browning (OD ₄₂₀)	0,63±0,015 ^{aA}	0,45±0,009 ^{aB}	0,45±0,021 ^{aB}

*: Titratable acidity was expressed in terms of malic acid in apples, ^{A-C}: Uppercase superscripts indicate significant differences among the same day of different treatments ($p<0.05$), ^{a-h}: Lowercase superscripts indicate significant differences among the different days of the same treatment ($p<0.05$).

4.4.1.3. (1). Infection Rate

Natural decay development in cv. Starking Delicious apples was determined in the control trial during the 45-days of storage (conducted at 20 °C and about 40-50% of humidity level) and the effect of coating the apple surfaces with yeast solutions (*Aureobasidium pullulans* GE17 and mixed culture) on the

natural infection rate of apple fruits was investigated. Table 4.10. shows the number of infected and non-infected fruits and also the infection rate (%) by days during storage time.

According to the results obtained after 45 days of storage, the highest infection rate was determined in the control group apples as 28.8%, followed by the apples sprayed with mixed culture with the infection ratio of 11.11%. On the other hand, the lowest number of infected fruits (infection rate 8.33%) was detected in the apples inoculated with *Aureobasidium pullulans* GE17. In comparison to the control group, the infection rate of apples was decreased by 20.47% and 17.69% at the end of the storage period with application of single culture of *Aureobasidium pullulans* GE17 and mixed culture, respectively. These results indicate that; significant economic losses dependent on fruit infection during storage could be avoided by using antagonistic yeasts for large scale fruit-vegetables storing companies without need for the application of chemical pesticides.

It is important to mention that there was zero infected fruit out of 180 apples during the first 25 days of storage in the apples sprayed with *Aureobasidium pullulans* GE17, while only one infected apple was determined in the group coated with mixed culture. However, although the infection rate was determined in low numbers (5%) in the control group during the first 20 days of storage, afterwards rapid increase was observed in the rotting of apples and it increased to 10% at the 30th day, then reached 28.8% at the end of 45 days. As a result, it can be inferred that application of *Aureobasidium pullulans* GE17 and its combination with *Meyerozyma guilliermondii* KL3 could be helpful in reducing apple losses during especially long term storage.

Table 4.10. Number of infected apples and infection rate during 45-days of storage

Storage Time	Analyses	Control	<i>Aureobasidium pullulans</i> GE17	Mixed Culture
0. Day	Intact (Pieces)	180	180	180
	Infected (Pieces)	0	0	0
	I.R. (%)	0	0	0
5. Day	Intact (Pieces)	180	180	180
	Infected (Pieces)	0	0	0
	I.R. (%)	0	0	0
10. Day	Intact (Pieces)	178	180	180
	Infected (Pieces)	2	0	0
	I.R. (%)	1,11	0	0
15. Day	Intact (Pieces)	175	180	179
	Infected (Pieces)	5	0	1
	I.R. (%)	2,77	0	0,55
20. Day	Intact (Pieces)	171	180	179
	Infected (Pieces)	9	0	1
	I.R. (%)	5	0	0,55
25. Day	Intact (Pieces)	169	180	179
	Infected (Pieces)	11	0	1
	I.R. (%)	6,11	0	0,55
30. Day	Intact (Pieces)	162	178	176
	Infected (Pieces)	18	2	4
	I.R. (%)	10	1,11	2,22
35. Day	Intact (Pieces)	153	176	172
	Infected (Pieces)	27	4	8
	I.R. (%)	15	2,22	4,44
40. Day	Intact (Pieces)	142	171	164
	Infected (Pieces)	38	9	16
	I.R. (%)	21,11	5	8,88
45. Day	Intact (Pieces)	128	165	160
	Infected (Pieces)	52	15	20
	I.R. (%)	28,8	8,33	11,11

I. R.: Infection rate

4.4.1.3. (2). Weight Loss

Weight is one of the most important quality parameters of fruits and vegetables. Farmers and commercial companies supplying fruits-vegetables to the market desire their products to lose as little weight as possible during storage time. The most important reason for this is that as the weight loss in the amount of the product increases during storage period, the amount of profit that the seller will earn from the sale of this product directly decreases. Weight loss analysis was always conducted on the same apples that were previously selected and labelled by measuring their mass on the precision balance (with sensitivity of ± 0.0001 g) at the beginning and per five days until the end of the storage. The changes occurring in the weight of apples by five-day intervals is shown in Figure 4.14.

As it can be understood from the results, the apples belonging to the control group lost the most weight (7.88 %) at the end of the storage period followed by treatments of single culture of *Aureobasidium pullulans* GE17 (6.97 %) and mixed culture (6.90 %), respectively. It was determined that each apple belonging to control, mixed culture and *Aureobasidium pullulans* GE17 groups lost mass in averages of 1.127 g, 1.094 g and 1.046 g per five-day intervals, respectively. Statistical expressions in Table 4.9 indicate that the means of weight loss values between the analysis days of each treatment were insignificant ($p > 0.05$) while the differences were significant when the treatments were compared with each other on a daily basis ($p < 0.05$). As a result, the weight falls in apples during storage at ambient condition (20 ± 2 °C and 50 ± 5 % RH) was detected to be slower in the apples sprayed with particularly mixed culture (*Aureobasidium pullulans* GE17 + *Meyerozyma guilliermondii* KL3) and also *Aureobasidium pullulans* GE17 than the control (inoculated with SDW) group.

Cao et al (2013) reported that antagonist treatment had no significant effect on weight loss of apples after 35 days of storage at 20 °C and the same weight loss value (2.78 %) was determined in control and antagonist treated apples. However, after 45 days of storage at 4 °C and a further 15 days at 20 °C, yeast antagonist treatment led to a decrease in weight loss value from 3.62% (control) to 3.55% (treatment).

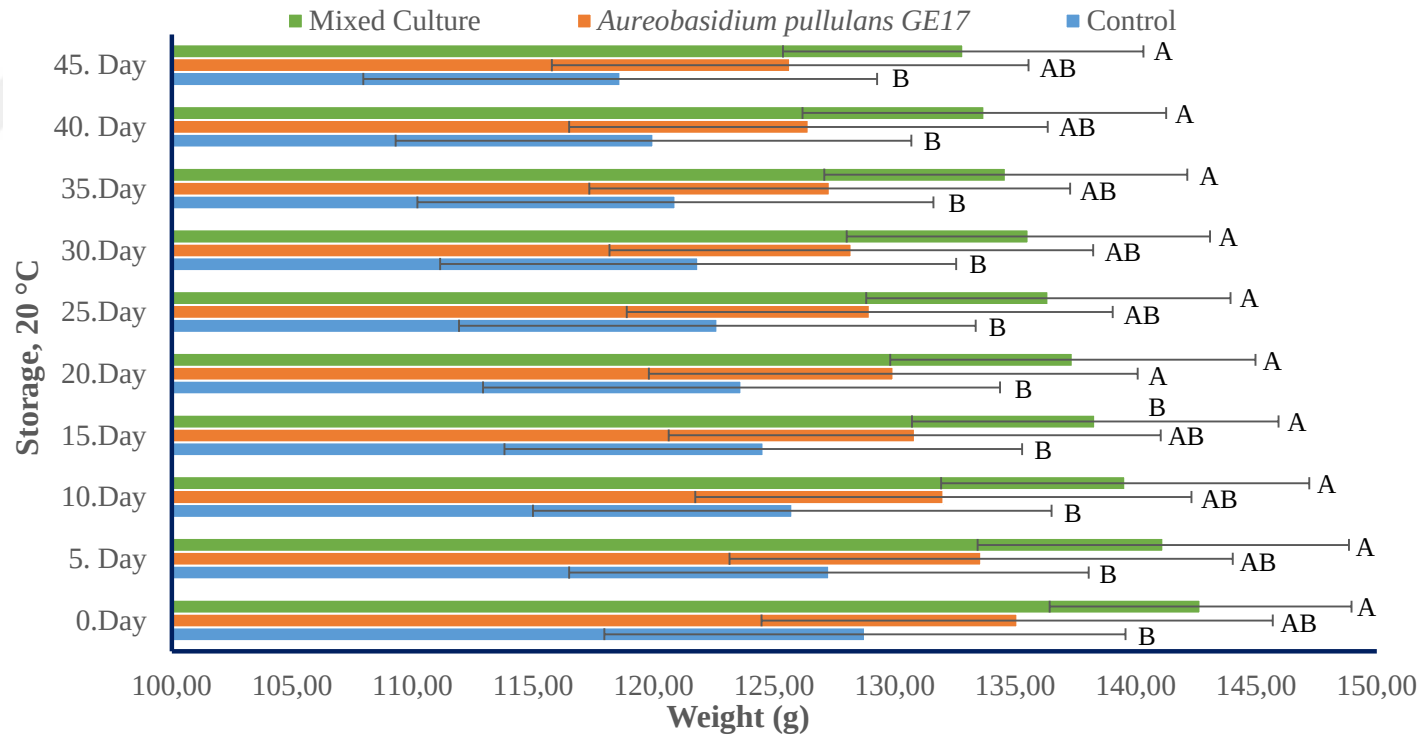


Figure 4.14. Examination of weight loss in apples during 45 days of storage (Bars represent the standard deviations).

4.4.1.3.(3). Brix (Total Soluble Solids)

The water soluble part of the total dry matter is called total soluble solids (TSS), brix or refractometer value. In other words, brix is the sugar content of an aqueous solution. One degree brix is 1 gram of sucrose in 100 grams of solution and represents the strength of the solution as percentage by mass. Brix value is one of the most important quality parameters that are noted in the export process of apple puree and apple juice as well as fresh apple trade. High brix value emerges as an important criterion especially in the production and export of concentrated apple juice. In parallel with the increasing demands for concentrated apple juice in the world, the exportation amount of Turkey has also increased. Approximately 95% of total apple juice exportation of Turkey is composed of apple juice having at least 20% or higher brix value (Aras, 2015). In addition, it is stated in the Turkish Food Codex Communiqué on Fruit Juice and Related Products (2014/34) that the water soluble dry matter value for apple (*Malus domestica* Borkh.) juice should be at least 11.2 % (GTHB, 2014). Changes in brix values of apple juices on the course of storage for 45 days at 20 °C is shown in Figure 4.15.

There is a positive correlation between weight loss and brix values. Brix value increases depending on the decrease in the water content of apple fruits. Differences between the means of brix values were found to be statistically significant in terms of both different treatments and different storage days ($p < 0.05$). Brix values of all apples belonging to different treatments were increased during storage time. The highest brix value was determined as 15.93 % in the control group apples after 45 days of storage. Brix content of apples inoculated with single culture of *Aureobasidium pullulans* GE17 increased from 13.30 % to 15.20 % at the end of the storage, while increased from 12.70 % to 15.20 % in apples treated with mixed culture. The brix values of apple juices obtained from both control groups and different yeast applications were found to be in accordance with the lowest value reported by Turkish Food Codex Communiqué on Fruit Juice and Related Products (2014/34).

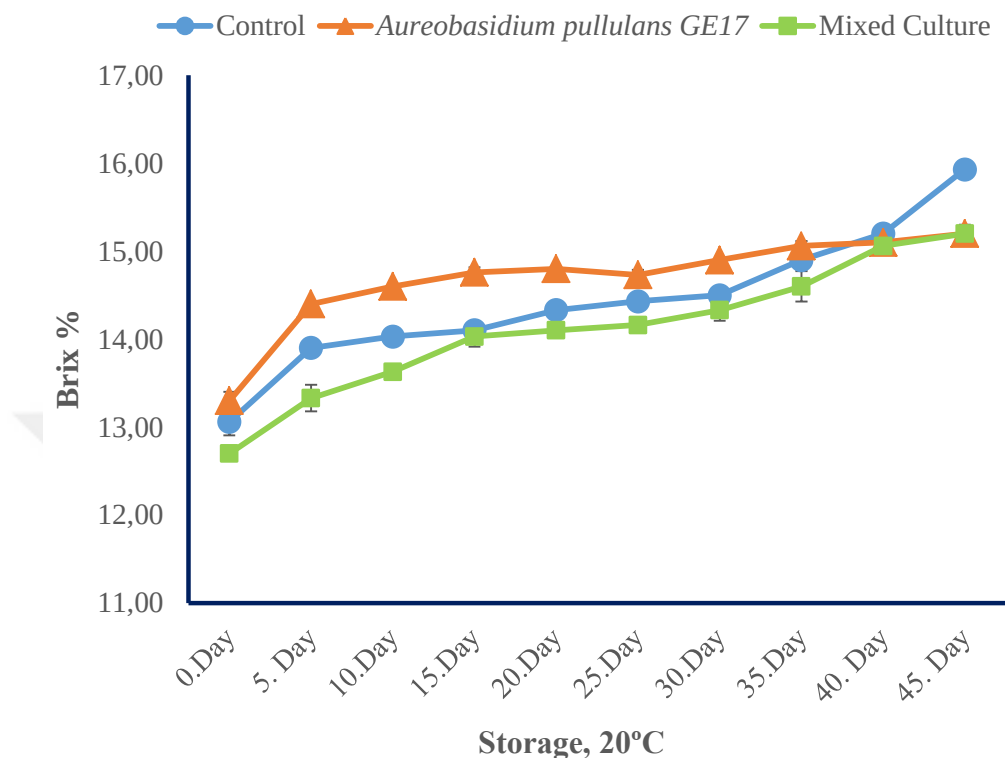


Figure 4.15. Changes in brix values of different treatments during 45 days of storage (Bars represent the standard deviations).

TSS values of juices obtained from cv. Fuji apples were reported as 13.23 % and 12.91 % after storage (45 days at 4 °C followed by 15 days at 20 °C) for control and antagonist treatments, respectively (Cao et al, 2013).

4.4.1.3. (4). pH and Titratable Acidity

The change in pH and titratable acidity values of apples were presented in Figure 4.16 as a combination chart. The differences between the means of pH and titratable acidity results among both storage time and different treatments were found to be significant at the level of 95 % ($p < 0.05$) (Table 4.9). According to the results, pH values tended to increase in all treatments while titratable acidity levels were determined to decrease during the 45 days of storage.

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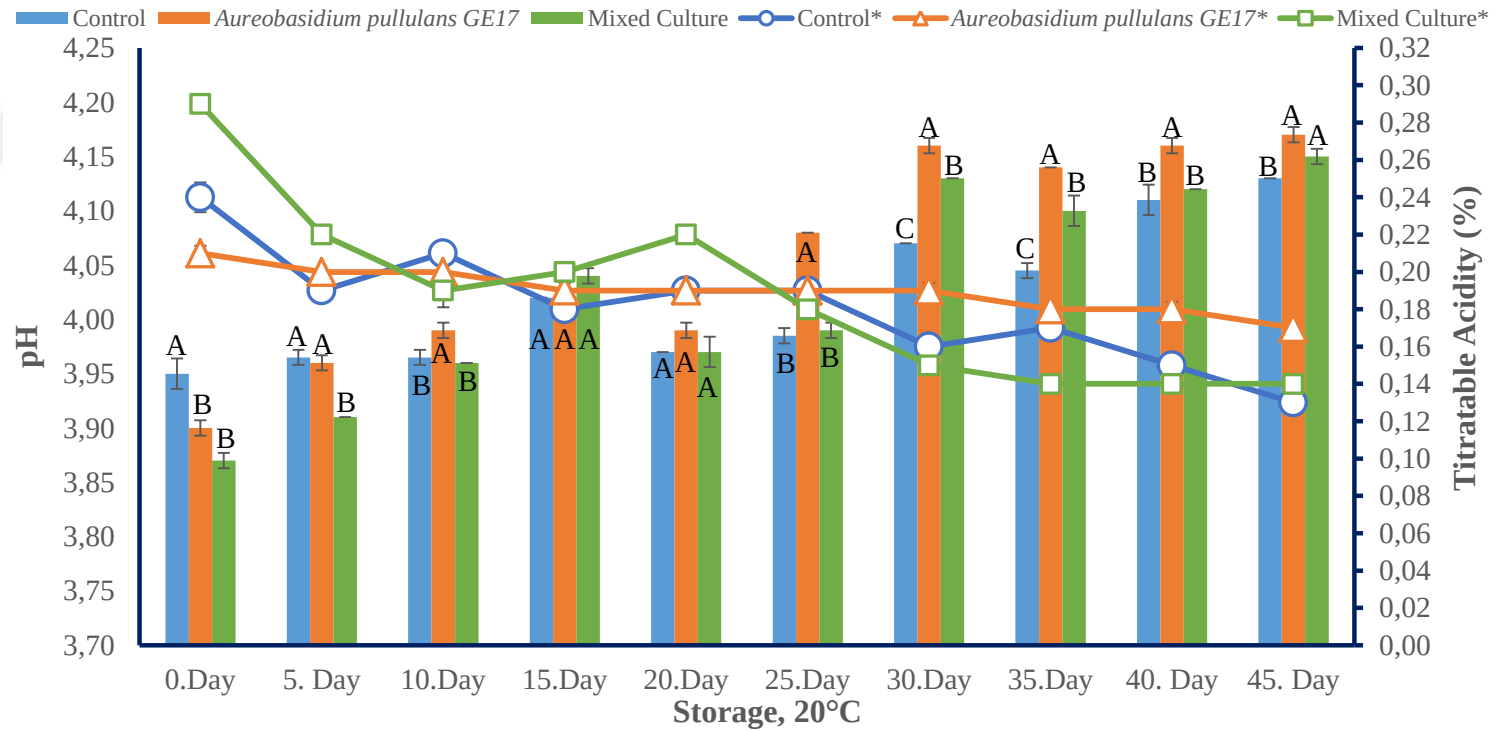


Figure 4.16. Changes in pH and titratable acidity levels of cv. Starking Delicious apples during 45 days of storage
*indicates titratable acidity in terms of malic acid equivalent.

pH values of apple juices were changed between 3.87-3.95 and 4.13-4.17 at the beginning and end of the storage, respectively. Similarly, titratable acidity level of apple juices in terms of malic acid, the predominant organic acid in apples, ranged from 0.21 % to 0.29 % and 0.13 % to 0.17 % at initial and after 45 days of storage at 20 °C, respectively. At the end of the storage period, the lowest pH and acidity content was determined in control treatment while the highest values were detected in apples coated with the yeast strain of *Aureobasidium pullulans* GE17. The acidity level of apples treated with mixed culture were almost similar to the control group apples with the value of 0.14 % after 45 days of storage. In short, yeast application to the apples during storage was not negatively influenced by the pH and titratable acidity levels of the fruits. In addition, apples inoculated with a single culture of *Aureobasidium pullulans* GE17 preserved 81% of the initial acid content after 45 days of storage while this rate was only 54% in control group apples.

Mahunu et al (2016) examined the effect of spraying *Pichia caribbica* alone or in combination with phytic acid on quality parameters of apples after storage period. Titratable acidity levels (as malic acid) of the control and other treatments were found between 0.15 % - 0.18% and 0.15% - 0.16% after 35 days of storage at 20 °C and 45 days at 4 °C followed by 15 days at 20 °C, respectively. Therefore, it was reported that fruits treated with either *Pichia caribbica* or phytic acid, or the two combined, showed no significant effect on the quality parameters of apples after 35 days at 20 °C or 45 days at 4 °C followed by 15 days at 20 °C of storage.

In another comparative study, the pH of apples was measured in various treatments as follows: apple treated with *Sporidiobolus pararoseus* Y16 alone, *Sporidiobolus pararoseus* Y16 amended with glycine betaine or treated fruits with distilled water as a control. The pH of treated fruits ranged from 3.8 - 4.0 after 7 days of incubation at 20 °C and the lowest pH value was determined in apples belonging to control treatment (Abdelhai et al, 2019).

4.4.1.3.(5). Browning Index

Browning is the process of food turning brown due to chemical reactions that take place within. The process of browning is desired in some food products (i.e. coffee, tea, cocoa beans and dried fruits such as figs, raisins and plum) due to developing colour and flavour, while it is accepted as one of the factors that reduce the quality of many other foods (i.e. apples, banana, peach, pear, avocado, mushroom, lettuce, potatoes). The interior part of the apple is desired to be light colour. Therefore, browning is one of the criteria that negatively affects the quality of both fresh apple and its juice.

There are two main mechanisms of browning reactions; enzymatic browning (by the catalytic action of the enzyme polyphenol oxidase (PPO)) (Mishra and Gautam, 2016), non-enzymatic browning (maillard reaction, caramelization and ascorbic acid oxidation) (Yu et al, 2018). It is necessary to keep these changes as minimum as possible until apples reach the consumers. Within the scope of this thesis, determination of browning levels of cv. Starking Delicious apples depending on storage time at 20 ° C, and the effect of spraying the apple surfaces with yeasts (*Aureobasidium pullulans* GE17 and mixed culture) on browning of the fruits were investigated. Other quality parameters (weight loss, brix, pH, titratable acidity and fruit firmness) were examined during 45 days of storage. However, storage time of apples was extended to 165 days until a distinct browning was detected in the inner colour of apples due to the determination that the internal browning level of apples was very low during the first 45 days of storage. For this purpose, browning analysis in the apples was performed once a month during 4 extra months after completion of the first 45 days of storage. According to the results obtained, the development of browning depending on the storage period (in a room with 20 ± 2 °C and 50 ± 5 % RH) is shown in Figure 4.17.

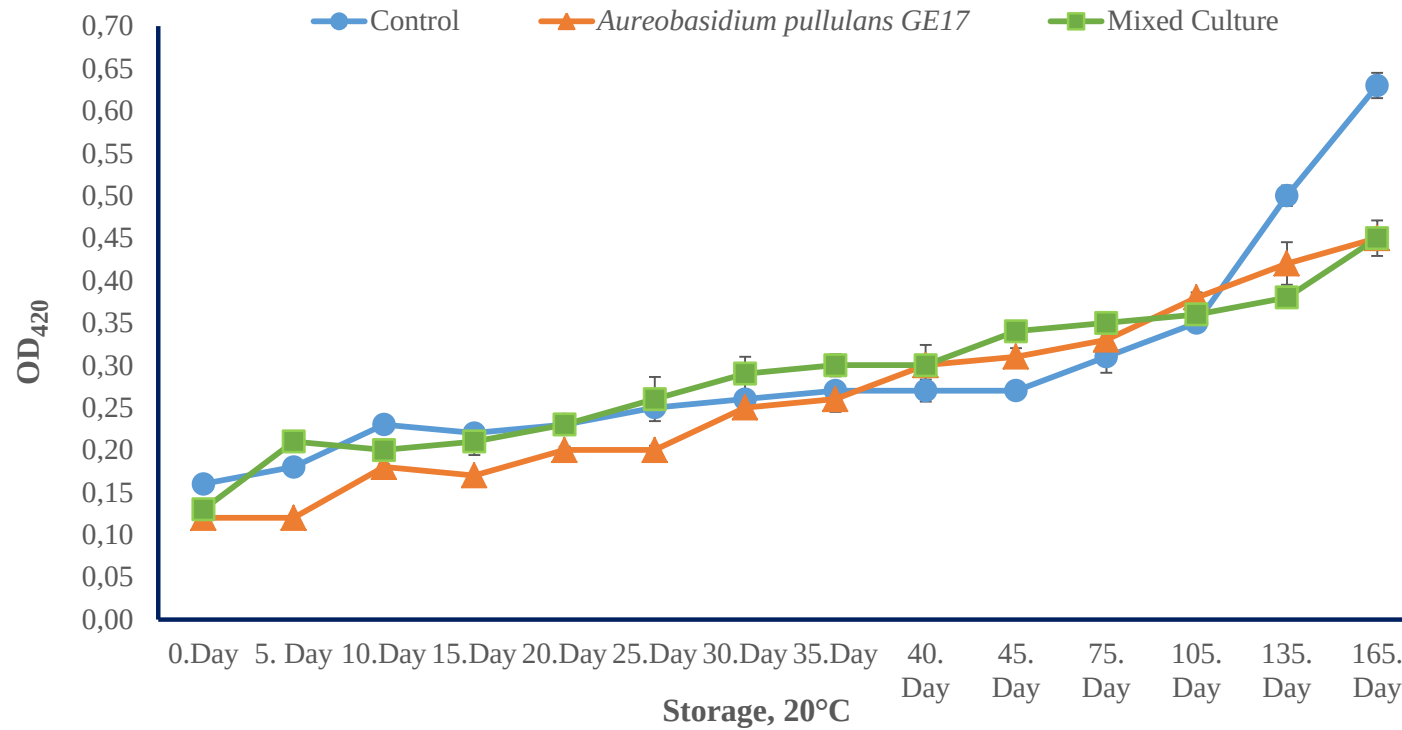


Figure 4.17. Development of browning in apples during 45 days of storage (Bars represent the standard deviations).

It is observed that OD₄₂₀ values of apples belonging to all treatments regularly increased during the storage period. However, sometimes fluctuations were determined in ABS values of apples in the form of slight decreases. The reason for these decreases is thought to be due to the necessity of using new apples (since the previous apple juices cannot be stored and thus cannot be used for the next analysis) in each analysis day. In the control group apples, the browning (OD₄₂₀) value increased from 0.16 ABS to 0.63 ABS at the end of 165 days, and this change was between 0.12 ABS- 0.45 ABS in the apple treated with *Aureobasidium pullulans* GE17 alone and 0.13 ABS – 0.45 ABS in the treatment of mixed culture. According to the statistical data presented in Table 4.9, the differences of ABS values among the treatments and also among the storage days of each treatment itself was found statistically significant ($p<0.05$). A rapid increase in browning reactions was observed in the control group, especially after 45 days of storage. The lowest browning degree was determined in apples treated with *Aureobasidium pullulans* GE17 alone along all of the analyses performed during 35 days of storage (Figure 4.17). As a result, at the end of the storage period, the browning index of apples belonging to control group (sprayed with SDW) is much higher than that of inoculated with *Aureobasidium pullulans* GE17 alone and in combination with the strain of *Meyerozyma guilliermondii* KL3. Therefore, application of these two native yeast strains isolated within the scope of this thesis led to delay of the browning process in apples and have a positive effect on the quality of cv. Starking Delicious apples. However, specific studies are needed to clarify the mechanism/s and explain the retardant effect of yeasts on browning reactions.

As far as it is known, there is not any comparative study conducted with such long-term storage on apples. Furthermore, studies in the literature only share data at the end of storage period, and changes in quality parameters occurring during storage time are ignored. There are several studies which reported the browning degree (OD₄₂₀) of apples after 35 days of storage at 20 °C as 0.165

(control) and 0.157 (yeast antagonist) (Cao et al, 2013), 0.240 (control) and 0.186 (yeast antagonist) (Mahunu et al, 2016) and 0.207 (control) and 0.170 (yeast antagonist) (Abdelhai et al, 2019). As can be clearly understood from the results of these studies, the browning degree of apples was reduced as a result of antagonist yeast application, as in the results presented in this thesis.

4.4.1.3.(6). Firmness Values of cv. Starking Delicious Apples

Texture is one of the important features that consumers pay attention to while purchasing and consuming various food products, and which affects the taste of the product. Texture profile analysis gives information about some parameters related to the structure of the product such as hardness, firmness, cohesiveness, chewiness, elasticity and springiness. Each of these terms are textural features and have different meanings. For instance, a soft product defines a product with little resistance to deformation, a tight product with moderate resistance to deformation, a hard product with significant resistance to deformation. In addition, these features can vary from person to person. Although it is known that apples with a hard texture are generally preferred by consumers, some consumers prefer apples with tight texture that are not too hard. However, soft apples are not accepted by consumers and softness is considered as a quality defect by manufacturers and sellers. The softening of an apple indicates that the fruit has been harvested for a long time ago or that it has been kept in cold storage for a long time. Moreover, softening in the texture of apples negatively affects the sensory sensation. In this context, the changes in the firmness value of cv. Starking apples stored 45 days at 20 °C were followed and the effect of yeast application on firmness properties of apples was evaluated. The results are presented in Table 4.9 and Figure 4.18.

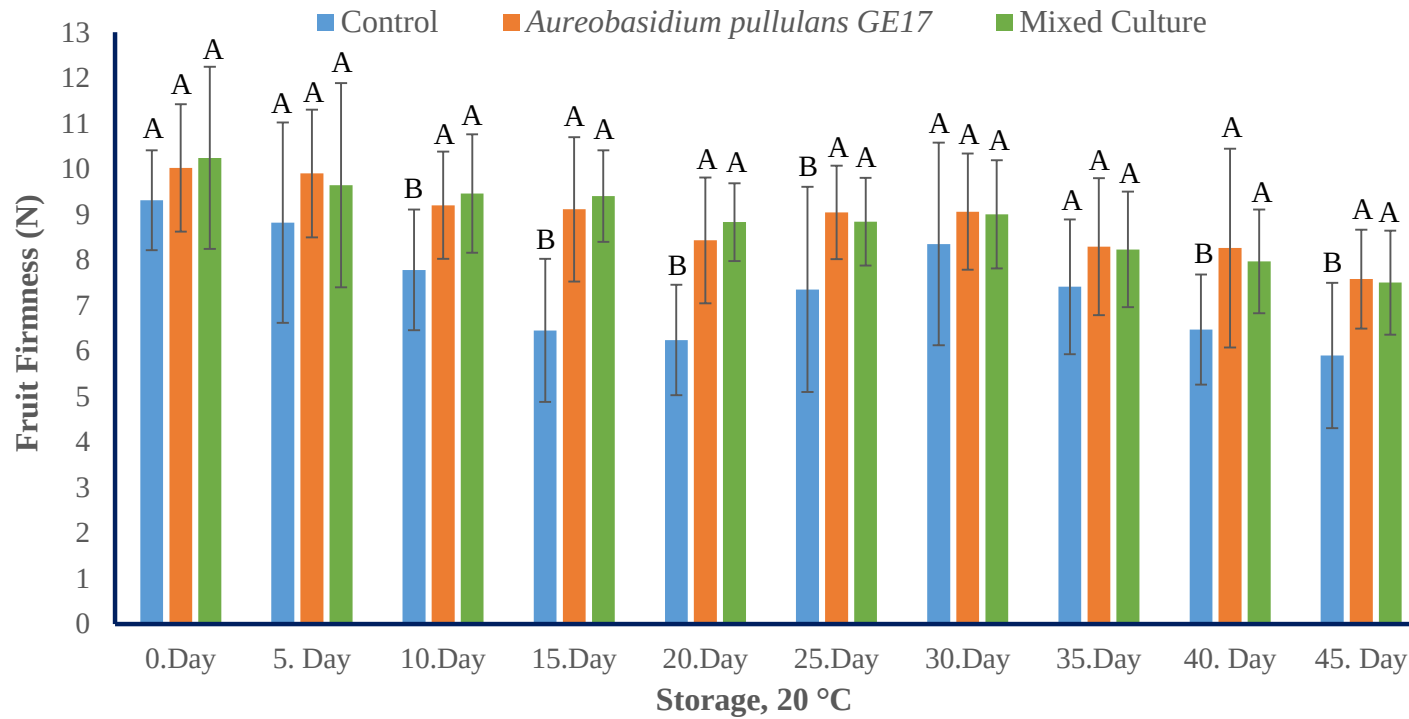


Figure 4.18. Changes in firmness of apples during 45 days of storage (Bars represent the standard deviations).

According to the results, all treatments exhibited the same pattern in terms of firmness as decline was determined in all treatments during the first 20 days of storage, then a slight increase was observed on the 25th and 30th days, and a decrement was detected again until the end of the storage period. It is considered that the tissue of apples softened during the first 20 days as a result of the decrease in water content of the apples as previously mentioned in the section of weight loss. Afterwards, this water loss caused the hardening of the skins of apples and thus a slight increase in the firmness value of apples. The hardening in skins of apples continued for 10 days and then softening started again by the possible activity of pectinase enzyme until the end of the storage period. Measurement of firmness in apples is shown in Figure 4.19.

At the beginning and end of the storage process, the firmness value of apples decreased from 9.31 N to 5.90 N for control group, from 10.02 N to 7.58 N for the treatment of *Aureobasidium pullulans* GE17 alone and from 10.24 N to 7.50 N for the treatment of mixed culture. It was remarkable that the firmness value of apples belonging to the control group was lower during all storage days than the apples which were inoculated with antagonist yeasts (Figure 4.18). The apples treated with *Aureobasidium pullulans* alone and mixed culture had close to the same results and the highest firmness values as 7.58 N and 7.50 N at the last day of storage, respectively. The apples treated with yeast antagonists had significantly higher firmness than that of the control apples (5.90 N) at the end of the storage period ($p<0.05$). As a result, the apple fruits treated with *Aureobasidium pullulans* GE17 alone and mixed culture kept firmness during 45 days of storage at 20 °C.

Consistent with the results mentioned above, higher firmness values in apples treated with antagonist yeasts compared to control groups have been reported in the literature. After 35 days of storage at 20 °C, the firmness values of the control group and the apples sprayed with *Pichia caribbica* were reported as 15.50 N and 15.63 N (Cao et al, 2013), 15.33 N and 23.22 N (Mahunu et al, 2016), respectively. Conversely, the firmness value of control group apples (13.96 N) was

determined to be higher than the apples treated with yeast antagonist *Sporidiobolus pararoseus* Y16 (13.77 N) after storage at 20 °C for 35 days (Abdelhai et al, 2019).



Figure 4.19. Some images during texture analysis

4.4.1.4. Efficacy of Yeasts Against Blue and Green Moulds During Cold Room Storage of Apples

Efficacy of *Aureobasidium pullulans* GE17 alone and in combination with *Meyerozyma guilliermondii* KL3 in inhibiting blue mould (*Penicillium expansum* DSM62841) and green mould (*Penicillium digitatum* DSM2750) decay incidence, lesion diameter and disease severity in cv. Starking Delicious apples under commercial cold room storage conditions (4°C and 95% RH) during 3 months were evaluated. Development of lesion diameters during 3 months in cold room storage was presented in Table 4.11.

The lesion diameter of apples belonging to all treatments was 4 mm in day zero due to the fact that the apples were artificially wounded with a diameter of 4 mm by a sterile cork borer during the establishment of experiments. In the subsequent days, the lesion diameters of apples increased rapidly, particularly in control groups. Although the apples treated with yeasts and the control groups had close values of lesion diameter in the first 15 days, application of single and mixed cultures of yeasts provided a significant inhibition in lesion diameters caused by *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750 after 30 days of storage ($p<0.05$). Since control group apples of both pathogens completely rotted after 60 days, lesion measurements could not be performed for control treatments on the last month of storage period. However, apples treated with yeasts completely decayed after additional 30 days of storage. After 60 days of cold room storage, yeast strain *Aureobasidium pullulans* GE17 prevented the lesion formation caused by *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 by 48.98 % and 45.99%, and application of mixed culture of two yeasts prevented the same pathogens by 48.32% and 48.70%, respectively. It was observed that development of lesion diameters slowed down during the next 75 and 90 days of storage period.

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Table 4.11. Development of lesion diameters (mm) in apples during 90 days of cold room storage

Treatments	0. Day	15. Day	30. Day	45. Day	60. Day	75. Day	90. Day
P1 (Control)	4,00±0,000 ^{dA}	34,43±1,920 ^{cA}	46,94±5,175 ^{bA}	56,03±5,512 ^{bA}	113,47±17,988 ^{aA}	C. R.	C. R
P2 (control)	4,00±0,000 ^{dA}	29,11±2,826 ^{cAB}	44,41±1,134 ^{bA}	49,90±5,072 ^{bAB}	108,87±9,956 ^{aA}	C. R.	C. R
Y2-P1	4,00±0,000 ^{eA}	27,02±4,589 ^{dABC}	27,77±2,222 ^{dB}	46,94±5,193 ^{cABC}	57,89±3,548 ^{bB}	59,77±3,511 ^{bA}	67,87±7,274 ^{aAB}
Y2-P2	4,00±0,000 ^{fA}	20,11±6,535 ^{eC}	28,51±3,099 ^{dB}	38,22±6,776 ^{cC}	58,79±3,078 ^{bB}	61,18±2,260 ^{abA}	66,91±3,390 ^{aB}
X-P1	4,00±0,000 ^{eA}	20,69±11,410 ^{dC}	28,25±0,781 ^{cdB}	38,10±16,901 ^{cC}	58,63±3,049 ^{bB}	61,16±3,531 ^{abA}	69,95±4,076 ^{aAB}
X-P2	4,00±0,000 ^{gA}	22,00±5,196 ^{fBC}	28,96±1,107 ^{eB}	40,08±4,488 ^{dBC}	55,84±3,071 ^{cB}	63,21±3,314 ^{bA}	73,37±4,565 ^{aA}

C.R.: Completely rotten, Y2: *Aureobasidium pullulans* GE17, P1: *Penicillium digitatum* DSM2750, P2: *Penicillium expansum* DSM62841, X: Mixed culture (*Meyerozyma guilliermondii* KL3 + *Aureobasidium pullulans* GE17), ^{A-C}: Uppercase superscripts indicate significant differences among the same day of different treatments ($p<0.05$), ^{a-g}: Lowercase superscripts indicate significant differences among the different days of the same treatment ($p<0.05$).

According to results presented in Table 4.11, it can be concluded that application of *Aureobasidium pullulans* GE17 alone showed almost similar levels of efficacy on the inhibition of both pathogens in apples after 3 month of storage in a cold room. However, treatment of mixed culture exhibited higher inhibition on lesion development of *Penicillium digitatum* DSM2750 than *Penicillium expansum* DSM62841 at the end of storage period. Comparing the use of single culture and mixed culture, it was determined that the use of single culture of *Aureobasidium pullulans* GE17 was slightly more effective on the inhibition of both pathogens in cv. Starking Delicious apples at the end of cold room storage for 90 days. The differences in lesion diameter measurements between different storage days of within each treatment or between the same days of different treatments were found to be significant ($p<0.05$).

Disease severity and infection rate (%) were also followed during cold room storage for 90 days (Table 4.12). Apples were considered infected when the first lesion was seen on the fruits. Infection rate was determined as 100 % for control treatments on the 6th day of cold room storage because infection formation was observed in all apples. Over the same period, the infection rate was determined as 75 % in the treatment Y2P1, 80 % in the treatment Y2P2, 60 % in the treatment XP1 and 85 % in the treatment XP2. It is important to observe that there was a lower infection rate in all treatments which utilized a single or mixed culture of yeasts than in the control groups. The fact that no infection was determined in 40 % of the apples in treatment XP1 and 15 -25 % of the apples belonging to the other treatments proved the effectiveness of yeast strains used in this study, although the apples employed for cold room storage were already artificially wounded and inoculated with pathogen at the beginning of the storage. In particular, utilization of mixed culture appears to be highly effective (40 %) in inhibiting infection caused by *Penicillium digitatum* DSM2750 in apples during industrial storage conditions.

Table 4.12. Infection rate and disease severity measurements on apples during cold room storage

Treatments	Disease Severity (%)						Infection Rate (%)
	15. Day	30. Day	45. Day	60. Day	75. Day	90. Day	Day 6 th
P1 (Control)	15,7	47,14	63,57	78,57	83,57	85,71	100
P2 (control)	15,7	45	55,71	80,71	85	85,71	100
Y2-P1	14,2	20,3	27,14	57,5	65,71	85,71	75
Y2-P2	13,75	16,78	25,71	43,39	72,14	84,82	80
X-P1	13,9	17,5	26,42	44,1	77,85	85,17	60
X-P2	14,1	15,5	24,28	40	81,42	85,71	85

P1: *Penicillium digitatum* DSM2750, P2: *Penicillium expansum* DSM62841, Y2: *Aureobasidium pullulans* GE17, X: Mixed culture (*Meyerozyma guilliermondii* KL3 + *Aureobasidium pullulans* GE17).

The infection rate indicates the amount of infected apples as a percentage, while the level of this infection was determined by disease severity value according to Townsend-Heuberger scale (Table 3.6). Once the apples were infected, the development of infection was followed with the disease severity measurement during 3 months of cold room storage (Table 4.12). At the end of 15 days in the storage, the formation of medium rot started in control group apples while apples treated by yeasts had not yet begun the slight rot phase. When the first month of storage had completed, moulds were started to form mycelium on apples belonging to control groups, but apples treated with yeast strains were still at the medium rot level. The mould reached the sporulation stage in the control group apples which were treated with *Penicillium digitatum* DSM2750, while the mould continued to form mycelium in the control trial treated with *Penicillium expansum* DSM62841 at the end of 45 days. Apples treated with yeasts were in medium rotting phase during the 45 days of storage period. At the end of the first two months of storage, it was determined that approximately all of the fruits belonging to the control treatments completely decayed, but the apples were in high rot stage and did not start the mycelium formation in treatments Y2P2, XP1 and XP2. Conversely, Y2P1 was the only yeast applied treatment that started to form mycelium during the same period. It can be inferred from the results that utilization of *Aureobasidium pullulans* GE17 alone emerged more effective results in inhibition of *Penicillium expansum* DSM62841 than *Penicillium digitatum* DSM2750. Besides, application of mixed culture during cold storage resulted in almost similar efficacy against both pathogens. In short, the use of *Aureobasidium pullulans* GE17 alone or as a mixed culture with *Meyerozyma guilliermondii* KL3 reduced the lesion diameter and disease severity caused by blue and green moulds, the most destructive postharvest pathogens in apples, on cv. Starking Delicious apples by almost half during two months of storage in cold room with 95 % RH and 4 °C. Some photos taken during the cold room storage assay are presented in Figure 4.20.



Figure 4.20. Some images during the experimentation of apples in cold room storage

In contrast to the 3-month storage (at 4°C and 95 % relative humidity) on apples performed in this thesis, shorter storage periods are generally encountered in the literature. In addition, those studies focused on the quality parameters (i.e. weight loss, firmness, acidity) of apples and population dynamics of yeasts during the cold storage period (Cao et al, 2013; Mahunu et al, 2016; Abdelhai et al, 2019). Therefore, there is no comparative study that examines the infection rate, disease severity and lesion diameter values of apples during cold storage for 3 months.

4.4.2. Lemon Treatment

4.4.2.1. Population Studies of Yeasts in Lemon Wounds

Yeasts display some mechanisms that account for the protective effect they convey against postharvest diseases. Among these proposed mechanisms, the ability to rapidly colonize wounds (Mercier and Wilson, 1994) and the ability to compete with other microorganisms for space and nutrients are the most significant (Lima et al, 1997; Ippolito et al, 2011).

The population dynamics of the single culture of *Aureobasidium pullulans* GE17 and its mixture with *Meyerozyma guilliermondii* KL3 in cv. Kutdiken lemon wounds are shown in Figure 4.21 and Table 4.13. A photo taken during the population studies analysis of yeasts is presented in Figure 4.22.

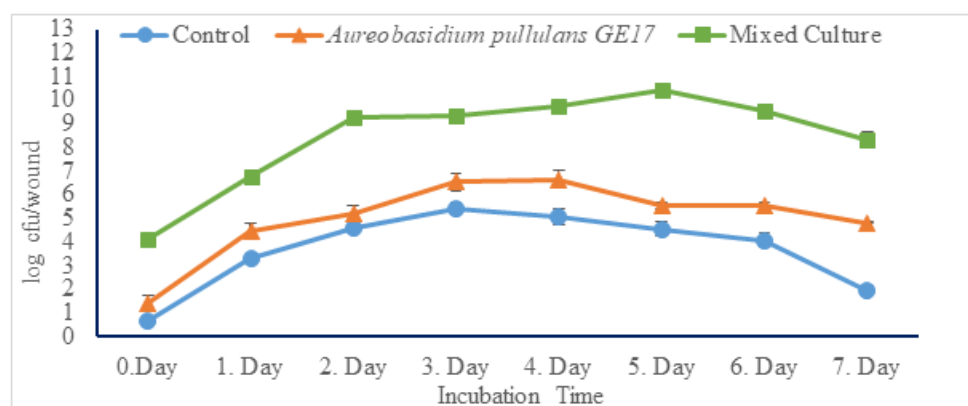


Figure 4.21. Population dynamic of control and two selected yeasts in lemon wounds. (Bars represent the standard deviations).

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Table 4.13. Population density of yeasts in artificially wounded lemons as log cfu/wound

Treatments	0. Day	1. Day	2. Day	3. Day	4. Day	5. Day	6. Day	7. Day
Control	0,67±0,177 ^{cB}	3,32±0,075 ^{abCB}	4,60±0,154 ^{abB}	5,43±0,269 ^{ab}	5,05±0,343 ^{ab}	4,55±0,286 ^{abB}	4,08±0,282 ^{abB}	1,97±0,177 ^{bcC}
<i>Aureobasidium</i>	1,40±0,317 ^{bB}	4,44±0,343 ^{ab}	5,21±0,333 ^{ab}	6,54±0,389 ^{ab}	6,66±0,378 ^{ab}	5,55±0,148 ^{ab}	5,54±0,126 ^{ab}	4,83±0,061 ^{ab}
<i>pullulans</i> GE17								
Mixed Culture	4,09±0,194 ^{dA}	6,80±0,074 ^{cA}	9,26±0,069 ^{abA}	9,36±0,217 ^{abA}	9,73±0,203 ^{abA}	10,45±0,237 ^{aA}	9,57±0,275 ^{abA}	8,31±0,326 ^{bA}

^{A-C} : Uppercase superscripts indicate significant differences within the same column ($p<0.05$), ^{a-d} : Lowercase superscripts indicate significant differences within the same row ($p<0.05$).

As it can be understood from the results, the yeasts *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 quickly adapted and colonized the lemon wounds as well as those of the apple. After 2 h of inoculation the total yeast number was determined in lemons belonging to the control group, single culture (*Aureobasidium pullulans* GE17), and mixed culture as 0.67 log cfu/wound, 1.40 log cfu/wound and 4.09 log cfu/wound, respectively. The lowest yeast number was determined in the control group throughout 7 days of incubation (Figure 4.21). Although single culture of *Aureobasidium pullulans* GE17 exhibited acceptable colonization ability in cv. Kutdiken lemons by reaching 6.66 log cfu/wound on the fourth day of incubation, results of population study clearly demonstrated that when this yeast utilized in combination with *Meyerozyma guilliermondii* KL3 they reached excellent population numbers on wounded lemons. For instance, the yeast number in lemons treated with mixed culture was found to be almost two times higher than that of inoculated with single culture (*Aureobasidium pullulans* GE17) on the 5th, 6th and 7th days of incubation (Table 4.13). This result indicates that when these two yeast present together on lemons, they do not compete for space and nutrition.

Yeast population in the control group lemons started to diminish from the 3rd day of incubation while it began to decrease after 4th and 5th days of incubation for experiments of single and mixed culture, respectively. The highest number of yeast was determined in lemons treated with mixed culture with the value of 10.45 log cfu/wound after the 5th day of incubation. Even at the end of the incubation period, there was a considerable number (8.31 log cfu/wound) of yeasts in lemon wounds inoculated with both yeasts. After the same incubation time, yeast counts in control and single culture trials were detected at lower amounts of 1.97 log cfu/wound and 4.83 log cfu/wound, respectively.

The colonization capability of the two yeasts studied on wounded parts of cv. Starking Delicious apples and cv. Kutdiken lemons differed. Although yeast count on apples inoculated with a single culture of *Aureobasidium pullulans* GE17

was slightly higher than the treatment of mixed culture, both yeasts exhibited fairly close growth character on wounded apples. On the other hand, yeast population in wounded lemons inoculated with mixed culture was found to be noticeably higher than that of those inoculated with single culture of *Aureobasidium pullulans* GE17. In addition, the yeast population was quite higher in apples treated with both single and mixed culture than the control group, while it was very close in lemons belonging to control and single culture of *Aureobasidium pullulans* GE17 treatments.

As a result, the combined use of these two yeasts, *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3, results in the special ability to colonize and grow rapidly in wounded tissues of lemon. Moreover, the population dynamics of the mixed culture of these two yeasts in wounded tissues of lemons were higher by far than reported studies in the literature.

Perez et al (2019) assessed the wound colonization ability of *Clavispora lusitaniae* 146 in lemons. After the initial inoculation of wounds at 25 °C, growth of the yeast was exponential until 72 h. Subsequently, the rate of yeast growth declined and then stabilized at 120 h, time at which the population density reached 7.03 log cfu/mL. Also, the colonization ability of yeast in lemon wounds was tested at low temperatures (8 °C during 40 days). When lemons were incubated at 8 °C, *Clavispora lusitaniae* 146 exhibited an irregular pattern of growth over time. After inoculation, the yeast population increased over a period of 10 days, then was stationary until 20 days, after which the population again increased over the next 10 days, stabilizing again at 30 days. After 30 days, the population level remained stationary, maintaining a population of 7.34 log cfu/mL until at least day 40.

Arras et al (1998) examined the population dynamics of the isolate *Pichia guilliermondii* 5A on wounded oroblanco fruits at concentrations ranging from 10^4 to 10^8 cells per mL. The yeast was applied alone or with 25 µL of a spore suspension of *Penicillium italicum* (10^5 spores/mL) to oroblanco wounds. Twenty-four hours after treatment, cfu of isolate *Pichia guilliermondii* 5A, applied at 10^4

cells/mL increased about ten-fold in oroblanco wounds without the pathogen: after 6 days the yeast concentration ranged up to 2.3×10^5 cfu/wound. When co-inoculated with the pathogen, the yeast number again increased swiftly, up to about 60-fold within 24 h. When oroblanco grapefruit wounds were treated with the yeast at a concentration of 10^8 cells/mL (25 μ L) the yeast population increased progressively up to 1.7×10^7 cfu/wound after 6 days of incubation at 20 °C.

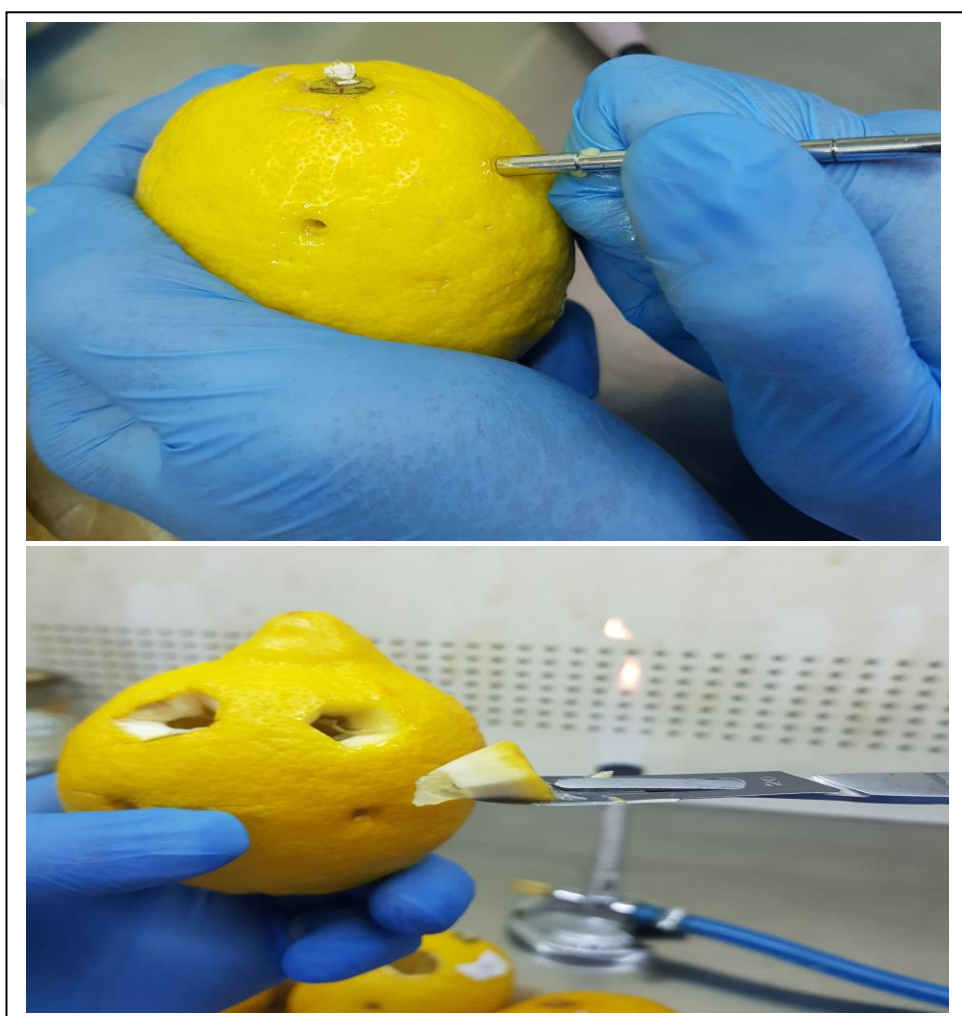
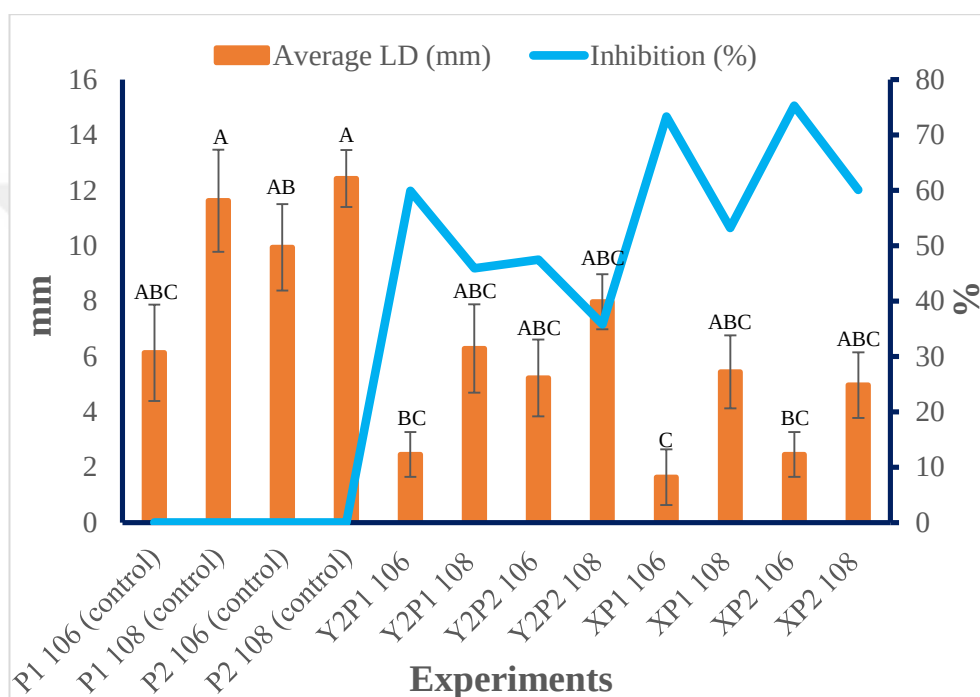


Figure 4.22. An image from wound size colonization assay of lemons

4.4.2.2. *In Vivo* Efficacy of the VOCs in Controlling Blue and Green Mold Rot of Lemons

The efficacy of VOCs produced by testing two yeast strains on the growth of blue and green moulds in cv. Kutdiken lemons is demonstrated in Figure 4.23.



(LD: Lesion diameter, Y2: *Aureobasidium pullulans* GE17, P1: *Penicillium digitatum* DSM2750, P2: *Penicillium expansum* DSM62841, X: Mixed culture, mean values with different letters indicate significant differences among the experiments according to Duncan's multiple range test at $p < 0.05$.)

Figure 4.23. Effect of VOCs on the mycelial diameter (mm) and percent radial growth inhibition of pathogens.

As can be seen from Figure 4.23, the least radial growth and the highest percent inhibition values were determined in treatment which used the combination of *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 yeast strains. Results obtained proved that application of mixed culture of two antagonistic yeast isolates obviously led to secretion of volatile organic substances that inhibited the growth of both *Penicillium digitatum* DSM2750 and *Penicillium*

expansum DSM62841 at level of 73.30% and 75.28%. On the other hand, the utilization of single culture of *Aureobasidium pullulans* GE17 exhibited lesser inhibition activity in all treatments in comparison to mixed culture. Inhibition percentage of single culture of *Aureobasidium pullulans* GE17 against *Penicillium digitatum* DSM2750 (1×10^6 conidia/mL and 1×10^8 conidia/mL) and *Penicillium expansum* DSM62841 (1×10^6 conidia/mL and 1×10^8 conidia/mL) were 59.92 (%), 45.89 (%), 47.48 (%) and 35.82 (%), respectively. However, those ratios were elevated to 73.30 (%), 53.18 (%), 75.28 (%) and 60.06 (%) by combining Y2 with *Meyerozyma guilliermondii* KL3, respectively (Figure 4.23). Also it is clear that increasing the concentration of pathogens led to a decrease in the inhibition activity of yeasts. The increase of concentration of *Penicillium digitatum* DSM2750 from 10^6 to 10^8 conidia/mL caused reduction in the percent of inhibition values of mixed culture from 73.30 (%) to 53.18 (%). Similarly, an increment in the concentration of *Penicillium expansum* DSM62841 led to lessening of inhibition ratio achieved by mixed culture from 75.28 (%) to 60.06 (%). Besides their action mechanisms, the application of yeasts in the appropriate concentrations where they can be more effective against fungal pathogens is also a crucial factor in controlling postharvest diseases. Therefore, the determination of specific concentrations of yeasts against different concentrations of postharvest pathogens belonging to various genera is required for establishing the maximum inhibitory effect of yeasts.

Although application of mixed culture exhibited close inhibition ability against both pathogens, it was slightly higher against *Penicillium expansum* DSM62841. Conversely, the inhibition ratio exhibited by use of a single culture of *Aureobasidium pullulans* GE17 was higher against *Penicillium digitatum* DSM2750 compared to *Penicillium expansum* DSM62841. It is remarkable that radial growth inhibition of *Penicillium digitatum* DSM2750 was achieved at the ratio 59.92 (%) through application of *Aureobasidium pullulans* GE17 alone (Figure 4.23).

Effects of VOCs produced by single culture of *Aureobasidium pullulans* GE17 and its combination with *Meyerozyma guilliermondii* KL3 yeast strain on disease severity (DS), disease incidence (DI), percent inhibition (%I) and lesion diameter (LD) caused by *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 are presented in Table 4.14.

Table 4.14. Effects of VOCs produced by tested yeasts on LD, DI and DS

Experiments	Average LD (mm)	Inhibition (%)	DI (%)	DS (%)
P1 (10^6)	6,13±1,736 ^{ABC}	control	50	7,14
P1 (10^8)	11,62±1,846 ^A	control	83,33	11,9
P2 (10^6)	9,94±1,560 ^{AB}	control	83,33	11,9
P2 (10^8)	12,42±1,031 ^A	control	100	14,28
Y2 - P1 (10^6)	2,45±0,807 ^{BC}	59,92	33,33	4,76
Y2 - P1 (10^8)	6,28±1,594 ^{ABC}	45,89	50	7,14
Y2 - P2 (10^6)	5,22±1,387 ^{ABC}	47,48	50	7,14
Y2 - P2 (10^8)	7,97±0,994 ^{ABC}	35,82	83,33	11,9
X - P1 (10^6)	1,63±1,009 ^C	73,30	16,66	2,38
X - P1 (10^8)	5,44±1,315 ^{ABC}	53,18	50	7,14
X - P2 (10^6)	2,45±0,808 ^{BC}	75,28	33,33	4,76
X - P2 (10^8)	4,96±1,189 ^{ABC}	60,06	33,33	4,76

(LD: Lesion diameter, DI: Disease incidence, DS: Disease severity, Y2: *Aureobasidium pullulans* GE17, P1: *Penicillium digitatum* DSM2750, P2: *Penicillium expansum* DSM62841, X: Mixed culture, mean values with different letters in each column indicate significant differences among the experiments according to the least significant difference test $p<0.05$.)

Data presented in Table 4.14 clearly indicated that increasing the concentration of pathogens from 10^6 conidia/mL to 10^8 conidia/mL led to an increment in LD, DI and DS values. Regarding the VOCs effects against *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 (Table 4.14), utilization of mixed culture in the experiments of X-P1(10^6) and X-P2 (10^6) resulted in the highest *in vivo* efficacy in reducing infections (DI, DS and LD values) on wounded lemon (cv. Kutdiken) fruits followed by treatments of X-P1

(10^8) and X-P2 (10^8). Single culture of the *Aureobasidium pullulans* GE17 strain provided lesser efficacy in controlling blue and green mold decay on lemon fruits. Among all treatments, the second highest inhibition (73.30%) and the lowest LD, DI and DS measurements were detected against *Penicillium digitatum* DSM2750 (10^6 conidia/mL) in the lemons treated with mixed culture. The results presented in Table 4.14 undoubtedly showed that VOCs produced by both antagonistic yeasts significantly decreased the disease incidence, disease severity and lesion diameters caused by blue and green moulds on cv. Kutdiken lemons compared to lemons without yeast application (control). As can be inferred from the results, postharvest losses of lemons caused by two prevalent pathogenic fungi (*Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841) could be reduced significantly by application of the particularly mixed culture of *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3. Results of VOCs assay emphasized that production of VOCs represents one of the key action mechanisms of *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 antagonistic yeasts that they are employed against various postharvest pathogenic fungi. Some photos taken during the VOCs assay are presented in Figure 4.24.

In terms of VOCs production mechanism, effectiveness of *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 yeast strains against *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 pathogens was higher in lemon fruits compared to apples. When the results of VOCs assays in apples (Table 4.8) and in lemons (Table 4.14) are compared, it can be seen that lower DI, DS and LD values and also higher inhibition (%) ratio were determined in the lemon assay in all treatments.

Wang et al (2021) aimed to control blue mold caused by *Penicillium italicum* in postharvest citrus fruits through VOCs produced by *Pseudomonas fluorescens* ZX. Treatments with pure individual components of the VOCs were also conducted. VOCs produced by *Pseudomonas fluorescens* ZX colonized on healthy fruits significantly ($p<0.05$) inhibited the development of *Penicillium*

italicum on citrus fruits. Compared to the control conditions, citrus fruits fumigated with VOCs from *Pseudomonas fluorescens* ZX colonized on healthy fruits reduced the disease incidence from approximately 100% to below 75% and lesion diameter from about 40 mm to less than 30 mm. *In vitro* testing of the individual pure compounds comprising the VOCs suggested that organic acids and sulfur compounds are the active components of VOCs, with dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) exhibiting the highest antifungal activity: concentrations of only 100 and 10 µL/L, respectively, afforded complete inhibition of blue mold on citrus fruits *in vivo*.

Liu et al (2019) evaluated the *in vitro* antagonistic activity of VOCs produced by five yeasts (*Metschnikowia citriensis* FL01 and FL02, *Candida oleophila* FL14, *Pseudozyma antarctica* FL17 and *Pichia membranaefaciens*) isolated from the surface of citrus leaves toward *Penicillium digitatum* and *Penicillium italicum*. All five tested yeasts exhibited weak inhibitory effect on the mycelial growth of both pathogens. Mycelial growth inhibition of *Penicillium digitatum* ranged from 2.80 (%) to 12.15 (%) while it was between 0.82 (%) and 11.32 (%) for *Penicillium italicum*.

Wang et al (2020b) reported that VOCs produced by *Metschnikowia citriensis* had no inhibitory effect on mycelial growth of *Geotrichum citri-aurantii* when compared to the control at three different pH values. The reason for this result might be that the spores and hyphae of the *Geotrichum citri-aurantii* were insensitive to quantities sufficient VOCs released by *Metschnikowia citriensis*.



Figure 4.24. An image from *in vivo* VOCs assays of cv. Kutdiken lemons

4.4.2.3. The Effect of Yeast Coating on the Quality Parameters of Lemons During Storage

The surfaces of pre-selected healthful cv. Kutdiken lemons were inoculated by spraying them with a suspension of single culture of *Aureobasidium pullulans* GE17 (1×10^8 cells/mL) or mixed culture (1×10^8 cells/mL), using sterile distilled water as the control. Yeast-coated lemons were stored in a standard room at ambient condition (20 ± 2 °C and 50 ± 5 % RH) for 45 days. Natural decay development (infection rate) and quality characteristics (weight loss, pH, brix, titratable acidity, fruit firmness and browning) of lemons were determined at the beginning and every five days during storage period. All results obtained during 45 days of storage are presented in Table 4.15.

Table 4.15. Changes of quality characteristics of lemons during 45 days of storage

Storage Time	Analyses	Control	<i>Aureobasidium pullulans</i> GE17	Mixed Culture
0. Day	Weight (g)	96,39±7,483 ^{aA}	92,81±9,506 ^{aA}	93,90±8,650 ^{aA}
	Brix (%)	8,08±0,057 ^{gA}	8,05±0,173 ^{gA}	8,03±0,000 ^{hA}
	pH	2,35±0,007 ^{cB}	2,38±0,007 ^{aA}	2,36±0,007 ^{abAB}
	T. Acidity (g/L) *	68,71±0,000 ^{aA}	63,10±0,000 ^{dB}	67,23±0,000 ^{aA}
	Firmness (N)	15,69±1,752 ^{eA}	17,18±1,533 ^{eA}	16,52±2,768 ^{gA}
	Browning (OD ₄₂₀)	0,031±0,002 ^{fA}	0,032±0,002 ^{dA}	0,030±0,002 ^{gA}
5. Day	Weight (g)	93,49±8,735 ^{aA}	90,56±9,205 ^{aA}	91,69±8,456 ^{aA}
	Brix (%)	8,50±0,000 ^{gA}	8,20±0,173 ^{gB}	8,16±0,057 ^{gB}
	pH	2,37±0,015 ^{bcA}	2,38±0,007 ^{aA}	2,36±0,007 ^{abA}
	T. Acidity (g/L) *	66,18±0,000 ^{aB}	71,31±0,000 ^{aA}	64,38±0,362 ^{bc}
	Firmness (N)	16,93±1,258 ^{eC}	18,02±1,285 ^{eB}	19,49±1,716 ^{fgA}
	Browning (OD ₄₂₀)	0,039±0,001 ^{efA}	0,036±0,001 ^{cdB}	0,035±0,001 ^{fB}
10. Day	Weight (g)	88,33±8,615 ^{abA}	85,82±8,659 ^{abA}	87,00±8,123 ^{abA}
	Brix (%)	8,76±0,057 ^{fA}	8,36±0,057 ^{fB}	8,33±0,057 ^{fgB}
	pH	2,36±0,000 ^{bcA}	2,36±0,007 ^{bA}	2,37±0,007 ^{aA}
	T. Acidity (g/L) *	67,71±0,000 ^{aB}	70,28±0,000 ^{bA}	61,81±0,362 ^{cC}
	Firmness (N)	18,58±2,854 ^{eA}	19,23±2,672 ^{eA}	20,47±2,225 ^{efA}
	Browning (OD ₄₂₀)	0,041±0,001 ^{efA}	0,038±0,003 ^{cdA}	0,037±0,002 ^{efA}
15. Day	Weight (g)	85,00±8,617 ^{bcA}	82,85±8,443 ^{bA}	84,11±7,957 ^{bA}
	Brix (%)	8,83±0,152 ^{fA}	8,53±0,057 ^{efB}	8,43±0,057 ^{efB}
	pH	2,40±0,007 ^{aA}	2,35±0,007 ^{bB}	2,37±0,007 ^{aB}
	T. Acidity (g/L) *	64,29±1,029 ^{bA}	66,23±0,343 ^{cA}	57,99±1,029 ^{dB}
	Firmness (N)	26,63±3,186 ^{dA}	20,06±3,334 ^{eB}	20,80±2,393 ^{efB}
	Browning (OD ₄₂₀)	0,045±0,003 ^{deA}	0,042±0,002 ^{bcdAB}	0,039±0,002 ^{deB}

Table 4.15. Continued

Storage Time	Analyses	Control	<i>Aureobasidium pullulans</i> GE17	Mixed Culture
20. Day	Weight (g)	80,04±8,781 ^{cdA}	78,30±8,180 ^{bcA}	79,67±7,784 ^{bcA}
	Brix (%)	9,10±0,100 ^{eA}	8,60±0,100 ^{eB}	8,50±0,000 ^{defB}
	pH	2,41±0,007 ^{aA}	2,30±0,007 ^{dC}	2,33±0,000 ^{dB}
	T. Acidity (g/L) *	62,96±0,171 ^{bA}	56,29±0,000 ^{fC}	60,65±0,000 ^{cB}
	Firmness (N)	34,77±4,113 ^{cA}	20,54±2,730 ^{eB}	21,76±3,203 ^{efB}
	Browning (OD ₄₂₀)	0,049±0,001 ^{cdeA}	0,044±0,001 ^{bcdB}	0,041±0,001 ^{dC}
25. Day	Weight (g)	75,33±8,965 ^{deA}	73,74±7,945 ^{cdA}	75,06±7,731 ^{cdA}
	Brix (%)	9,26±0,057 ^{dA}	8,70±0,100 ^{deB}	8,60±0,100 ^{deB}
	pH	2,36±0,007 ^{bcA}	2,29±0,007 ^{dB}	2,30±0,000 ^{gB}
	T. Acidity (g/L) *	58,47±0,709 ^{cB}	52,53±0,266 ^{hC}	61,62±0,686 ^{cA}
	Firmness (N)	36,37±5,368 ^{bcA}	24,73±3,197 ^{dB}	23,41±2,809 ^{deB}
	Browning (OD ₄₂₀)	0,051±0,003 ^{cdeA}	0,046±0,003 ^{bcA}	0,046±0,001 ^{cA}
30. Day	Weight (g)	72,83±9,002 ^{defA}	71,18±7,767 ^{cdeA}	72,51±7,657 ^{cdeA}
	Brix (%)	9,33±0,057 ^{dA}	8,90±0,173 ^{cdB}	8,66±0,115 ^{cdB}
	pH	2,30±0,010 ^{dB}	2,38±0,000 ^{aA}	2,32±0,000 ^{eB}
	T. Acidity (g/L) *	48,85±1,029 ^{fC}	60,17±0,686 ^{eA}	55,56±0,343 ^{eB}
	Firmness (N)	37,97±4,576 ^{abcA}	28,93±2,471 ^{cB}	26,02±2,944 ^{cdB}
	Browning (OD ₄₂₀)	0,056±0,004 ^{bcdA}	0,049±0,003 ^{bB}	0,048±0,000 ^{bcB}
35. Day	Weight (g)	69,96±8,840 ^{efA}	68,06±7,420 ^{deA}	69,36±7,528 ^{defA}
	Brix (%)	9,50±0,100 ^{cA}	9,10±0,100 ^{bcB}	8,80±0,000 ^{cC}
	pH	2,39±0,011 ^{abA}	2,32±0,007 ^{cC}	2,36±0,000 ^{bB}
	T. Acidity (g/L) *	54,35±1,372 ^{eA}	54,35±0,000 ^{gA}	55,80±0,686 ^{eA}
	Firmness (N)	41,18±3,894 ^{abA}	31,03±2,362 ^{cB}	27,31±2,974 ^{bcB}
	Browning (OD ₄₂₀)	0,062±0,002 ^{bcA}	0,051±0,004 ^{abB}	0,050±0,002 ^{bB}

Table 4.15 Continued

Storage Time	Analyses	Control	<i>Aureobasidium pullulans</i> GE17	Mixed Culture
40. Day	Weight (g)	67,77±8,553 ^{efA}	65,59±7,097 ^{eA}	67,88±7,443 ^{efA}
	Brix (%)	9,63±0,057 ^{bA}	9,30±0,173 ^{bB}	9,00±0,000 ^{bC}
	pH	2,36±0,007 ^{bcA}	2,35±0,007 ^{bA}	2,31±0,000 ^{fB}
	T. Acidity (g/L) *	56,77±0,000 ^{dA}	49,98±0,000 ^{iC}	52,40±0,686 ^{fB}
	Firmness (N)	41,63±4,713 ^{abA}	34,46±5,371 ^{bB}	30,33±6,364 ^{bB}
	Browning (OD ₄₂₀)	0,067±0,002 ^{bA}	0,052±0,002 ^{abB}	0,051±0,002 ^{bB}
45. Day	Weight (g)	65,99±8,238 ^{fA}	63,95±6,874 ^{eA}	65,81±7,480 ^{fA}
	Brix (%)	9,80±0,000 ^{aA}	9,40±0,100 ^{aB}	9,13±0,251 ^{aC}
	pH	2,36±0,012 ^{bcB}	2,39±0,007 ^{aA}	2,34±0,000 ^{cB}
	T. Acidity (g/L) *	48,25±0,343 ^{fB}	52,65±1,029 ^{hA}	51,92±0,000 ^{fA}
	Firmness (N)	42,30±5,878 ^{aA}	37,82±6,224 ^{aAB}	33,93±4,126 ^{aB}
	Browning (OD ₄₂₀)	0,090±0,004 ^{aA}	0,060±0,003 ^{aAB}	0,056±0,000 ^{aB}

*: Titratable acidity was expressed in terms of citric acid in lemons, ^{A-C}: Uppercase superscripts indicate significant differences among the same day of different treatments ($p<0.05$), ^{a-i}: Lowercase superscripts indicate significant differences among the different days of the same treatment ($p<0.05$).

4.4.2.3. (1). Infection Rate

Ratio of natural decay development in cv. Kutdiken lemons was determined in the treatment without yeast application (control) during the 45-days of storage (conducted at 20 ± 2 °C and 50 ± 5 % humidity level) and the effect of coating the lemon surfaces with yeast solutions (*Aureobasidium pullulans* GE17 alone and mixed culture) on the natural infection rate of lemon fruits was investigated. Table 4.16. demonstrates the number of infected and non-infected fruits and also infection rate (%) by days during storage time.

According to the results obtained after 45 days of storage, the highest infection rate was determined in the lemons belonging to the control group as

8.23%, followed by the lemons sprayed with mixed culture with the infection ratio of 2.35%. Conversely, the lowest number of infected fruits (infection rate 0.58%) was detected in the lemons inoculated with single culture of *Aureobasidium pullulans* GE17. In comparison to the control group, the infection rate of lemons decreased by 7.65% and 5.88% at the end of the storage period with the application of a single culture of *Aureobasidium pullulans* GE17 and mixed culture, respectively. These results indicate that; significant economic losses dependent on fruit infection during storage could be prevented by using antagonistic yeasts for large scale fruit-vegetables storing companies without need for application of chemical pesticides.

Application of a single culture of *Aureobasidium pullulans* GE17 strain was greatly protected by the cv. Kutdiken lemons during 45 days of storage at ambient condition (20 ± 2 °C and 50 ± 5 % RH). It is noteworthy that only one out of the 170 lemons was infected at the end of the 45 days of storage in the lemon group which was surface-coated with a single culture of *Aureobasidium pullulans* GE17. In addition, utilization of mixed culture also exhibited powerful efficacy on natural decay development of cv. Kutdiken lemons with limited infected fruit numbers, only to 4 after 45 days of storage. Consequently, the results of 45 days of storage at 20 °C elicited that application of particularly *Aureobasidium pullulans* GE17 alone and its combination with *Meyerozyma guilliermondii* KL3 could be helpful in the reduction of lemon losses during especially long term storage.

When comparing the infection rate of apples and lemons after 45 days of storage at ambient condition (20 ± 2 °C and 50 ± 5 % RH), the percentage of infected fruit was determined to be considerably lower in lemon fruit. However, prospering results were obtained on the inhibition of natural decay formation on cv. Starking Delicious apples during 45 days of storage at 20 °C. Infection rates of apples belonging to control, single culture and mixed culture treatments were detected as 28.8 %, 8.33 % and 11.11 %, respectively. This result indicates not only that apple spoils faster than lemon at ambient conditions (20 ± 2 °C and 50 ± 5 % RH), but also demonstrates that the single or mixed culture of yeasts used were more effective in controlling the natural decay formation on lemons compared to

the apples. It is considered that having a high acidity level and quite thick shell are the inherent supporter protective factors of lemons against fungal pathogens and results in delaying the spoilage of lemon fruit.

Table 4.16. Number of infected lemons and infection rate during 45-days of storage

Storage Time	Analyses	Control	<i>Aureobasidium</i>	Mixed
0. Day	Intact (Pieces)	170	170	170
	Infected (Pieces)	0	0	0
	I.R. (%)	0	0	0
5. Day	Intact (Pieces)	169	170	170
	Infected (Pieces)	1	0	0
	I.R. (%)	0,58	0,00	0,00
10. Day	Intact (Pieces)	165	169	167
	Infected (Pieces)	5	1	3
	I.R. (%)	2,94	0,58	1,76
15. Day	Intact (Pieces)	165	169	167
	Infected (Pieces)	5	1	3
	I.R. (%)	2,94	0,58	1,76
20. Day	Intact (Pieces)	165	169	167
	Infected (Pieces)	5	1	3
	I.R. (%)	2,94	0,58	1,76
25. Day	Intact (Pieces)	163	169	167
	Infected (Pieces)	7	1	3
	I.R. (%)	4,11	0,58	1,76
30. Day	Intact (Pieces)	162	169	167
	Infected (Pieces)	8	1	3
	I.R. (%)	4,70	0,58	1,76
35. Day	Intact (Pieces)	160	169	167
	Infected (Pieces)	10	1	3
	I.R. (%)	5,88	0,58	1,76
40. Day	Intact (Pieces)	158	169	167
	Infected (Pieces)	12	1	3
	I.R. (%)	7,05	0,58	1,76
45. Day	Intact (Pieces)	156	169	166
	Infected (Pieces)	14	1	4
	I.R. (%)	8,23	0,58	2,35

I. R.: Infection rate

4.4.2.3.(2). Weight Loss

Weight loss, one of the most important quality parameters that affects the economic value of fruits, was followed on cv. Kutdiken lemons treated with or without yeasts during 45 days of storage at 20 °C. The major problems limiting postharvest life of lemon is weight loss, which results mainly from transpiration and respiration and yellowing of peel colour (Kaewsuksaeng et al, 2011). The changes which occurred in the weight of lemons by five-day intervals are presented in Figure 4.25.

Lemon fruits lost a considerable amount of weight during 45 days of storage. As it can be understood from the results, the lemons belonging to the control group lost the most weight (31.54 %) at the end of the storage period followed by treatments of single culture of *Aureobasidium pullulans* GE17 (31.09 %) and mixed culture (29.91 %), respectively. It was determined that each lemon fruit belonging to groups of control, *Aureobasidium pullulans* GE17 and mixed culture lost an average mass of 3.37 g, 3.21 g and 3.12 g per five-day intervals, respectively. Statistical expressions in Table 4.15 indicate that the means of weight loss values between the analysis days of each treatment were significant ($p < 0.05$) while the differences were insignificant when the treatments were compared with each other on a daily basis ($p > 0.05$). Although weight loss in cv. Kutdiken lemons was slowed down by spraying them with *Aureobasidium pullulans* GE17 alone or its combination with *Meyerozyma guilliermondii* KL3, rapid weight loss was determined in lemon fruits. Therefore, it is clear that long-term storage at 20 °C is not convenient for lemon fruit and if it happens it will cause problems in terms of quality and financial.

The weight loss measurement in cv. Starking Delicious apples, which had the highest weight loss value as 7.88 % in the control trial, are quite low compared to lemons. This result is due to the fact that lemon fruits have a much higher water content than apples. Weight loss was caused by evaporation of water from the fruit (Amarante et al, 2001). Typically, lemon packinghouses use wax coatings to

reduce weight loss, shrinkage, and improve fruit appearance. Such wax-based compounds are commonly mixed with synthetic fungicides to provide control of fungal postharvest diseases (Palou et al, 2016).

Nasrin et al (2020) conducted an experiment to assess the influence of coconut oil and beeswax coating and modified atmospheric packaging (MAP) on postharvest storage quality of lemon at ambient storage (21 ± 2 °C and 50 ± 5 % RH) for 18 days. Weight loss of fruits, in general, increased with the advancement in storage period. Maximum weight loss occurred in uncoated open lemon, whereas MAP lemons coated or uncoated preserved their weight nicely throughout the storage period. Around 27.22% weight loss happened on the 18th day of storage in uncoated open lemons stored at ambient condition (21 ± 2 °C). On the other hand, coconut oil-beeswax (both formulations, 90:10 and 80:20) coated lemons without MAP also have a good capacity to retain their weight; around 9% weight loss occurred on the 15th day of storage, while MAP lemons coated or uncoated lost their weight even less than 5% on the 18th day of storage. Coating on fruits and MAP creates a barrier between fruit skin and outer atmosphere which controls movement of O₂, CO₂, moisture, and solute movement, thereby reducing respiration, water loss, and oxidation rates.

Sun et al (2019) investigated the effects of three different maturity stages (green, green-yellow and yellow stages) and cold storage periods (30, 60 and 90 days) at 10 °C on Eureka lemon fruit quality. The fruit weight loss was significantly ($p \leq 0.05$) affected by the harvest maturity stages and cold storage periods. After 90 days of storage, the weight loss during the cold storage periods was significantly higher in the fruit harvested at green stage (15.85%) when compared to those harvested at green-yellow stage (14.25%) and yellow stage (11.78%). The mean fruit weight loss was significantly higher with the extension of cold storage periods from 30 days (6.75%) to 90 days (13.96%).

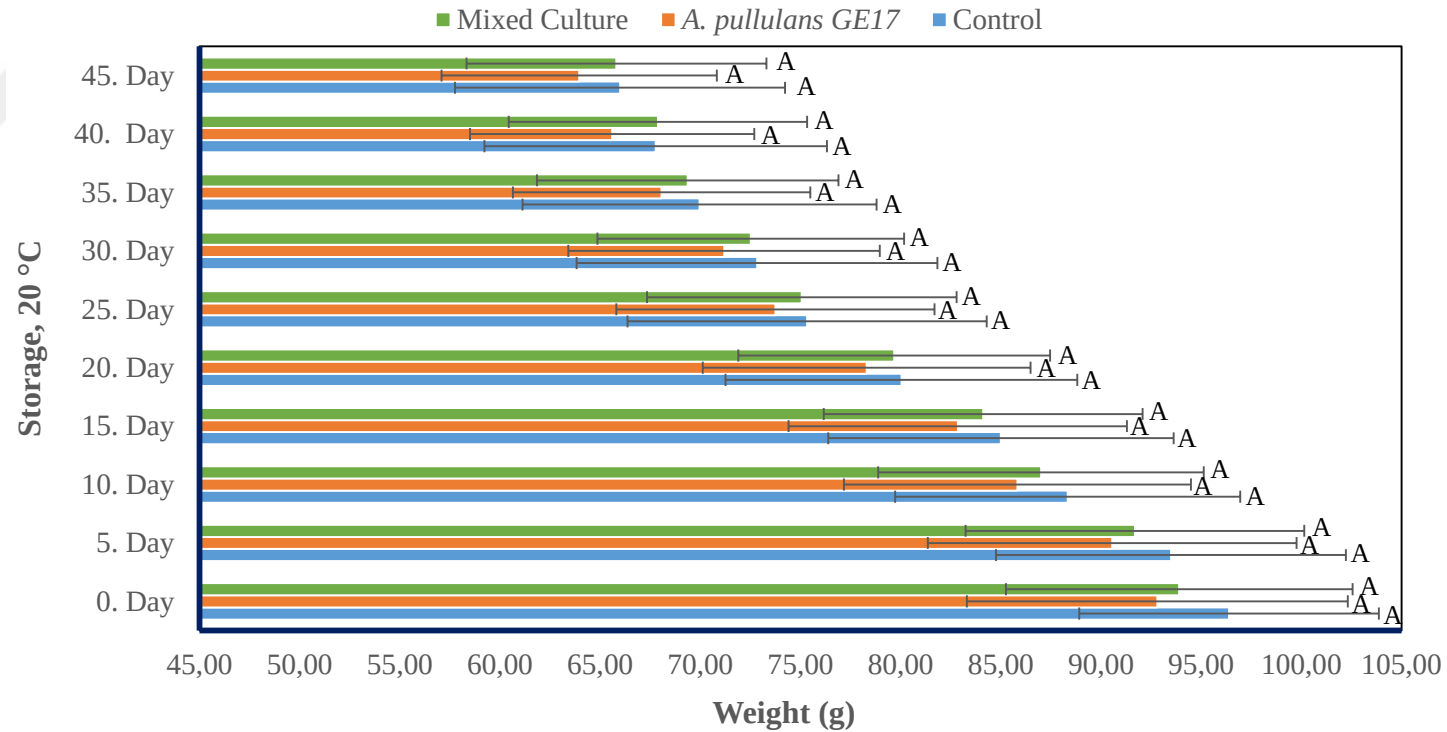


Figure 4.25. Examination of weight loss in lemons during 45 days of storage (Bars represent the standard deviations).

4.4.2.3.(3). Brix (Total Soluble Solids)

Brix value of lemon (*Citrus limon* (L.)) juice obtained from concentrate should be at least 8.0 % according to the Turkish Food Codex Communiqué on Fruit Juice and Related Products (2014/34) (GTHB, 2014). TSS values of fruit juices closely related with the moisture content of corresponding fruit. During storage, TSS value of lemon juices increased in all treatments with parallel to the increment of weight loss. Changes of brix values in cv. Kutdiken lemon juices during the storage at 20 °C for 45 days was demonstrated in Figure 4.26.

The mean TSS value at the end of the storage period at ambient condition (20 ± 2 °C and 50 ± 5 % RH) was significantly highest in the control group (9.80 %) when compared to those treated with single culture of *Aureobasidium pullulans* GE17 (9.40 %) and mixed culture (9.13 %). In the beginning of the storage period (day zero), there were no significant differences ($p > 0.05$) between the mean TSS values of lemon juices belonging to the different treatments, and quite close (between 8.03 % – 8.08 %) TSS values were measured. As soon as storage was started, the mean TSS value of the control group increased rapidly and statistically distinguished from both yeast treatments ($p < 0.05$) (Table 4.15). According to the statistical expressions dependent on the post-hoc test, there was no statistical difference in terms of mean TSS value among the yeast applications during the first 30 days although these treatments were significantly differentiated ($p < 0.05$) after the 35th day until the end of the storage (Table 4.15).

TSS parameter of lemon juices was significantly affected by yeast treatments. *Aureobasidium pullulans* GE17 alone or in combination with *Meyerozyma guilliermondii* KL3 maintained a lower TSS content in the lemon fruit than control treatment throughout the storage. From the beginning to the end of the storage, TSS levels increased by 21.28 %, 16.77 % and 13.69 % in the control, single and mixed culture treatments, respectively. The loss of soluble solids during the storage period was in natural sugars which are the primary

constituent of the soluble solids content of a product, consumed by respiration and used for the metabolic activities of the fruits (Taghinezhad and Ebadollahi, 2017). So lemon fruits with yeast coatings may have the lowest respiration and moisture loss. Therefore, yeast application using *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 has protected the fruit quality.

These findings were in agreement with the reported observations by Geng et al (2011) who examined the effects of *Kluyveromyces marxianus* alone or in combination with sodium bicarbonate (SBC) on postharvest quality parameters of citrus fruit. Level of TSS was determined to be similar (13.83 %) in control and combination (*Kluyveromyces marxianus* + SBC) treatments after 14 days of storage at 20 °C. However, TSS content was decreased to 13.27 % in the citrus fruit by the application of *Kluyveromyces marxianus* alone. As a result of individual application of SBC, the TSS value was 13.70 % in citrus fruit.

Results of Geng et al (2011) contradicted with the findings of Zhou et al (2014) who investigated the effects of *Pichia membranaefaciens* and salicylic acid (SA) on quality parameters of citrus fruit after 14 days of storage at 20 °C. The lowest TSS value was reported in the control treatment (10.13 %) when compared to those treated with single culture of *Pichia membranaefaciens* (10.90 %), SA alone (10.47 %) and their combination (11.17 %).

Uzun et al (2009a) determined the variations for some agronomical traits and genetic markers among eight “Kutdiken” clones derived from selections. Significant differences for TSS value were not determined among the 8 lemon clones and TSS content of lemons ranged from 8.6 % to 9.1 %.

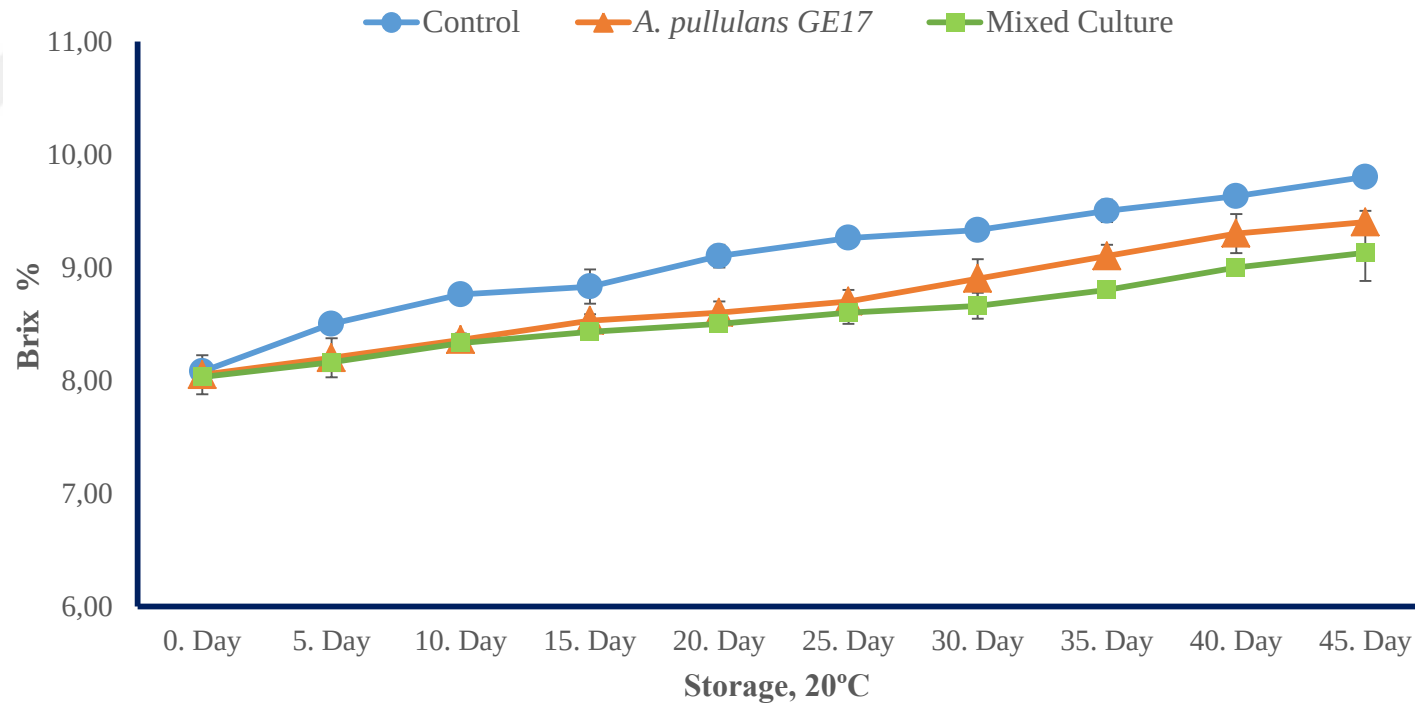


Figure 4.26. Changes in brix values of different treatments during 45 days of storage (Bars represent the standard deviations).

4.4.2.3.(4). pH and Titratable Acidity

Lemon (*Citrus limon*) is an important horticultural crop for their unique flavour and acidity and a good source of functional compounds like vitamin C, phenols and flavonoids (Nasrin et al, 2020). High acidity level is found in lemon fruit. However, a decrease generally occurs during storage in lemons as well as limes. This decrease in acidity during storage of fruits and vegetables is explained by the consumption of acids present in the fruit during the aging stages as a result of respiration. The rapid decrease in acidity means that respiration is fast or external conditions have an effect on the increment of respiration. For lemon fruit, respiration is generally slow and therefore there is usually no big change in acidity amounts. It is known that anything that slows the metabolism and reduces respiration (low temperature, low oxygen and high carbon dioxide) also reduces acid loss (Canan et al, 2015).

Combined representation of the results of pH and titratable acidity (in terms of citric acid) during storage at ambient condition (20 ± 2 °C and 50 ± 5 % RH) was presented in Figure 4.27. Although there was no big change in pH level of lemon, titratable acidity content was significantly changed during storage period ($p < 0.05$). The pH range of all treatments at the beginning and end of the storage was measured between 2.35-2.38 and 2.34-2.39, respectively. During storage, acidity content of lemon decreased. Comparing acidity values at the beginning and end of storage, the highest acidity loss was recorded in the control group (29.77 %) followed by mixed culture (22.77 %) and single culture of *Aureobasidium pullulans* GE17 (16.56 %). Similar findings of the increased acidity loss with the extension of storage periods were observed in the lemon fruit by Piga et al (1997) and Nasrin et al (2020). Initially, acidity level was 6.68 % and then decreased to 5.32 % in lemons stored at ambient condition (21 ± 2 °C and 50 ± 5 % RH) (Nasrin et al, 2020). Piga et al (1997) reported that after harvest lemon fruit retained acidity 7.33 (%) and during storage the acidity value decreased slightly.

It can be concluded from the results that yeast treatment did not affect the pH level of cv. Kutdiken lemon during 45 days of storage at ambient condition. In addition, the spraying of lemons with especially single or mixed culture had a positive effect to decrease the acidity loss during ambient storage. It is strongly possible that acid loss could be further decreased by storage of yeast treated lemon at lower temperatures.

Canan et al (2015) monitored the acidity loss of cv. Kutdiken lemons for 7 months by storing them in an artificially (by refrigerant gas i.e. ammonia or freon) cooled modern cold room and in a natural cooled traditional cave. Acidity amounts of 6.57% at the beginning of the storage period became 7.18% in the 3rd month of the storage, 6.91% in the 4th month, 6.23% in the 5th month, 4.03% in the 6th month and 4.39% in the 7th month.

In various studies on quality parameters of cv. Kutdiken lemons, titratable acidity content in terms of citric acid of fresh lemons varied between 6.00% and 7.46% (Uzun et al, 2009a, 2009b; Yılmaz et al, 2015).

Sun et al (2019) studied the influence of harvest maturity stages on the postharvest quality of lemon fruit during storage. A gradual reduction in titratable acidity content has been noted with the advancement of the maturation process in lemon fruit (*Citrus limon* (L.) Burm. F. cv. Eureka). The mean titratable acidity in the juice of cold stored (10 ± 2 °C and 95 ± 5 % RH) fruit which was harvested at the green stage was significantly ($p \leq 0.05$) higher (6.69%) compared to those harvested at green-yellow stage (6.26%) and yellow stage (5.99%).

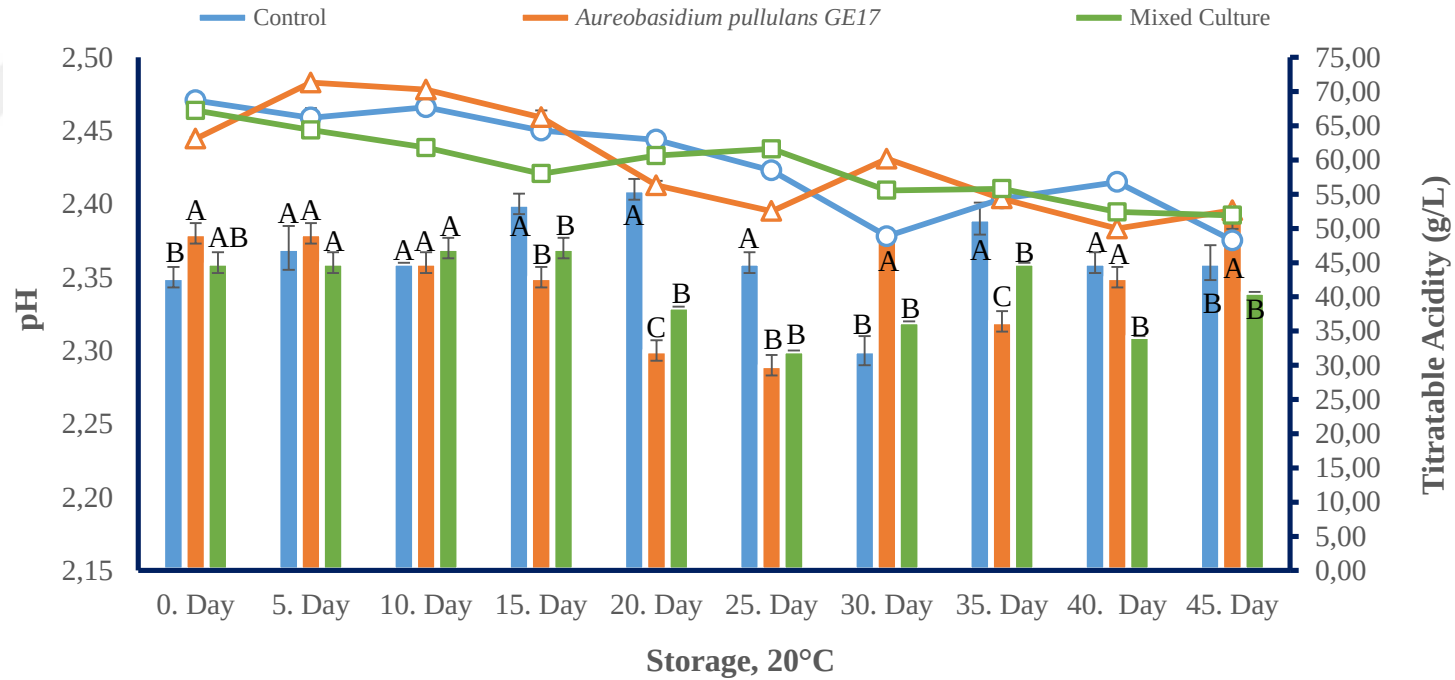


Figure 4.27. Changes in pH and titratable acidity levels of cv. Kutdiken lemon during 45 days of storage
*indicates titratable acidity in terms of citric acid equivalent.

4.4.2.3.(5). Browning Index

The browning of fruit juices such as lemon juice, orange juice, and strawberry juice, etc. during storage is one of the main quality deteriorations which cause a big challenge in the juice industry (Paravisini and Peterson, 2016; Pham et al, 2020). Juice browning can occur through enzymatic and non-enzymatic pathways. Enzymatic browning results from the oxidation of phenolic compounds into quinones by the polyphenol oxidase enzyme (PPO) (Martinez and Whitaker, 1995). The use of additives and/or heat treatment can suppress enzymatic browning during juice processing (Campos et al, 1996; Sanchez-Vega et al 2009). However, the mechanisms of the non-enzymatic browning reaction are complex and have not been adequately characterized, challenging the development of effective mitigation strategies. Browning index values of fresh lemon juices stored at ambient condition (20 ± 2 °C and 50 ± 5 % RH) for 45 days were given in Figure 4.28.

The 45 days of storage of lemon fruit brought significant changes in the browning index values, with their mean values varying between 0.030 (OD₄₂₀) and 0.090 (OD₄₂₀) ($p < 0.05$). The highest increase in browning index was determined in the control group lemon juices, whose absorbance value increased almost 3 times (0.031 to 0.090) compared to the beginning. The samples treated with single and mixed cultures of yeasts demonstrated significantly ($p < 0.05$) lower mean browning than the control samples. During storage period, the absorbance value of lemon juices treated with a single culture of *Aureobasidium pullulans* and mixed culture increased approximately at the same ratio (1.87 times). Despite the increase in absorbance values, a noticeable colour change was not observed in lemon juices due to the short term storage (45 days). However, the increase determined in OD₄₂₀ values during this 45-day storage period indicates that this increment may have potential for causing brown coloration of juice due to chemical reactions continued during long term storage. Intensification of browning in juice may be attributed to the accumulation of degradation products of sugars, ascorbic acids and proteins during storage.

The browning reactions in orange juice during storage are affected by product-related factors such as composition and pH as well as by the storage conditions (Kennedy et al, 1990; Solomon et al, 1995). Among the many compounds present in citrus juice, ascorbic acid, sugars, and amino acids play a key role in browning and are considered the main non-enzymatic browning (NEB) precursors (Bharate and Bharate, 2014). The degradation of NEB precursors is highly interdependent and is affected by other factors. For instance, it has been shown that the presence of amino acids accelerates the degradation of ascorbic acid and the breakdown of reducing sugars to produce brown-coloured compounds (Kennedy et al, 1990; Shinoda et al, 2005). Among the amino acids, arginine was reported to be the most reactive one in the browning formation of citrus juice model systems (Wolf from et al, 1974; Shinoda et al, 2005).

In a recent study, extracted juice of lemon fruits was treated with potassium metabisulphite, sodium benzoate and thermal processing. Treated and untreated samples of juice were stored at room and refrigeration temperature for 90 days and analyzed for chemical properties at 15 days of interval during storage. Findings explained above were consistent with earlier study of Sindhu and Khatkar (2018) who reported that the browning of lemon juice in different treatments was highly affected by storage temperature, showing noticeable changes at room temperature storage as compared to refrigeration temperature storage. It was reported that the mean OD₄₂₀ value of lemon juices stored at room temperature increased from 0.022 to 0.057 while it varied from 0.022 to 0.030 on juices stored at refrigeration temperature after 90 days of storage. Maximum mean browning (0.042 OD₄₂₀) was noticed in pasteurized samples stored at room temperature while minimum mean browning (0.022 OD₄₂₀) was observed in potassium metabisulphite treated samples stored at refrigeration temperature (Sindhu and Khatkar, 2018).

Uçan et al (2016) extracted concentrate lemon juice from cv. Interdonato lemons and monitored the browning index value of them during storage at - 25 °C for 180 days by 2 month intervals. Browning value of juices increased with

extension of storage time. Absorbance (OD_{420}) value of lemon juices was reported as 0.139 (day 0), 0.143 (day 60), 0.170 (day 120) and 0.177 (day 180).

Paravisini and Peterson (2019) investigated the function of reactive carbonyl species (RCS) which originated from the maillard reaction and ascorbic acid degradation on brown colour formation in orange juice during storage under refrigerated (4 °C) and accelerated conditions (35 °C). According to the reported results, the maillard reaction was supported as a chief mechanism of orange juice browning, with fructose determined as the principal precursor of RCS showing a causative impact on brown colour generation. The primary RCS of brown colour generation consisted of 3-deoxyglucosone and acetol, as well as, glyoxal and methylglyoxal indirectly via synergistic relations. In conclusion, amino acids affected colour formation by altering both the amount and the composition of RCS.

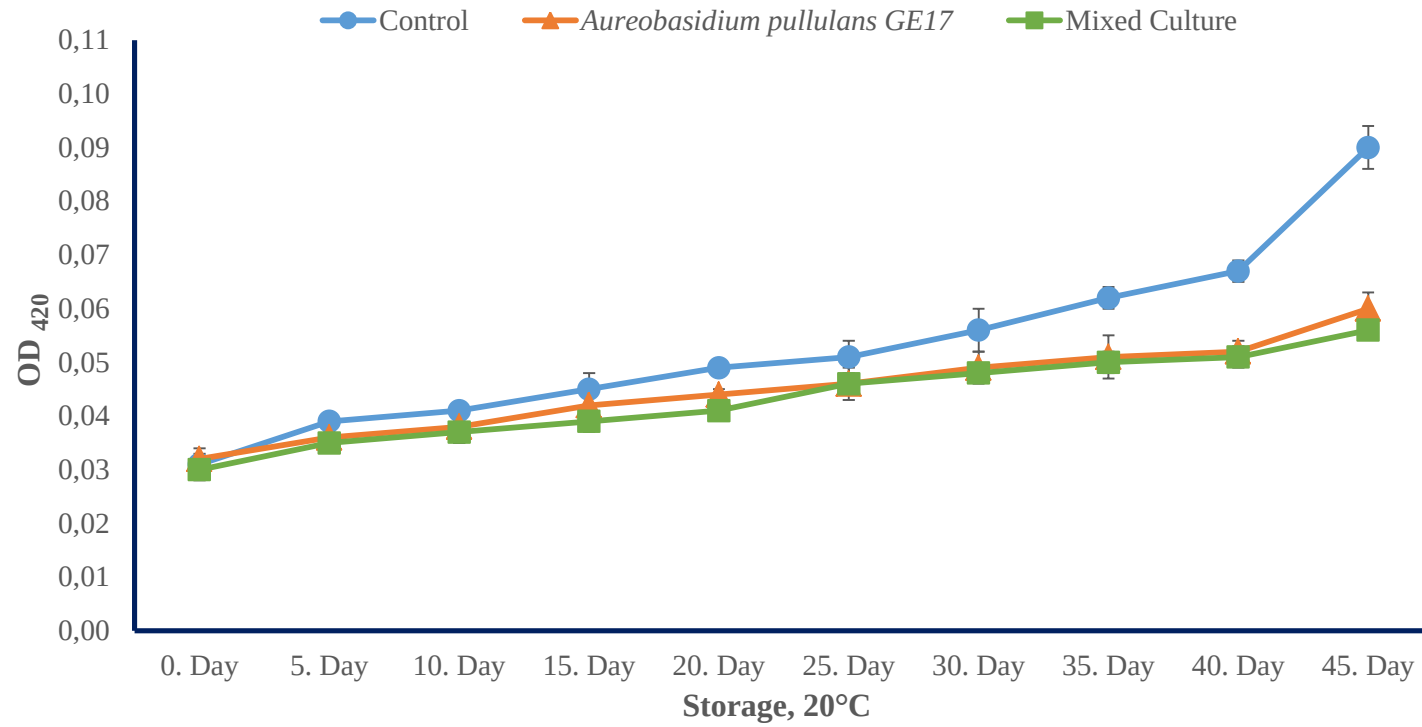


Figure 4.28. Development of browning in lemons during 45 days of storage (Bars represent the standard deviations).

4.4.2.3.(6). Firmness Values of cv. Kuldiken Lemons

Firmness is one of the most important indicators used to assess the quality of many fruits. Loss of fruit firmness during storage shows that there are water losses and metabolic changes in fruits (Tzortzakis, 2007). Degradation of insoluble protopectin to the more soluble pectic acid and pectin contributes to a decrease of firmness in many fruits. These changes occur relatively slowly and are less prominent to citrus fruits as compared to climacteric fruits. However, some softening of fruits also occurs due to the change of turgor pressure, and/or respiratory loss of dry matters during growth, development and senescence (Nasrin et al, 2020).

Figure 4.28 illustrates the effect of epiphytic yeast coating during ambient (20 ± 2 °C and 50 ± 5 % RH) storage on the firmness of lemon. Initially the firmness values of lemon were determined between 15.69 N and 17.18 N ($p>0.05$) in all treatments. Lemon fruit firmness significantly increased with the extension of storage periods in both yeast treated and untreated lemon ($p<0.05$). Highest firmness value on the forty-fifth day was detected in the control treatment (42.30 N) followed by a single culture of *Aureobasidium pullulans* GE17 (37.82 N), while in treatment of mixed culture firmness value was determined as lowest (33.93 N). According to the results monitored during 45 days of storage, firmness of lemon fruits increased gradually in all treatments, but in yeast treated lemons it was less than the control (Table 4.15, Figure 4.29). Therefore, it can be concluded that yeast coating on the surface of lemons slowed down the development of hardness. The texture of lemons is not desired to be hard. The lesser increment in the firmness character of lemon treated with yeast could be attributed to the formation of water-vapour barriers by yeast coating technique on the surface of the lemon (Arnon et al, 2014). These results are in harmony with those obtained by Shadid and Abbasi (2011) who demonstrated that the coatings not only prevented the moisture loss but improved the texture and the general appearance of the fruits for a long period of

storage. Measurement of firmness in cv. Kutdiken lemons is demonstrated in Figure 4.30.

When firmness value of cv. Starking Delicious apples and cv. Kutdiken lemons was compared during 45 days of storage at ambient condition (20 ± 2 °C and 50 ± 5 % RH), the firmness value of apples decreased (by 24.35% to 36.62 %) while that of lemons increased (by 105.38% to 169.59%) over time. One of the reasons for this is that lemon has a quite thick peel compared to apples. Also, lemon lost a reasonably higher amount of weight (29.91 % to 31.54 %) than apples (6.90% to 7.88%) during 45 days of storage. Higher water loss and thicker peel are considered to cause an increase in the firmness value of lemons during storage.

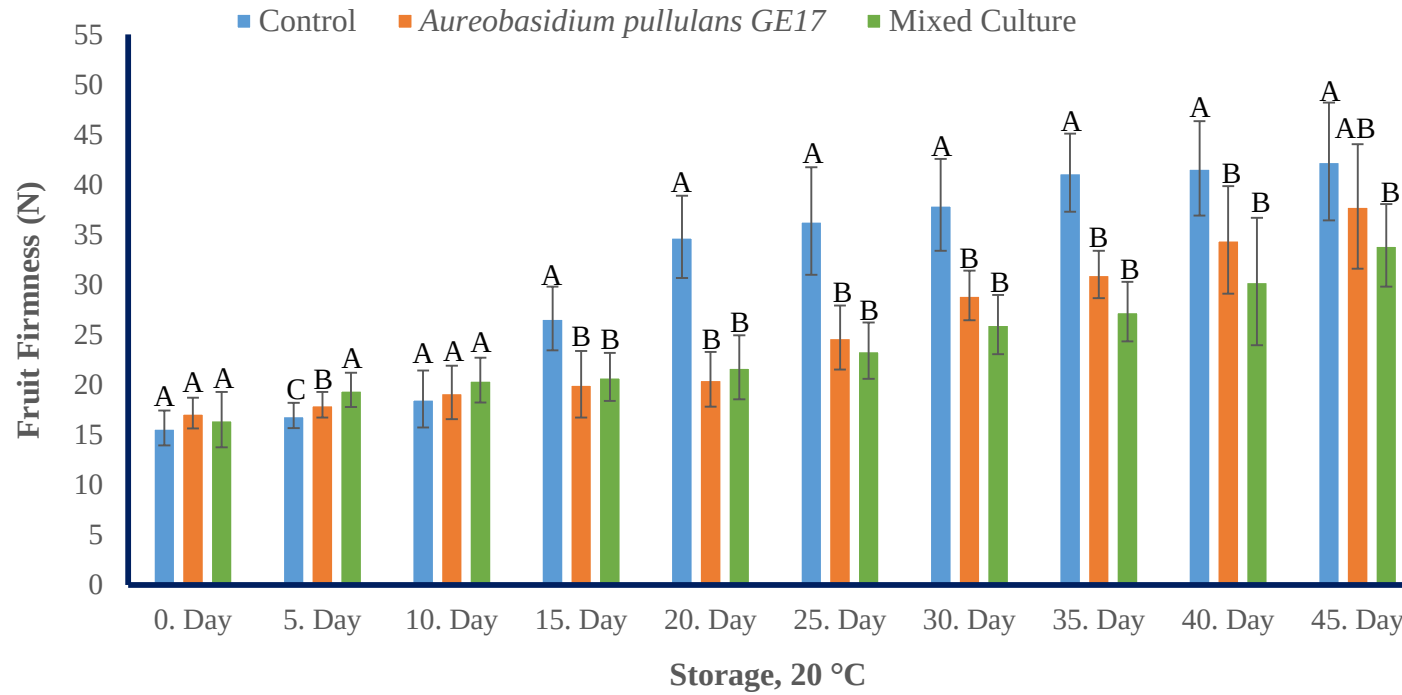


Figure 4.29. Changes in firmness of lemons during 45 days of storage (Bars represent the standard deviation).

In a comparative study, Barakat et al (2012) subjected the Washington Navel orange fruits (*Citrus sinensis* Osbeck) to the following treatments by dipping in wax only (control), imazalil 1500 ppm for 3 minutes, *Trichoderma album* 1500 ppm for 3 or 5 minutes and *Bacillus megatrium* 1500 ppm for 3 or 5 minutes. Then all fruits air-dried and stored at 10 °C and 85-90 RH for 168 days and some physical and chemical properties of orange fruits were determined. Results clearly indicate that orange fruit firmness significantly decreased with the prolongation of storage periods. Postharvest treatments of imazalil and BCAs significantly decreased orange fruit firmness during storage compared with untreated fruits. Furthermore, firmness of fruits treated with BCAs were significantly lower than those treated with imazalil during the two seasons.

Habiba et al (2019) examined the effect of twenty-five epiphytic isolates of yeast isolated from the healthy fresh fruit and vegetables including lemon, orange, mango etc. on firmness in kinnow on 0, 5, 10 and 15 day of experiment, artificially sprayed with *Penicillium digitatum* and stored at room temperature. They reported a decreasing pattern in the firmness of kinnow at all stages of storage. However, this loss in firmness was less in fruits treated with epiphytic yeast as compared to control.

Sumalan et al (2020) assessed the activity of essential oils (mint (MEO), basil (BEO) and lavender (LEO)) acting as the vapour-phases in antifungal protection against *Penicillium digitatum* and also their effect on lemon fruit quality after storage for 7 days. Essential oils (EOs) were tested at two different concentrations as C1 and C2 (C1 represents dose of EOs corresponding to the IC50 value, and C2 is the half of it). Loss of fruit firmness, assessed by the penetration power of lemon fruits, was influenced by type and concentration of EO vapour. In this regard, the treatment LEO-C2 (65.1 N) and MEO-C1 (59.0 N) showed significant differences compared with the control (46.6 N). In contrast, treatments with BEO, at both concentrations (C1 and C2), did not provide significant differences versus the control.

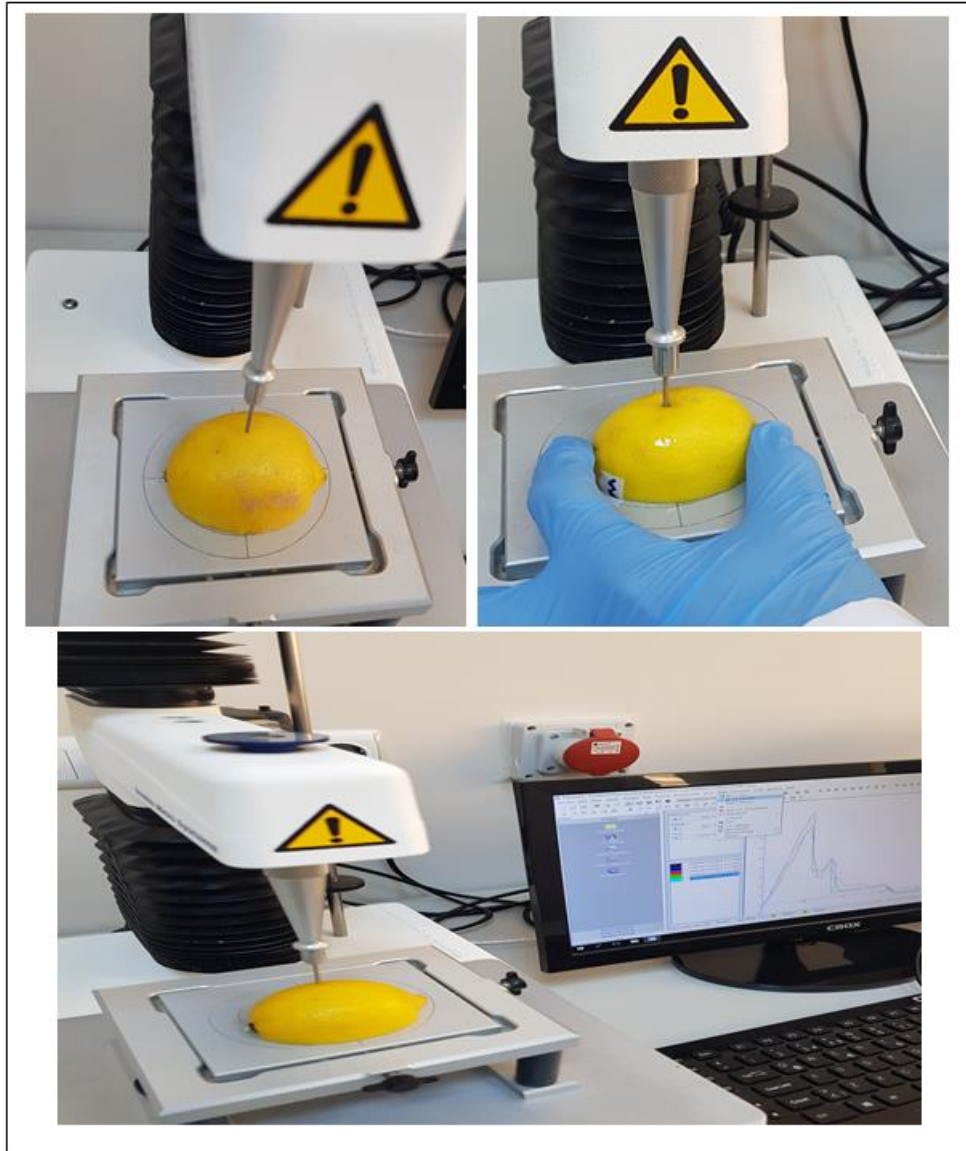


Figure 4.30. Some images during texture analysis

4.4.2.4. Efficacy of Yeasts Against Blue and Green Moulds During Cold Room Storage of Lemons

Efficacy of *Aureobasidium pullulans* GE17 alone and in combination with *Meyerozyma guilliermondii* KL3 in inhibiting blue mould (*Penicillium expansum* DSM62841) and green mould (*Penicillium digitatum* DSM2750) decay incidence, lesion diameter and disease severity in cv. Kutdiken lemons under commercial cold room storage conditions (10°C and 90% RH) during 3 months were evaluated. Development of lesion diameters during 3 months in cold room storage is presented in Table 4.17.

The lesion diameter of lemons belonging to all treatments was 4 mm in day zero because the lemons were artificially wounded with a diameter of 4 mm by a sterile cork borer during the establishment of experiments. In the subsequent days, the lesion diameters of lemon fruits increased rapidly, particularly in control groups. Significant differences were determined both among the different treatments and storage days ($p < 0.05$). The highest lesion diameter value after 90 days of storage was determined in lemons treated with *Penicillium expansum* DSM62841 (control) as 74.89 mm followed by treatments of *Penicillium digitatum* DSM2750 (control) with diameter value of 71.22 mm. The inhibition activity of yeasts on radial development of pathogens in lemons decreased with extension of the storage period. For example, utilization of the yeast strain of *Aureobasidium pullulans* GE17 alone reduced the lesion formation of *Penicillium digitatum* DSM2750 (P1) and P2 *Penicillium expansum* DSM62841 (P2) by 45.95 (%) and 57.96 (%) after 30 days of storage, respectively. Similarly, inoculation of mixed cultures of yeasts with pathogens into artificial wounds on lemons resulted in an inhibition on the radial growth of P1 and P2 by 52.01 (%) and 44.22 (%) at one month of storage, respectively. However, after 90 days of cold room storage, the inhibition ratio of yeasts for growth of P1 decreased to between 22.79 % – 23.95 % while this reduction was between 15.44 % – 23.97 % for P2.

4. RESULTS AND DISCUSSION

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Table 4.17. Development of lesion diameters (mm) in lemons during 90 days of cold room storage

Treatments	0. Day	15. Day	30. Day	45. Day	60. Day	75. Day	90. Day
P1 (Control)	4,00±0,000 ^{fA}	15,26±2,783 ^{eA}	27,05±5,461 ^{dA}	40,98±5,038 ^{cA}	57,36±6,115 ^{bA}	69,95±5,811 ^{aA}	71,22±4,149 ^{aA}
P2 (control)	4,00±0,000 ^{eA}	13,80±2,882 ^{fA}	26,48±6,320 ^{eA}	46,09±8,432 ^{dA}	58,35±7,869 ^{cA}	66,75±5,046 ^{bA}	74,89±3,396 ^{aA}
Y2-P1	4,00±0,000 ^{eA}	7,48±4,018 ^{eBC}	14,62±3,312 ^{dB}	27,84±8,617 ^{cB}	36,48±11,046 ^{bC}	48,83±5,655 ^{aC}	54,16±3,524 ^{aC}
Y2-P2	4,00±0,000 ^{fA}	4,74±4,189 ^{fC}	11,13±5,466 ^{eB}	28,36±4,526 ^{dB}	44,01±7,385 ^{cB}	50,11±3,454 ^{bC}	56,94±6,267 ^{aC}
X-P1	4,00±0,000 ^{fA}	8,02±3,749 ^{fB}	12,98±2,174 ^{eB}	29,21±5,599 ^{dB}	42,69±6,666 ^{cBC}	48,44±2,800 ^{bC}	54,99±7,907 ^{aC}
X-P2	4,00±0,000 ^{eA}	9,15±1,349 ^{fB}	14,77±2,448 ^{eB}	32,12±3,748 ^{dB}	43,61±3,710 ^{cB}	56,88±7,463 ^{bB}	63,33±4,047 ^{aB}

C.R.: Completely rotten, Y2: *Aureobasidium pullulans* GE17, P1: *Penicillium digitatum* DSM2750, P2: *Penicillium expansum* DSM62841, X: Mixed culture (*Meyerozyma guilliermondii* KL3 + *Aureobasidium pullulans* GE17), ^{A-C}: Uppercase superscripts indicate significant differences among the same day of different treatments ($p<0.05$), ^{a-g}: Lowercase superscripts indicate significant differences among the different days of the same treatment ($p<0.05$).

According to the results presented in Table 4.17, application of single culture of *Aureobasidium pullulans* GE17 and in combination with *Meyerozyma guilliermondii* KL3 generally exhibited close inhibition activity for both blue and green postharvest pathogens. However, it can conclude that utilization of single culture is more effective in controlling the *Penicillium expansum* DSM62841 compared to *Penicillium digitatum* DSM2750 although the opposite is true for the treatment of mixed culture.

Disease severity and infection rate (%) of lemon fruits were also monitored during cold room storage for 90 days (Table 4.18). Lemons were considered to be infected when the first lesion was seen on the fruits. Infection rate was determined to be 100 % for control treatments on the 9th day of cold room storage due to the fact that infection formation was determined in all lemons. Over the same period, the infection rate was determined to be 60 % in the treatment Y2P1, 45 % in the treatment Y2P2, 65 % in the treatment XP1 and 75 % in the treatment XP2. It is important to observe a lower infection rate in all treatments which utilized a single or mixed culture of yeasts than control groups. Under industrial conditions, application of yeasts proved to be effective against decay development on lemons, caused by blue and green moulds, since the fact that no infection was determined in the 55 % and 40 % of lemons belonging to the treatments of Y2P2 and Y2P1, respectively. The ratio of uninfected fruit was lower in lemons inoculated with mixed culture compared to single culture and percentage of healthy fruit was determined as 35% and 25% in treatments of XP1 and XP2, respectively. As a result, utilization of single culture of *Aureobasidium pullulans* GE17 appears to be highly effective for inhibiting infection caused by *Penicillium expansum* DSM62841 (55 %) and *Penicillium digitatum* DSM2750 (40 %) on lemons during industrial storage conditions (10 °C and 90% RH). On the other hand, inoculation of lemon wounds with mixed culture partly (25 % to 35 %) inhibited the infection formation in lemon fruits. Preserving fruits at these ratios during storage means overcoming enormous economic losses.

Table 4.18. Infection rate and disease severity measurements on lemons during cold room storage

Treatments	Disease Severity (%)						Infection Rate (%)
	15. Day	30. Day	45. Day	60. Day	75. Day	90. Day	Day 9 th
P1 (Control)	14,85	23,42	33,71	54,85	72,57	81,14	100
P2 (control)	15,42	25,14	34,85	57,14	72,57	82,85	100
Y2-P1	14,28	18,28	23,42	34,28	50,85	71,42	60
Y2-P2	12	13,71	18,85	38,85	50,85	79,42	45
X-P1	14,28	17,14	22,28	42,85	55,42	73,71	65
X-P2	14,28	17,14	22,85	44	61,71	80	75

P1: *Penicillium digitatum* DSM2750, P2: *Penicillium expansum* DSM62841, Y2: *Aureobasidium pullulans* GE17, X: Mixed culture (*Meyerozyma guilliermondii* KL3 + *Aureobasidium pullulans* GE17).

Once the lemons were infected, the development of infection was followed with the disease severity measurement according to the Townsend-Heuberger scale (Table 3.6) during 3 months of cold room storage (Table 4.18). At the end of 15 days in the storage, the slight rot phase was examined in all treatments. When the first month of storage was completed, medium rot level was determined in all treatments except Y2P2, which was still in the slight rot phase. After 45 days of storage in the cold room, high rot phase was determined in lemon fruits belonging to both control groups, while medium rot phase was not yet complete in the yeast treated lemons. At the end of the 2 months of storage, moulds were starting to form mycelium on lemons belonging to control groups, but lemon fruits treated with yeast strains were still at the high rot grade. At the end of 75 days, the mould reached the sporulation stage in both control groups (P1 and P2) and the XP2 treatment, while moulds continued to form mycelium in the Y2P1, Y2P2 and XP1 trials. After 3 months of storage, lemon fruits belonging to both control trials (P1 and P2), Y2P2 and XP2 treatments completely decayed, but sporulation of *Penicillium digitatum* DSM2750 continued in the treatments of Y2P1 and XP1 during the same period.

During the first 45 days of storage, close disease severity results were obtained by the utilization of *Aureobasidium pullulans* GE17 alone and in combination with *Meyerozyma guilliermondii* K3. However, lower DS values were determined on lemons treated with *Aureobasidium pullulans* GE17 alone against both pathogens in the last 45 days of storage.

Comparing the cold storage results of apples and lemons, the lesion diameter, disease severity and infection ratio values of lemons are significantly lower than apples. These results indicate that the yeasts (single and mixed culture) used in this study are more successful in preventing the spoilage of lemons than apples during storage under industrial conditions.

Some photos taken during the cold room storage assay are presented in Figure 4.31.



Figure 4.31. Some images during the experimentation of lemon in cold room storage

In a comparative study similar findings were reported; Zhou et al (2014) investigated the separate or combined effects of *Pichia membranaefaciens* and salicylic acid (SA) on the control of blue and green mold decay in citrus fruits. They applied two different methods to evaluate the effects of *Pichia membranaefaciens*, SA, or their combination on the postharvest quality parameters of citrus fruits. Firstly, the fruits were surface-disinfected with 2% (v/v) sodium hypochlorite, washed with tap water, and air-dried at room temperature and then wounded with a sterile borer. Each wound was inoculated with 30 µl of (1) sterile distilled water as the control; (2) the cell suspensions of *Pichia membranaefaciens* (1×10^8 cells/mL); (3) 1, 10, or 100 µg/mL SA solution; or (4) *Pichia membranaefaciens* suspensions (1×10^8 cells/mL) in combination with 1, 10, or 100 µg/mL SA. After 4 h, 20 µl of 1×10^4 spores/mL suspension of *Penicillium italicum* or *Penicillium digitatum* was inoculated into each wound. After air drying, all fruits were separately packed in plastic bags and incubated at 20 °C and 85–90% RH. Secondly, intact fruits were drenched for 2 min in (1) sterile distilled water as the control; (2) the cell suspensions of *Pichia membranaefaciens* (1×10^8 cells/mL); (3) an SA solution (10 µg/mL); and (4) *Pichia membranaefaciens* (1×10^8 cells/mL) combined with SA (10 µg/mL). The treated fruits were air-dried, separately packed in plastic bags, and stored for 14 d at 20 °C and 85–90% RH.

According to the results of the first application, SA alone did not effectively reduce disease incidence but slightly inhibited the lesion diameters of blue and green moulds *in vivo* compared with the control when it was utilized at the highest concentrations. A single application of *Pichia membranaefaciens* (1×10^8 cells/mL) on fruit wounds inoculated with *Penicillium italicum* or *Penicillium digitatum* resulted in a significantly ($p < 0.05$) lower disease incidence and smaller lesion diameters of blue and green moulds after 6 d or 8 d of incubation at 20 °C. A higher disease control efficacy was achieved when *Pichia membranaefaciens* was used with SA. Compared with *Pichia membranaefaciens* alone, the combined treatment of *Pichia membranaefaciens* and 10 µg/mL SA was more remarkable, as

indicated by the significantly decreased disease incidence caused by *Penicillium italicum* and *Penicillium digitatum* in citrus fruits from 83.33% to 72.22% and from 88.89% to 77.56%, respectively, after 6 d of incubation at 20 °C. The lesion diameters of blue and green mold decay were 38.17 and 70.4 mm, respectively, which were significantly ($p < 0.05$) lower than those in yeast-inoculated fruit after 8 d of incubation at 20 °C.

The second application resulted with *Pichia membranaefaciens*, as a single biocontrol agent, decreasing the lesion diameters and disease incidence of blue and green moulds in citrus fruits compared with the control. As the storage period was prolonged, the control efficacy of yeast decreased. SA alone had no significant inhibitory effect on blue and green mould decay in citrus fruits. However, the application of SA, specifically at 10 µg/mL, significantly ($p < 0.05$) improved the biocontrol efficacy of *Pichia membranaefaciens*. When *Pichia membranaefaciens* was combined with 10 µg/mL SA, the disease incidences induced by *Penicillium italicum* and *Penicillium digitatum* reduced to 86.33% and 94.44%, respectively, compared with those of 94.45% and 100% in yeast-dipped fruits after 6 d of storage at 20 °C.



5. CONCLUSION

The aim of this study to control two common postharvest pathogens, blue mold (*Penicillium expansum* DSM62841) and green mold (*Penicillium digitatum* DSM2750), through yeasts which possess antagonist effect as an alternative to chemical control procedures. For this purpose, the potential antagonist yeasts to be used in this study were isolated from three different varieties of apple (cv. Golden Delicious, cv. Starking Delicious, cv. Amasya) and lemon (cv. Kutdiken, cv. Interdonato, cv. Meyer). All isolates identified by molecular methods were subjected to preliminary screening to determine the strains which possess higher antagonistic capability against blue and green moulds. Afterwards, selected strains were tested *in vitro* to determine their action mechanisms via analyses including production of VOCs, extracellular lytic enzymes activity (chitinase, protease, gelatinase, β -1,3 Glucanase, pectinase), iron depletion, effect on spore germination of fungal pathogens and percent (%) inhibition of mycelial growth. Patulin degradation by yeasts was also assessed *in vitro*. During *in vitro* analyses, single and mixed culture combinations of selected yeasts with different cell concentrations were investigated. According to the results, the yeast/s and cell concentration with the highest efficacy were specified and *in vivo* analyses continued with them. Within the scope of *in vivo* analyses, apple and lemon fruits were stored either ambient condition and cold room. The ambient conditions were 20 ± 2 °C and 50 ± 5 % RH for storage in a standard room. Industrial storage specifications were applied during cold room storage (4°C and 95 % RH for apple; 10°C and 90 % RH for lemon). During 45 days of storage at ambient condition, quality parameters such as pH, total acidity, brix, weight loss, fruit firmness and browning were followed per five days. Infection rate, disease severity and lesion diameter values were determined every fifteen days during the three month of cold room storage. In addition, *in vivo* colonization ability of the selected yeast strains

on wound parts of the fruits and the effect of VOCs produced by studied yeasts in controlling blue and green mold directly on apples and lemons were examined.

In short, this thesis composed of three main parts; (i) isolation and identification of potential antagonistic yeasts, (ii) evaluation of selected yeasts *in vitro* and (iii) application of selected treatments (yeast/cell number) *in vivo* (on cv. Starking Delicious apples and cv. Kutdiken lemons). Obtained results were briefly presented below.

Results of molecular identification of yeasts:

- A total of 69 yeasts were isolated from the surface of apples and lemons belonging to five different genera and six different species.
- These species were; *Saccharomyces cerevisiae*, *Saccharomyces paradoxus*, *Aureobasidium pullulans*, *Meyerozyma guilliermondii*, *Rhodotorula mucilaginosa* and *Wickerhamomyces anomalus*.
- *Saccharomyces cerevisiae* was the only species isolated from all the three varieties of apples and lemons with the highest frequency (40%).
- *Aureobasidium pullulans* was the second dominant species with the frequency of 30%. This species was present in all varieties of apple but none of the lemons.
- Conversely, *Meyerozyma guilliermondii* was isolated from all the lemon varieties while not found in any of the apple variety. It was the third (12%) most frequently isolated species.

Results of *in vitro* analyses:

- During preliminary screening of 69 isolated yeasts by dual culture test, only two yeast strains (*Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3) restricted the mycelial growth of *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750 over 50%.

- The highest inhibition (%) values on mycelial growth of both pathogens (*Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750) were obtained via VOCs produced in the case of utilization of mixed culture rather than their usage alone.
- Studied two yeast strains (*Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3) demonstrate the same characteristics with regard to extracellular lytic enzyme production. Although they were able to produce β -1,3 glucanase, protease and gelatinase enzymes, they were incapable of producing pectinase and chitinase.
- It was detected that iron depletion mechanism was not present in the mode of actions of both yeasts.
- *Aureobasidium pullulans* GE17 strain reduced the germination ratio of *Penicillium digitatum* DSM2750 by 88.13%, while mixed culture (*Aureobasidium pullulans* GE17 + *Meyerozyma guilliermondii* KL3) inhibited the germination of *Penicillium expansum* DSM62841 by 94.69%.
- Although acceptable results were obtained by utilization of *Meyerozyma guilliermondii* KL3 alone, its efficacy was lower compared to treatment of single culture of *Aureobasidium pullulans* GE17 and mixed culture.
- When the results of the percent inhibition of radial growth by selected yeasts evaluated, the highest level of percentage inhibition of *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 was recorded by using the *Aureobasidium pullulans* GE17 alone as 74.66% and 83.37, respectively.
- Inhibition (%) capability of mixed culture against blue (between 48.41 % - 58.19 %) and green (between 50.86 % - 52.40 %) moulds were higher than *Meyerozyma guilliermondii* KL3, whereas lower than *Aureobasidium pullulans* GE17.

- It is obviously determined that, increasing the yeast concentrations from 10^6 cells/mL to 10^8 cells/mL resulted in an improvement on the biocontrol activity of yeasts in all *in vitro* tests.
- Successful biodegradation of patulin by strain KL3, GE17 and their mixed culture was proved. Only after 24 h of incubation, 91.46 % of initial patulin concentration was degraded in the presence of mixed culture and patulin was not detected at 40 h of incubation.
- Based on the findings from *in vitro* analyses, it was decided to continue *in vivo* analyses on apples and lemons using single culture (*Aureobasidium pullulans* GE17) and mixed culture (*Aureobasidium pullulans* GE17 + *Meyerozyma guilliermondii* KL3) of yeasts at the concentration of 10^8 cells/mL.

Results of *in vivo* analyses on cv. Starking Delicious apples;

- According to the results, both yeasts exhibited great colonization ability on wounded parts of cv. Starking Delicious apples. After one-week incubation at 25°C, the population density present in the apples treated with *Aureobasidium pullulans* GE17 alone and mixed culture reached to 10.14 log cfu/wound and 9.51 log cfu/wound, respectively.
- To the results of the *in vivo* VOCs analysis, the least radial growth and the highest inhibition (%) values were determined by utilization of mixed culture, as in *in vitro* results.
- Through VOCs produced by mixed culture, radial growth of *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 was suppressed by 44.83% and 51.58%, respectively.
- It is significant that almost the same efficacy of VOCs produced by mixed culture against growth of pathogens was detected both *in vitro* and *in vivo* experiments.

- The results of storage at ambient condition (20 ± 2 °C and 50 ± 5 % RH) indicated that yeast treatment did not impair quality parameters of apples during storage, including weight loss, pH, titratable acidity, brix, fruit firmness and browning index.
- After 45 days of storage at ambient condition, the highest infection rate (28.8 %) was determined in apples belong to the control group. Lower infection values were detected in apples treated with *Aureobasidium pullulans* GE17 and mixed culture as 8.33% and 11.11%, respectively.
- During storage at ambient condition, coating of apples with yeasts led to the deceleration of weight loss in comparison to control. In particular, the least weight loss in apples was achieved through application of mixed culture.
- Positive correlation was determined between weight loss and brix results. TSS values of apples belonging to all treatments increased during storage at ambient condition. The highest TSS value was detected in control group while apples belonging to the treatment of *Aureobasidium pullulans* GE17 and mixed culture had the same value at the end of the storage.
- pH values tended to increase in all treatments while titratable acidity levels were determined to decrease during 45 days of storage at ambient condition. Quite close results were obtained in yeast treatments and control group.
- It was determined that there is no negative impact of yeast application on pH and TA of apples.
- At the end of the storage period, the browning index of apples belonging to control group is much higher than that of inoculated with *Aureobasidium pullulans* GE17 alone and in combination with the strain of *Meyerozyma guilliermondii* KL3. It can be inferred that application of these two native yeast strains isolated within the scope of this thesis slowed down the browning process in cv. Starking Delicious apples.

- The application of yeast protected to the texture of apples compared to the control. At the end of the storage, the apples treated with *Aureobasidium pullulans* GE17 and mixed culture had close firmness values as 7.58 N and 7.50 N, respectively. However, the highest reduction in firmness value (5.90 N) was determined in apples belong to the control group.
- According to the experimentation in cold room (4°C and 95% RH), application of *Aureobasidium pullulans* GE17 and mixed culture exhibited close biocontrol activity against fungal pathogens and reduced the lesion formation by *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 almost by half at the 60th day of storage.
- When infection rate reached 100% in control group apples, it was determined as 60% in treatment of mixed culture against *Penicillium digitatum* DSM2750. Over the same period, 80% of total apples was infected by *Penicillium expansum* DSM62841 in the apples treated with *Aureobasidium pullulans* GE17.
- Remarkable results were obtained in the reduction of disease severity values in apples by yeast treatment, particularly during the first 2 month of storage in cold room.

Results of *in vivo* analyses on cv. Kutdiken lemons;

- According to the results, mixed culture of yeast showed better adaptation and colonization ability on lemon compared to *Aureobasidium pullulans* GE17. After one-week incubation at 25°C, 8.31 log cfu/wound yeast cells was present in wounded lemons inoculated with mixed culture. On the other hand, mean population density in each wound on lemons belonging to the *Aureobasidium pullulans* GE17 and control groups was determined as 4.83 log cfu/wound and 1.97 log cfu/wound, respectively.
- As the results of VOCs in the apples, the least pathogen growth, disease severity and disease incidence values were recorded in the treatment which

used mixed culture of two yeast. VOCs produced by the combined use of *Meyerozyma guilliermondii* KL3 and *Aureobasidium pullulans* GE17 restricted the growth of *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM 62841 by 73.30% and 75.28%.

- Through VOCs produced by mixed culture, reasonably low disease severity and disease incidence values were determined against lemons inoculated with green mold (DS: 2.38%, DI: 16.66%) and blue mold (DS: 4.76%, DI: 33.33%) compared to control.
- Coating of the surfaces of cv. Kutdiken lemons by yeasts contribute to protect quality parameters of fruit during 45 days of storage at ambient condition (20 ± 2 °C and 50 ± 5 % RH).
- During the 45-days of storage at ambient condition, the highest infection rate was determined in lemons belonging to control group (8.23%), followed by mixed culture (2.35%) and *Aureobasidium pullulans* GE17 (0.58%).
- As it can be seen from the results, postharvest losses of lemon fruits during storage at ambient conditions was significantly decreased via coating fruit surfaces with yeasts (especially *Aureobasidium pullulans* GE17) utilized in this thesis.
- Weight is undoubtedly one of the crucial quality properties that play a key role on the economic value of fruits. Weight loss in cv. Kutdiken lemons was slowed down by spraying them with antagonistic yeasts. The highest weight loss was determined in lemons without yeast treatment (control) followed by the groups of *Aureobasidium pullulans* GE17 and mixed culture.
- The TSS content of lemon belonging to the control group was significantly higher than those treated with *Aureobasidium pullulans* GE17 and mixed culture. It is mean that yeast coatings led to deceleration of fruit respiration

and therefore moisture loss. Slowing the respiration is an important factor to retard in preventing deterioration of fruits.

- Close pH and acidity results were obtained in control and yeast treatments. From beginning to the end of the storage, the highest acidity loss was detected in lemons belonging to the control group (29.77%), followed by mixed culture (22.77%) and *Aureobasidium pullulans* GE17 (16.56%).
- During storage of lemons at ambient condition, compared to the beginning, browning index of lemons belonging to control treatment increased almost 3 times while it is almost the same (1.87 times) for the treatments of mixed culture and *Aureobasidium pullulans* GE17.
- Firmness value significantly increased in lemons with the extension of storage periods. At the end of the storage, the highest firmness value was determined in the lemons belonging to the control treatment (42.30 N), followed by *Aureobasidium pullulans* GE17 (37.82 N) and mixed culture (33.93 N). Considering that the lemon texture is desired to be soft rather than hard, yeast treatment on the surface of lemon had positive effect for maintaining fruit texture close to the beginning as much as possible.
- At the end of the first month in cold room storage (10°C and 90 % RH), lesion diameters of *Penicillium digitatum* DSM2750 was reduced at the level of 45.95% – 52.01 %, while radial growth of *Penicillium expansum* DSM62841 was blocked by 44.22% to 57.96 %. Then inhibition activity of yeasts on growth of pathogens in lemons decreased with extension of the storage period.
- *Aureobasidium pullulans* GE17 and its combination with *Meyerozyma guilliermondii* KL3 exhibited close inhibition activity against blue and green moulds during cold room storage.
- When infection rate reached 100% in the control group lemons during storage at 10°C, it was reduced to 45% and 60% through inoculation

of *Aureobasidium pullulans* GE17 together *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750, respectively.

- Compared to the control group, lower disease severity values were obtained in lemons treated with yeasts throughout the cold storage.

To summarize briefly the results obtained within the scope of this thesis, the use of single culture of *Aureobasidium pullulans* GE17 and its combined use with *Meyerozyma guilliermondii* KL3 at a concentration of 10^8 cells/mL became quite effective in controlling *Penicillium expansum* DSM62841 and *Penicillium digitatum* DSM2750. However, it was determined that biocontrol activity of *Meyerozyma guilliermondii* KL3 is remain limited against blue and green moulds when it used alone. It was determined that antagonistic activity of two yeast strains studied depends on more than one mechanism including competition for space and nutrients, production of VOCs, secretion of extracellular lytic enzymes and inhibition of fungal spore germination. Conversely, iron competition mechanism was not detected in the mode of actions of yeasts studied. Both yeast strains and their mixed culture exhibited great biodegradation capability on patulin. In addition, the increase in the number of yeast cells resulted in higher biocontrol activity. Although the high efficacy of yeasts *in vitro* decreased slightly in *in vivo* conditions, promising results were obtained during *in vivo* analyses. Infection rate of cv. Starking Delicious apples and cv. Kutdiken lemons was significantly decreased by application of mainly *Aureobasidium pullulans* GE17 and also mixed culture during storage of both ambient conditions and cold storage. Accordingly, it can be said that postharvest losses of apples and lemons during storage was reduced in this study without chemical application, using epiphytic antagonistic yeasts. Also, yeast application helped preserve the quality parameters (pH, acidity, brix, weight loss, firmness, browning index) of cv. Starking Delicious apples and cv. Kutdiken lemons during storage for three months.

The following suggestions can be given for further studies based on the results of this thesis:

- In parallel to commonly reported findings in the literature, the yeasts tested in this thesis were not found as effective as chemical pesticides. The use of fungicides was still the primary method of controlling postharvest diseases.
- For that reason, the biocontrol activity of antagonistic microorganisms need to be improved by various methods including omic technique (genomic, transcriptomic or proteomic), manipulations in the physical and chemical environment during storage, addition of low doses of fungicides or salt additives in the microbial cultures, addition of nutrients and plant products in microbial cultures, use of the microbial cultures with other approaches/additives.
- VOCs produced by strains GE17 and KL3 should be identified and evaluated by performing specific biocontrol studies on each.
- The mechanisms by which microorganisms are most successful should be determined and improved through in-depth research.
- The fungal pathogen, commodity and concentration that each antagonist microorganism is most effective must be determined in order to develop appropriate formulations and application methods (i.e. spraying, dipping, drenching, coating and nebulization).

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CURRICULUM VITAE

The author was born in 1989 in the city of Dörtyol/Hatay, in the Mediterranean province of Turkey. He completed his first and second class of primary school in Dörtyol, then continued primary, secondary and high school education in Gaziantep. In 2011, he graduated from Erciyes University, Faculty of Engineering, Department of Food Engineering, Kayseri, Turkey. He has been working as a Research Assistant in the Department of Food Engineering of Çukurova University, Adana, Turkey, since 2012. In 2014, he successfully completed his MSc degree in Çukurova University, Department of Food Engineering, Adana, Turkey. His MSc thesis supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK) as a project under 1002-National Support Project Programme (Project Number: TÜBİTAK-TOVAG 113O818). In 2014, he was admitted into PhD programme in the Institute of Natural and Applied Science, Faculty of Agriculture, Department of Food Engineering, Çukurova University, Adana, Turkey. His PhD thesis supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK) as a project under 1001-National Support Project Programme (Project Number: TÜBİTAK-KBAG 118R020). In 2019, he undertook his military service in Amasya. He has been continuing his PhD education in the Department of Food Engineering in Çukurova University.



APPENDICES



Appendix 1. Sequence alignment of the D1/D2 domain of the 26S rRNA gene for identified yeast strains

(1) *Aureobasidium pullulans* AE2

TTCAGGCATTGCCTAGTACGGCGAGTGAAGCGGCAACAGCTCA
AATTTGAAAGCTAGCCTTCGGGTTCGCATTGTAATTTGTAGAGG
ATGATTTGGGGAAGCCGCCTGTCTAAGTTCCTTGGAACAGGACG
TCATAGAGGGTGAGAATCCCGTATGTGACAGGAAATGGCACCC
TATGTAAATCTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGC
TCTAAATGGGAGGTAAATTTCTTCTAAAGCTAAATATTGGCGAG
AGACCGATAGCGCACAAAGTAGAGTGATCGAAAGATGAAAAGCA
CTTTGGAAAGAGAGTTAAAAAGCACGTGAAATTGTTGAAAGGG
AAGCGCTTGCAATCAGACTTGTTTAAACTGTTTCGGCCGGTCTTCT
GACCGGTTTACTCAGTTTGGACAGGCCAGCATCAGTTTCGGCGG
CCGGATAAAGGCTCTGGGAATGTGGCCTCCACTTCGGTGGAGGT
GTTATAGC

(2) *Aureobasidium pullulans* AE3

AAACCAACAGGGATTGCCCTAGTAACGGCGAGTGAAGCGGCAA
CAGCTCAAATTTGAAAGCTAGCCTTCGGGTTCGCATTGTAATTT
GTAGAGGATGATTTGGGGAAGCCGCCTGTCTAAGTTCCTTGGA
CAGGACGTCATAGAGGGTGAGAATCCCGTATGTGACAGGAAAT
GGCACCTATGTAAATCTCCTTCGACGAGTCGAGTTGTTTGGGA
ATGCAGCTCTAAATGGGAGGTAAATTTCTTCTAAAGCTAAATAT
TGCGGAGAGACCGATAGCGCACAAAGTAGAGTGATCGAAAGATG
AAAAGCACTTTGGAAAGAGAGTTAAAAAGCACGTGAAATTGTT
GAAAGGGAAGCGCTTGCAATCAGACTTGTTTAAACTGTTTCGGCC
GGTCTTCTGACCGGTTTACTCAGTTTGGACAGGCCAGCATCAGT

TTCGGCGGCCGGATAAAGGCTCTGGGAATGTGGCCTCCACTTCG
GTGGAGGTGTTATAGCCCAGGGTGT

(3) *Saccharomyces cerevisiae* AE4

CAATGGCTTGTACGGCGAGTGAGCGGCAAAAGCTCAAATTTGA
AATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAA
CTTTGGGGCCGTTCCCTTGTCTATGTTCCCTTGAACAGGACGTCAT
AGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAA
AGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGT
GGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCG
ATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGA
AAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGC
ATTTGATCAGACATGGTGTTTTGTGCCCTCTGCTCCTTGTGGGTA
GGGGAATCTCGCATTTCACTGGGCCAGCATCAGTTTTGGTGGCA
GGATAAATCCATAGGAATGTAGCTTGCCCTCGGTAAGTATTATAG
CCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCGACGTAA
GTCAAGGATGCTGGCATAATGGTTATATGCCGCCC

(4) *Aureobasidium pullulans* AE8

CAGGGTTTAACCCTTAGTTCCGGCGAGTGAAGCGGCAACAGCTT
CCAAATTTTGAAAGCTAGCCTTTCGGGTTTCGCATTTGTAATTCTG
TAGAGGATGATTTCCGGGAAGCCGCCTGTCTAAGTTCCTTCGGA
ACAGGACGTCATAGAGGGTGAGAATCCCGTATGTGACAGGAAA
TGGCACCTATGTAAATCTCCTTCGACGAGTCGAGTTGTTTGGG
AATGCAGCTCTAAATGGGAGGTAAATTTCTTCTAAAGCTAAATA
TTGGCGAGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGAT
GAAAAGCACTTTGGAAAGAGAGTTAAAAAGCACGTGAAATTGT
TGAAAGGGAAGCGCTTGCAATCAGACTTGTTTAAACTGTTTCGGC

CGGTCTTCTGACCGGTTTACTCAGTTTGGACAGGCCAGCATCAG
TTTCGGCGGCCGGATAAAGGCTCTGGGAATGTGGCCTCCACTTC
GGTGGAGGTGTTATAGCCCAGGGTGTAAACGGCCAAGCCGGG
ACTGAGGTCCGCGCTTCGGCTAGGATGCTGGCGTAATGGTTGTA
AGCGACCCGTCTGA

(5) *Aureobasidium pullulans* AE13

CACCGGGGGCATGCCTTAGTACGGCGAGTGAAGCGGCAAAAGC
TCAAATTTGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTG
GAGAGGGCAACTTTGGGGCCGTTCTTGTCTATGTTCTTGAA
CAGGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGC
GGTTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAAT
GCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTG
GCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAA
AAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGA
AAGGGAAGGGCATTGATCAGACATGGTGTTTTGTGCCCTCTGC
TCCTTGTGGGTAGGGGAATCTCGCATTTCACTGGGCCAGCATCA
GTTTTGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGG
TAAGTATTATAGCCTGTGGGAATACTGCCAGCTGGGACTGAGGA
CTGCGACGTAAGTCAAGGATGCTGGCATAATGGTTATATGCCGC
CCGTCTTGAACACCGGGGAAAA

(6) *Aureobasidium pullulans* AE14

AACAGGGATTGCCCTAGTACGGCGAGTGAAGCGGCAACAGCTC
AAATTTGAAAGCTAGCCTTCGGGTTCGCATTGTAATTTGTAGAG
GATGATTTGGGGAAGCCGCCTGTCTAAGTTCCTTGGAACAGGAC
GTCATAGAGGGTGAGAATCCCGTATGTGACAGGAAATGGCACC

CTATGTAAATCTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAG
CTCTAAATGGGAGGTAAATTTCTTCTAAAGCTAAATATTGGCGA
GAGACCGATAGCGCACAAAGTAGAGTGATCGAAAGATGAAAAGC
ACTTTGGAAAGAGAGTTAAAAAGCACGTGAAATTGTTGAAAGG
GAAGCGCTTGCAATCAGACTTGTTTAAACTGTTCCGGCCGGTCTT
CTGACCGGTTTACTCAGTTTGGACAGGCCAGCATCAGTTTCGGC
GGCCGGATAAAGGCTCTGGGAATGTGGCCTCCACTTCGGTGGAG
GTGTTATAGCCCAGGGTGTAATACGGCCAGCCGGGACTGAGGTC
CGCGCTTCGGCTAGGATGCTGGCGTAATGGTTGT

(7) *Saccharomyces cerevisiae* AE19

GGCATGCCTTAGTACGGCGAGTGAAGCGGCAAAAGCTCAAATT
TGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGG
CAACTTTGGGGCCGTTCTTGTCTATGTTCTTGGAACAGGACGT
CATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTG
TAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTA
AGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGAC
CGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTT
GAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGG
GCATTTGATCAGACATGGTGTGTTTGTGCCCTCTGCTCCTTGTGGG
TAGGGGAATCTCGCATTTCCTGGGCCAGCATCAGTTTTGGTGG
CAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAGTATTAT
AGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCGACGT
AAGTCAAGGATGCTGGCATAATGGTTATATGCCGCCCGTCTAAA
AACCGGGAACC

(8) *Saccharomyces cerevisiae* AE19 (2)

AAGGGGCAATGCCTTAGTACGGCGAGTGAAGCGGCAAAAGCTC
AAATTTGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGA
GAGGGCAACTTTGGGGCCGTTCCCTTGTCTATGTTCCCTTGGAACA
GGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGG
TTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGAATGC
AGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGC
GAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAA
GAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAA
GGGAAGGGCATTGATCAGACATGGTGTTTTGTGCCCTCTGCTC
CTTGTGGGTAGGGGAATCTCGCATTTCACTGGGCCAGCATCAGT
TTTGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTA
AGTATT

(9) *Saccharomyces cerevisiae* IL9

GCCTTAGTAACGGCGAAGTGAAGCGGCAAAAGCTCAAATTTGA
AATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAA
CTTTGGGGCCGTTCCCTTGTCTATGTTCCCTTGGAACAGGACGTCAT
AGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAA
AGTGCCTTCGAAGAGTCGAGTTGTTTGGAATGCAGCTCTAAGT
GGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCG
ATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGA
AAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGC
ATTTGATCAGACATGGTGTTTTGTGCCCTCTGCTCCTTGTGGGTA
GGGGAATCTCGCATTTCACTGGGCCAGCATCAGTTTTTGGTGGCA
GGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAGTATTATAG
CCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCGACGTAA

GTCAAGGATGCTGGCATAATGGTTATATGCCCCCGTCTTGAAA
AA

(10) *Saccharomyces cerevisiae* ML11

TCACGGATGCTTAGTACGGCGAGTGAAGCGGCAAAAGCTCAAA
TTTGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAG
GGCAACTTTGGGGCCGTTTCCTTGTCTATGTTTCCTTGGAACAGGA
CGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCT
TTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCT
CTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGA
GACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAAC
TTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGA
AGGGCATTGATCAGACATGGTGTGTTTGTGCCCTCTGCTCCTTGT
GGGTAGGGGAATCTCGCATTTCCTGGGCCAGCATCAGTTTGG
TGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAGTAT
TATAGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCGAC
GTAAGTCAAGGATGCTGGCATAATGGTTATATGCCGCCCCGT

(11) *Saccharomyces cf. cerevisiae paradoxus* IL33

GGCGGGATTGCCTTAGTAACGGCGAAGTGAAGCGGCAAAAGCT
CAAATTTGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGG
AGAGGGCAACTTTGGGGCCGTTTCCTTGTCTATGTTTCCTTGGAAC
AGGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCG
GTTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATG
CAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGG
CGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAA
AGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAA

AGGGAAGGGCATTGATCAGACATGGTGTTTTGTGCCCTCTGCT
CCTTGTGGGTAGGGGAATCTCGCATTTCCTGGGCCAGCATCAG
TTTTGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGT
AAGTATTATAGCCTG

(12) *Meyerozyma guilliermondii* KL5

GTATTGTCTTAGTAGCGGCGAGTGAAGCGGCAAAAGCTCAAATT
TGAAATCTGGCGCCTTCGGTGTCCGAGTTGTAATTTGAAGATTG
TAACCTTGGGGTTGGCTCTTGTCTATGTTTCTTGGAACAGGACGT
CACAGAGGGTGAGAATCCCGTGCGATGAGATGCCCAATTCTATG
TAAGGTGCTTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTA
AGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGAC
CGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTT
GAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAG
GGTTTGAGATCAGACTCGATATTTTGTGAGCCTTGCCTTCGTGGC
GGGGTGACCCGCAGCTTATCGGGCCAGCATCGGTTTGGGCGGTA
GGATAATGGCGTAGGAATGTGACTTTACTTCGGTGAAGTGTTAT
AGCCTGCGTTGATGCTGCCTGCCTAGACCGAGGACTGCGATTTT
ATCAAGGATGCTGGCATAATGATCCCAAACCGCCCGTCTTGAAC
CACGGACTT

(13) *Meyerozyma guilliermondii* IL11

GCCATTGCCTTAGTAGCGGCGAGTGAAGCGGCAAAAGCTCAAA
TTTGAAATCTGGCGCCTTCGGTGTCCGAGTTGTAATTTGAAGATT
GTAACCTTGGGGTTGGCTCTTGTCTATGTTTCTTGGAACAGGACG
TCACAGAGGGTGAGAATCCCGTGCGATGAGATGCCCAATTCTAT
GTAAGGTGCTTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCT

AAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGA
CCGATAGCGAACAAGTACAGTGATGGAAAGTGAAAAGAACATT
GAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAG
GGTTTGAGATCAGACTCGATATTTTGTGAGCCTTGCCTTCGTGGC
GGGGTGACCCGCAGCTTATCGGGCCAGCATCGGTTTGGGCGGTA
GGATAATGGCGTAGGAATGTGACTTTACTTCGGTGAAGTGTTAT
A

(14) *Meyerozyma guilliermondii* KL3

GTCATGCCTTAGTAGCGGCGAGTGAAGCGGCAAAAGCTCAAAT
TTGAAATCTGGCGCCTTCGGTGTCCGAGTTGTAATTTGAAGATT
GTAACCTTGGGGTTGGCTCTTGTCTATGTTTCTTGGAACAGGAC
GTCACAGAGGGTGAGAATCCCGTGCGATGAGATGCCCAATTCTA
TGTAAGGTGCTTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTC
TAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAG
ACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAAGT
TTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAA
GGGTTTGAGATCAGACTCGATATTTTGTGAGCCTTGCCTTCGTG
GCGGGGTGACCCGCAGCTTATCGGGCCAGCATCGGTTTGGGCGG
TAGGATAATGGCGTAGGAATGTGACTTTACTTCGGTGAAGTGTT
ATAGCCTGCGTTGATGCTGCCTGCCTAGACCGAGGACTGCGATT
TTATCAAGGATGCTGGCATAATGATCCCAAACCGCCCGT

(15) *Meyerozyma guilliermondii* ML4

AAAGGCATTGCTTAGTAGCGGCGAGTGAGCGGCAAAAGCTCAA
ATTTGAAATCTGGCGCCTTCGGTGTCCGAGTTGTAATTTGAAGA
TTGTAACCTTGGGGTTGGCTCTTGTCTATGTTTCTTGGAACAGGA

CGTCACAGAGGGTGAGAATCCCGTGCGATGAGATGCCCAATTCT
ATGTAAGGTGCTTTCTGAAGAGTCGAGTTGTTTGGGAATGCAGCT
CTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGA
GACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAA
CTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGG
AAGGGTTTGAGATCAGACTCGATATTTTGTGAGCCTTGCCTTCGT
GGCGGGGTGACCCGCAGCTTATCGGGCCAGCATCGGTTTGGGCG
GTAGGATAATGGCGTAGGAATGTGACTTTACTTCGGTGAAGTGT
TATAGCCTGCGTTGATGCTGCCTGCCTAGACCGAGGACTGCGAT
TTTATCAAGGATGCTGGCATAATGATCCCAAACCGCCCGTCTTG
AAACACGGGGACC

(16) *Saccharomyces cerevisiae* GE1

GCGGGCATTGCCTTAGTACGGCGAGTGAAGCGGCAAAAGCTCA
AATTTGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAG
AGGGCAACTTTGGGGCCGTTCCCTTGTCTATGTTCCCTTGAACAG
GACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGT
TCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCA
GCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCG
AGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAG
AACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAG
GGAAGGGCATTGATCAGACATGGTGTTTTGTGCCCTCTGCTCCT
TGTGGGTAGGGGAATCTCGCATTTCACTGGGCCAGCATCAGTTT
TGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAG
TATTATAGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGC
GACGTAAGTCAAGGATGCTGGCATAATGGTTATATGCCGCCCCGT
CTGAAAAAA

(17) *Aureobasidium pullulans* GE11

ATCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGA
AAGCTAGCCTTCGGGTTTCGCATTGTAATTTGTAGAGGATGATT
TGGGGAAGCCGCCTGTCTAAGTTCCTTGGAACAGGACGTCATAG
AGGGTGAGAATCCCGTATGTGACAGGAAATGGCACCCCTATGTA
AATCTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAA
TGGGAGGTAAATTTCTTCTAAAGCTAAATATTGGCGAGAGACCG
ATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGG
AAAGAGAGTTAAAAAGCACGTGAAATTGTTGAAAGGGAAGCGC
TTGCAATCAGACTTGTTTAAACTGTTTCGGCCGGTCTTCTGACCGG
TTTACTCAGTTTGGACAGGCCAGCATCAGTTTCGGCGGCCGGAT
AAAGGCTCTGGGAATGTGGCCTCCACTTCGGTGGAGGTGTTATA
GCCCAGGGTGTAATACGGCCAGCCGGGACTGAGGTCCGCGCTTC
GGCTAGGATGCTGGCGTAATGGT

(18) *Aureobasidium pullulans* GE16

AGAGGGATTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTC
AAATTTGAAAGCTAGCCTTCGGGTTTCGCATTGTAATTTGTAGA
GGATGATTTGGGGAAGCCGCCTGTCTAAGTTCCTTGGAACAGGA
CGTCATAGAGGGTGAGAATCCCGTATGTGACAGGAAATGGCAC
CCTATGTAAATCTCCTTCGACGAGTCGAGTTGTTTGGGAATGCA
GCTCTAAATGGGAGGTAAATTTCTTCTAAAGCTAAATATTGGCG
AGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAG
CACTTTGGAAGAGAGTTAAAAAGCACGTGAAATTGTTGAAAG
GGAAGCGCTTGCAATCAGACTTGTTTAAACTGTTTCGGCCGGTCT
TCTGACCGGTTTACTCAGTTTGGACAGGCCAGCATCAGTTTCGG
CGGCCGGATAAAGGCTCTGGGAATGTGGCCTCCACTTCGGTGGA

GGTGTATAGCCCAGGGTGTAAATACGGCCAGCCGGGACTGAGG
TCCGCGCTTCGGCTAGGATGCTGGCGTAATGGTTGTAAGCGACC
CG

(19) *Aureobasidium pullulans* GE17

TTAAGGGCATTGCCTAGTACGGCGAGTGAAGCGGCAACAGCTC
AAATTTGAAAGCTAGCCTTCGGGTTTCGCATTGTAATTTGTAGAG
GATGATTTGGGGAAGCCGCTGTCTAAGTTCCTTGGAACAGGAC
GTCATAGAGGGTGAGAATCCCGTATGTGACAGGAAATGGCACC
CTATGTAAATCTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAG
CTCTAAATGGGAGGTAAATTTCTTCTAAAGCTAAATATTGGCGA
GAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGC
ACTTTGGAAGAGAGAGTTAAAAAGCACGTGAAATTGTTGAAAGG
GAAGCGCTTGCAATCAGACTTGTTTAAACTGTTTCGGCCGGTCTT
CTGACCGGTTTACTCAGTTTGGACAGGCCAGCATCAGTTTCGGC
GGCCGGATAAAGGCTCTGGGAATGTGGCCTCCACTTCGGTGGAG
GTGTTATAGCCCAGGGTGTAAATACGGCCAGCCGGGACTGAGGTC
CGCGCTTCGGCTAGGATGCTGGCGTAATGGTTGTAAGC

(20) *Saccharomyces cerevisiae* GE18

GCCTTAGTAACGGCGAAGTGAAGCGGCAAAAGCTCAAATTTGA
AATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAA
CTTTGGGGCCGTTTCCTTGTCTATGTTTCCTTGGAACAGGACGTCAT
AGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAA
AGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGT
GGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCG
ATAGCGAACAAGTACAGTGATGGAAGATGAAAAGAACTTTGA

AAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGC
ATTTGATCAGACATGGTGTGTTTTGTGCCCTCTGCTCCTTGTGGGTA
GGGGAATCTCGCATTTCACTGGGCCAGCATCAGTTTTGGTGGA
GGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAGTATTATAG
CCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCGACGTAA
GTCAAGGATGCTGGCATAATGGTTATATGCCGCCCCGTCTT

(21) *Rhodotorula mucilaginosa* IL1

GTTCCCCTAGTAGCGGCGAGCGAAGCGGGAAGAGCTCAAATTT
ATAATCTGGCACCTTCGGTGTCCGAGTTGTAATCTCTAGAAATG
TTTTCCGCGTTGGACCGCACACAAGTCTGTTGGAATACAGCGGC
ATAGTGGTGAGACCCCCGTATATGGTGCGGACGCCAGCGCTTT
GTGATACATTTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCA
AATTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGA
CCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAAGCACTT
TGAAAAGAGAGTTAACAGTACGTGAAATTGTTGGAAGGGAAAC
GCTTGAAGTCAGACTTGCTTGCCGAGCAATCGGTTTGCAGGCCA
GCATCAGTTTTCCGGGATGGATAATGGTAGAGAGAAGGTAGCA
GTTTCGGCTGTGTTATAGCTCTCTGCTGGATACATCTTGGGGGAC
TGAGGAACGCAGTGTGCCTTTGGCGGGGGTTTCGACCTCTTCAC
ACTTAGGATGCTGGTGAATGGCTTTAAACGACCCGTCT

(22) *Saccharomyces cf. cerevisiae paradoxus* IL7

GGGGCATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCA
AATTTGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAG
AGGGCAACTTTGGGGCCGTTTCCTTGTCTATGTTTCCTTGAACAG
GACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGT

TCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCA
GCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCG
AGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAG
AACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAG
GGAAGGGCATTGATCAGACATGGTGTTTTGTGCCCTCTGCTCCT
TGTGGGTAGGGGAATCTCGCATTTCACTGGGCCAGCATCAGTTT
TGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAG
TATTATAGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGC
GACGTAAGTCAAGGATGCTGGCATAATGGTTATATGCCGCCCCGT
CTGA

(23) *Saccharomyces cerevisiae* KL4

AACGGGGCATGCCTTAGTACGGCGAGTGAAGCGGCAAAAGCTC
AAATTTGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGA
GAGGGCAACTTTGGGGCCGTTCTTGTCTATGTTCTTGAACA
GGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGG
TTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGC
AGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGC
GAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAA
GAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAA
GGGAAGGGCATTGATCAGACATGGTGTTTTGTGCCCTCTGCTC
CTTGTGGGTAGGGGAATCTCGCATTTCACTGGGCCAGCATCAGT
TTTGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTA
AGTATTATAGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACT
GCGACGTAAGTCAAGGATGCTGGCATAATGGTTATATGCCGCCC
GTCTAAAAACCGGGACCAAAAA

(24) *Saccharomyces cf. cerevisiae paradoxus* KL6

GCAATGCCTTAGTAACGGCGAAGTGAAGCGGCAAAAGCTCAAA
TTTGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAG
GGCAACTTTGGGGCCGTTCCCTTGTCTATGTTCCCTTGGAACAGGA
CGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCT
TTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCT
CTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGA
GACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAAC
TTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGA
AGGGCATTGATCAGACATGGTGTGTTTGTGCCCTCTGCTCCTTGT
GGGTAGGGGAATCTCGCATTTCCTGGGCCAGCATCAGTTTTGG
TGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAGTAT
TATAGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCGAC
GTAAGTCAAGGATGCTGGCATAATGGTTA

(25) *Saccharomyces cerevisiae* ML3

CACAACGGGCATGCTTAGTACGGCGAGTGAGCGGCAAAAGCTC
AAATTTGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGA
GAGGGCAACTTTGGGGCCGTTCCCTTGTCTATGTTCCCTTGGAACA
GGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGG
TTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGC
AGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGC
GAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAA
GAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAA
GGGAAGGGCATTGATCAGACATGGTGTGTTTGTGCCCTCTGCTC
CTTGTGGGTAGGGGAATCTCGCATTTCCTGGGCCAGCATCAGT
TTTGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTA

AGTATTATAGCCTGTGGAATACTGCCAGCTGGGACTGAGGACT
GCGACGTAAGTCAAGGATGCTGGCATAATGGTTATATGCCGCCC
GTCTTGAAACGCGAAACCACAACA

(26) *Meyerozyma guilliermondii* KL22

CAGGGATTGCCTTAGTAGCGGCGAGTGAAGCGGCAAAAGCTCA
AATTTGAAATCTGGCGCCTTCGGTGTCCGAGTTGTAATTTGAAG
ATTGTAACCTTGGGGTTGGCTCTTGTCTATGTTTCTTGGAACAGG
ACGTCACAGAGGGTGAGAATCCCGTGCGATGAGATGCCCAATT
CTATGTAAGGTGCTTTCGAAGAGTCGAGTTGTTTGGGAATGCAG
CTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGA
GAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGA
ACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGG
GAAGGGTTTGAGATCAGACTCGATATTTTGTGAGCCTTGCCTTC
GTGGCGGGGTGACCCGCAGCTTATCGGGCCAGCATCGGTTTGGG
CGGTAGGATAATGGCGTAGGAATGTGACTTTACTTCGGTGAAGT
GTTATAGCCTGCGTTGATGCTGCCTGCCTAGACCGAGGACTGCG
ATTTTATCAAGGATGCTGGCATAATGATCCCAAACCGCCCGTCT
TGAAACACGGA

(27) *Rhodotorula mucilaginosa* SE1

TAAGGGCATTCCCTAGTAGCGGCGAGCGAGCGGGAAGAGCTCA
AATTTATAATCTGGCACCTTCGGTGTCCGAGTTGTAATCTCTAGA
AATGTTTTCCGCGTTGGACCGCACACAAGTCTGTTGGAATACAG
CGGCATAGTGGTGAGACCCCGTATATGGTGCGGACGCCACG
GCTTTGTGATACATTTTCGAAGAGTCGAGTTGTTTGGGAATGCA
GCTCAAATTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCG

AGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAA
GCACTTTGGAAAGAGAGTTAACAGTACGTGAAATTGTTGGAAG
GGAAACGCTTGAAGTCAGACTTGCTTGCCGAGCAATCGGTTTGC
AGGCCAGCATCAGTTTTCCGGGATGGATAATGGTAGAGAGAAG
GTAGCAGTTTCGGCTGTGTTATAGCTCTCTGCTGGATACATCTTG
GGGGACTGAGGAACGCAGTGTGCCTTTGGCGGGGGTTTCGACCT
CTTCACACTTAGGATGCTGGTGGAATGGCTTT

(28) *Wickerhamomyces anomalus* SE4

GCATTGCCTCAGTACGGCGAGTGAAGCGGCAAAAGCTCAAATTT
GAAATCTAGCACCTTCGGTGTTCGAGTTGTAATTTGAAGATGGT
AACCTTGGGTTTGGCTCTTGTCTATGTTTCCTTGGAACAGGACGTC
ATAGAGGGTGAGAATCCCGTCTGATGAGATGCCCATTCCTATGT
AAGGTGCTATCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAA
GTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACC
GATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTG
AAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGG
CATTAGATCAGACTTGGTGTTTTACGATTATCTTCTTCTTCTTGAG
TTGTGCACTCGTATTTCACTGGGCCAGCATCGATTCCGGATGGCA
AGATAATGGCAGTTGAATGTGGCTTCACTTCGGTGGAGTGTTAT
AGCTTCTGCTGATATTGCCTGTCTGGATCGAGGGCTGCGTCTTTT
GACTAGGATGCTGGCGTAATGATCTAATGCCGCCCGTCCAATT

(29) *Aureobasidium pullulans* SE5

ATCCTGGCATTGCCTAGTACGGCGAGTGAGCGGCAACAGCTCAA
ATTTGAAAGCTAGCCTTCGGGTTCGCATTGTAATTTGTAGAGGA
TGATTTGGGGAAGCCGCCTGTCTAAGTTCCTTGGAACAGGACGT

CATAGAGGGTGAGAATCCCGTATGTGACAGGAAATGGCACCCCT
ATGTAAATCTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCT
CTAAATGGGAGGTAAATTTCTTCTAAAGCTAAATATTGGCGAGA
GACCGATAGCGCACAAAGTAGAGTGATCGAAAGATGAAAAGCAC
TTTGAAAGAGAGTTAAAAAGCACGTGAAATTGTTGAAAGGGA
AGCGCTTGCAATCAGACTTGTTTAAACTGTTTCGGCCGGTCTTCTG
ACCGGTTTACTCAGTTTGGACAGGCCAGCATCAGTTTCGGCGGC
CGGATAAAGGCTCTGGGAATGTGGCCTCCACTTCGGTGGAGGTG
TTATAGCCCAGGGTGTAATACGGCCAGCCGGGACTGAGGTCCGC
GCTTCGGCTAGGATGCTGGCGTAATGGTTGTA

(30) *Saccharomyces cerevisiae* SE11

CACGGGCATTGCCTTAGTACGGCGAGTGAAGCGGCAAAAGCTC
AAATTTGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGA
GAGGGCAACTTTGGGGCCGTTCCTTGTCTATGTTTCCTTGGAACA
GGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGG
TTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGC
AGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGC
GAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAA
GAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAA
GGGAAGGGCATTGATCAGACATGGTGTTTTGTGCCCTCTGCTC
CTTGTTGGGTAGGGGAATCTCGCATTTCACTGGGCCAGCATCAGT
TTTGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTA
AGTATTATAGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACT
GCGACGTAAGTCAAGGATGCTGGCATAATGGTTATATGCCGCCC
GTCTGAAAAACGCG

(31) *Saccharomyces cerevisiae* SE18

GGCATGCCTTAGTACGGCGAGTGAAGCGGCAAAAGCTCAAATT
TGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGG
CAACTTTGGGGCCGTTCCCTTGTCTATGTTCCCTTGGAACAGGACGT
CATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTG
TAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTA
AGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGAC
CGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTT
GAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGG
GCATTTGATCACACATGGTGTGTTTGTGCCCTCTGCTCCTTGTGGG
TAGGGGAATCTCGCATTTCCTGAGGCGAGCATCAGTTTTGGTGG
CAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAGTATTAT
AGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCGACGT
AAGTCAAGGATGCTGGCATAATGGTTATATGCCGCC

(32) *Aureobasidium pullulans* SE18 (2)

GGTATTGCCCTAGTACGGCGAGTGAAGCGGCAACAGCTCAAATT
TGAAAGCTAGCCTTCGGGTTTCGCATTGTAATTTGTAGAGGATGA
TTTGGGGAAGCCGCCTGTCTAAGTTCCTTGGAACAGGACGTCAT
AGAGGGTGAGAATCCCGTATGTGACAGGAAATGGCACCCCTATG
TAAATCTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTA
AATGGGAGGTAAATTTCTTCTAAAGCTAAATATTGGCGAGAGAC
CGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTT
GGAAAGAGAGTTAAAAAGCACGTGAAATTGTTGAAAGGGAAGC
GCTTGCAATCAGACTTGTTTAAACTGTTTCGGCCGGTCTTCTGACC
GGTTTACTCAGTTTGGACAGGCCAGCATCAGTTTCGGCGGCCGG
ATAAAGGCTCTGGGAATGTGGCCTCCACTTCGGTGGAGGTGTTA

TAGCCCAGGGTGTAATACGGCCAGCCGGGACTGAGGTCCGCGC
TTCGGCTAGGATGCTGGCGTAATGGTTGTAAGCGACCCGT

(33) *Saccharomyces cf. cerevisiae paradoxus* SE19

CTAACGGCGAAGTGAAGCGGCCAAAAGCTCAAATTTGAAATCTG
GTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGG
GGCCGTTCCCTTGTCTATGTTCCCTTGGAACAGGACGTCATAGAGG
GTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAAAGTGC
CTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTG
GTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCG
AACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAG
AGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTGTA
TCAGACATGGTGTTTTGTGCCCTCTGCTCCTTGTGGGTAGGGGA
ATCTCGCATTTCACTGGGCCAGCATCAGTTTGGTGGCAGGATA
AATCCATAGGAATGTAGCTTGCCCTCGGTAAGTATTATAGCCTGT
GGGAATACTGCCAGCTGGGACTGAGGACTGCGACGTAAGTCAA
GGATGCTGGCATAATGGTTATATGCCGCCCGTCTA

(34) *Saccharomyces cerevisiae* SE19 (2)

TGCTTAGTACGGCGAGTGAGCGGCCAAAAGCTCAAATTTGAAATC
TGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTT
GGGGCCGTTCCCTTGTCTATGTTCCCTTGGAACAGGACGTCATAGA
GGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAAAGT
GCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGG
TGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAG
CGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAG
AGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATT

GATCAGACATGGTGTTTTGTGCCCTCTGCTCCTTGTGGGTAGGG
GAATCTCGCATTTCACTGGGCCAGCATCAGTTTTGGTGGCAGGA
TAAATCCATAGGAATGTAGCTTGCCTCGGTAAGTATTATAGCCT
GTGGGAATACTGCCAGCTGGGACTGAGGACTGCGACGTAAGTC
AAGGATGCTGGCATAATGGTTATATGCCGCCCGTCTGAAAAACC
GGGACCAAAAAAACCC

(35) *Saccharomyces cerevisiae* SE23

GGGGATGCCTTAGTACGGCGAGTGAAGCGGCAAAAGCTCAAAT
TTGAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGG
GCAACTTTGGGGCCGTTTCCTTGTCTATGTTTCCTTGGAACAGGAC
GTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTT
TGTAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTC
TAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAG
ACCGATAGCGAACAAGTACAGTGATGGAAGATGAAAAGAAGT
TTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAA
GGGCATTTGATCAGACATGGTGTTTTGTGCCCTCTGCTCCTTGTG
GGTAGGGGAATCTCGCATTTCACTGGGCCAGCATCAGTTTTGGT
GGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAGTATT
ATAGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCGAC
GTAAGTCAAGGATGCTGGCATAATGGTTATATGCCGCCCGTCTA
AAAACCGGGAACAAAAAT

(36) *Saccharomyces cerevisiae* ML5

GGGATGCCTTAGTACGGCGAGTGAGCGGCAAAAGCTCAAATTT
GAAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGC
AACTTTGGGGCCGTTTCCTTGTCTATGTTTCCTTGGAACAGGACGTC

ATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGT
AAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAA
GTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACC
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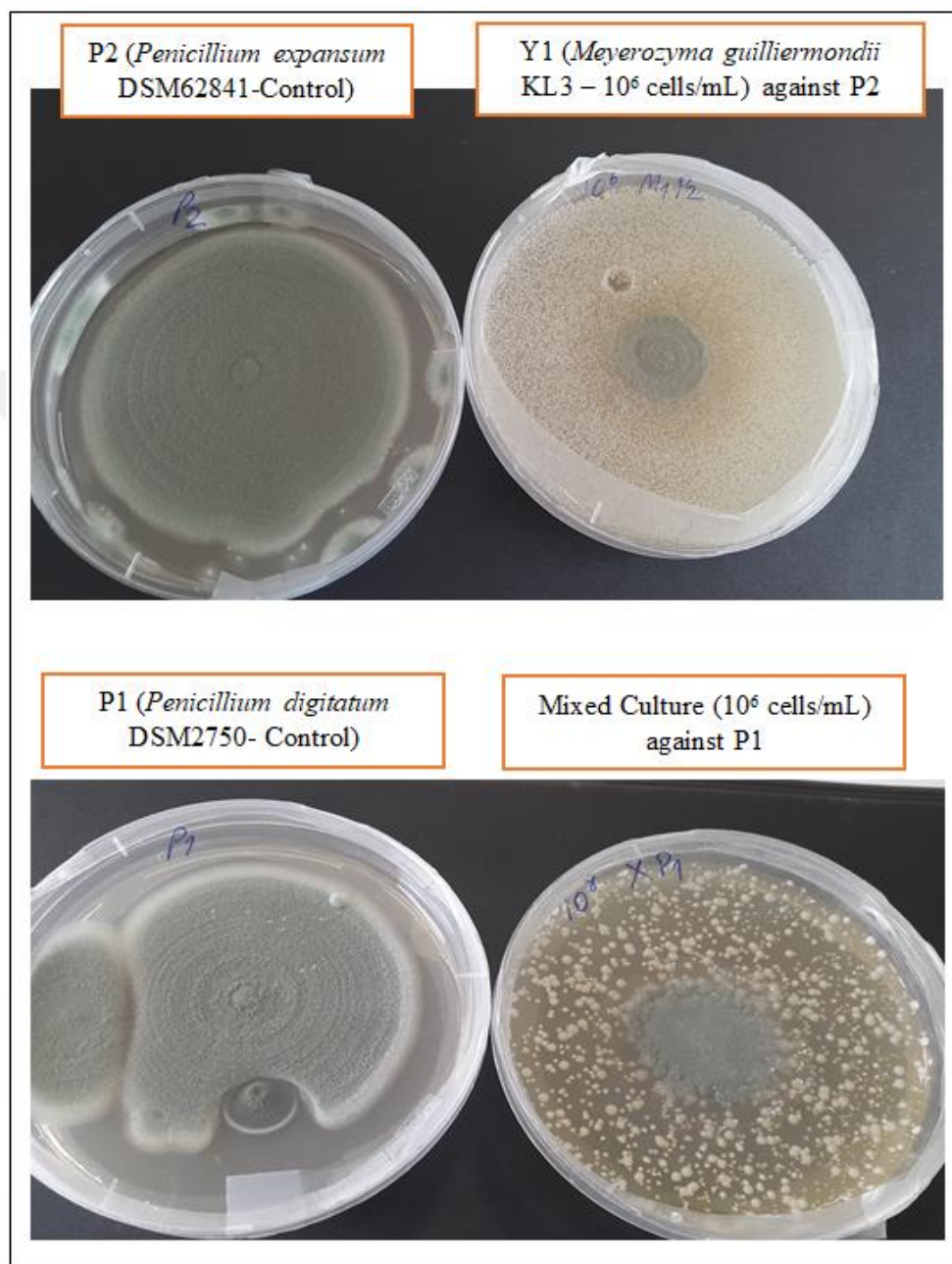
(37) *Saccharomyces cerevisiae* KL25

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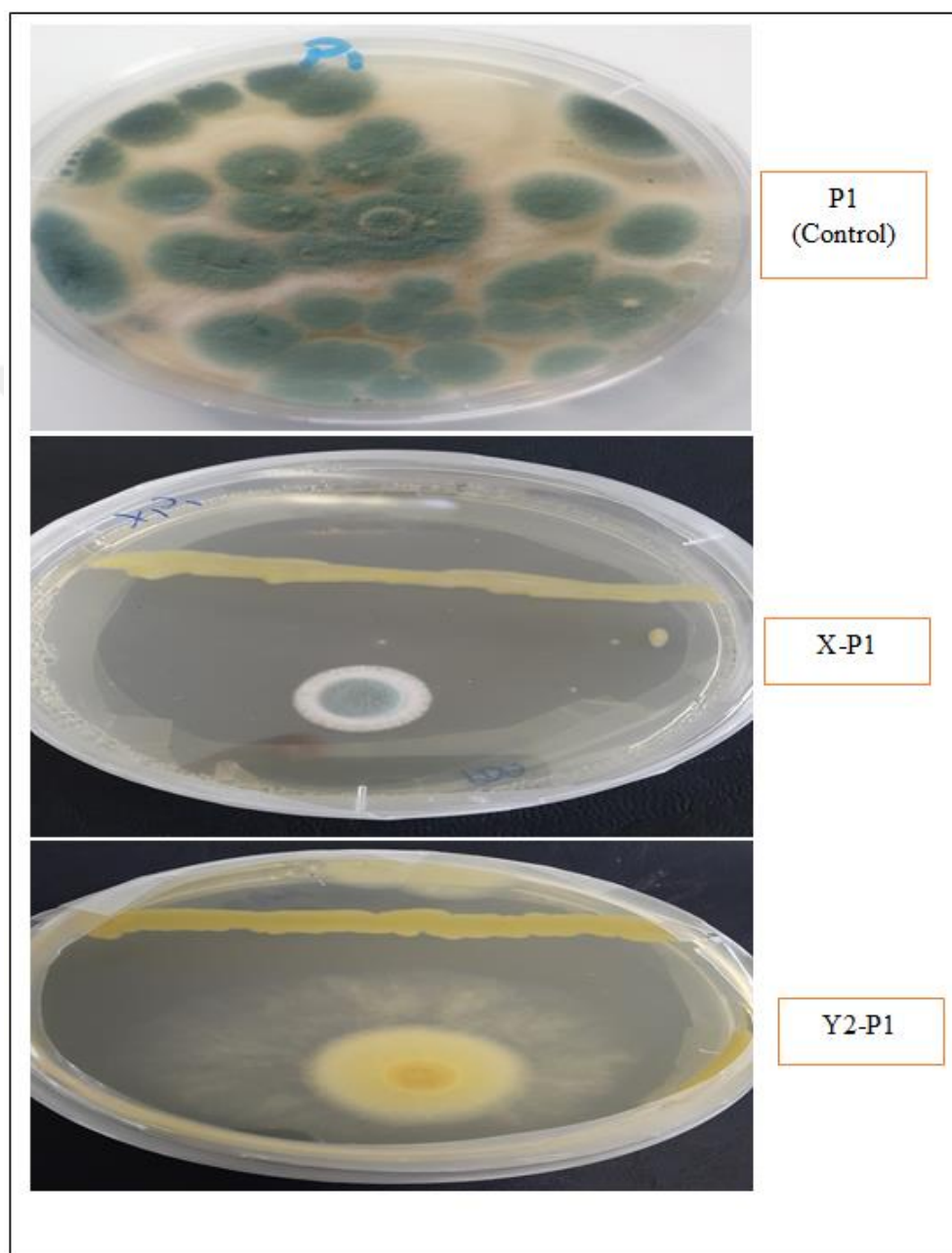
(38) *Saccharomyces cerevisiae* KL28

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Appendix 2. Some photos from *in vitro* % inhibition analyses



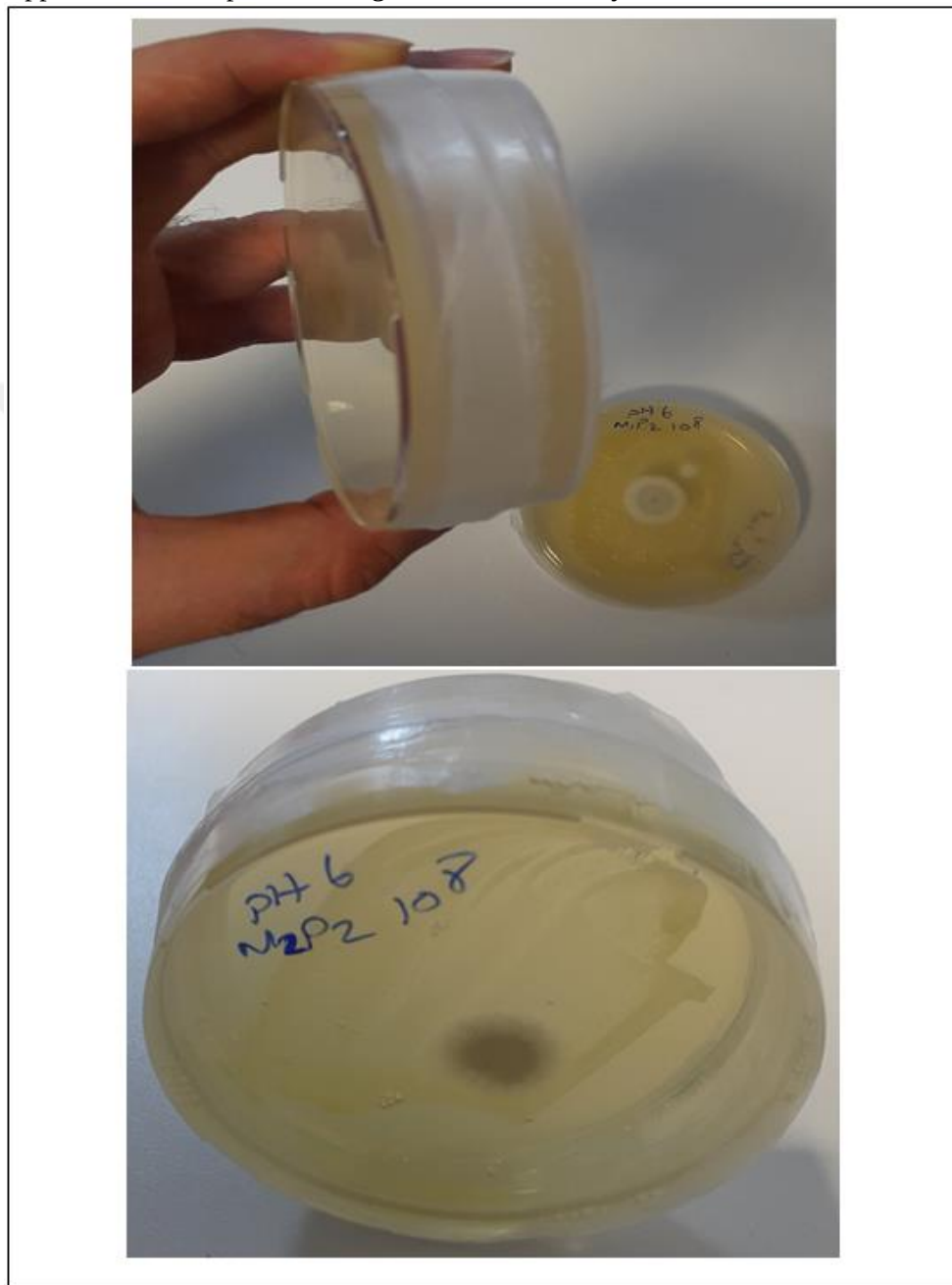
Appendix 3. Some images from *in vitro* dual culture assay



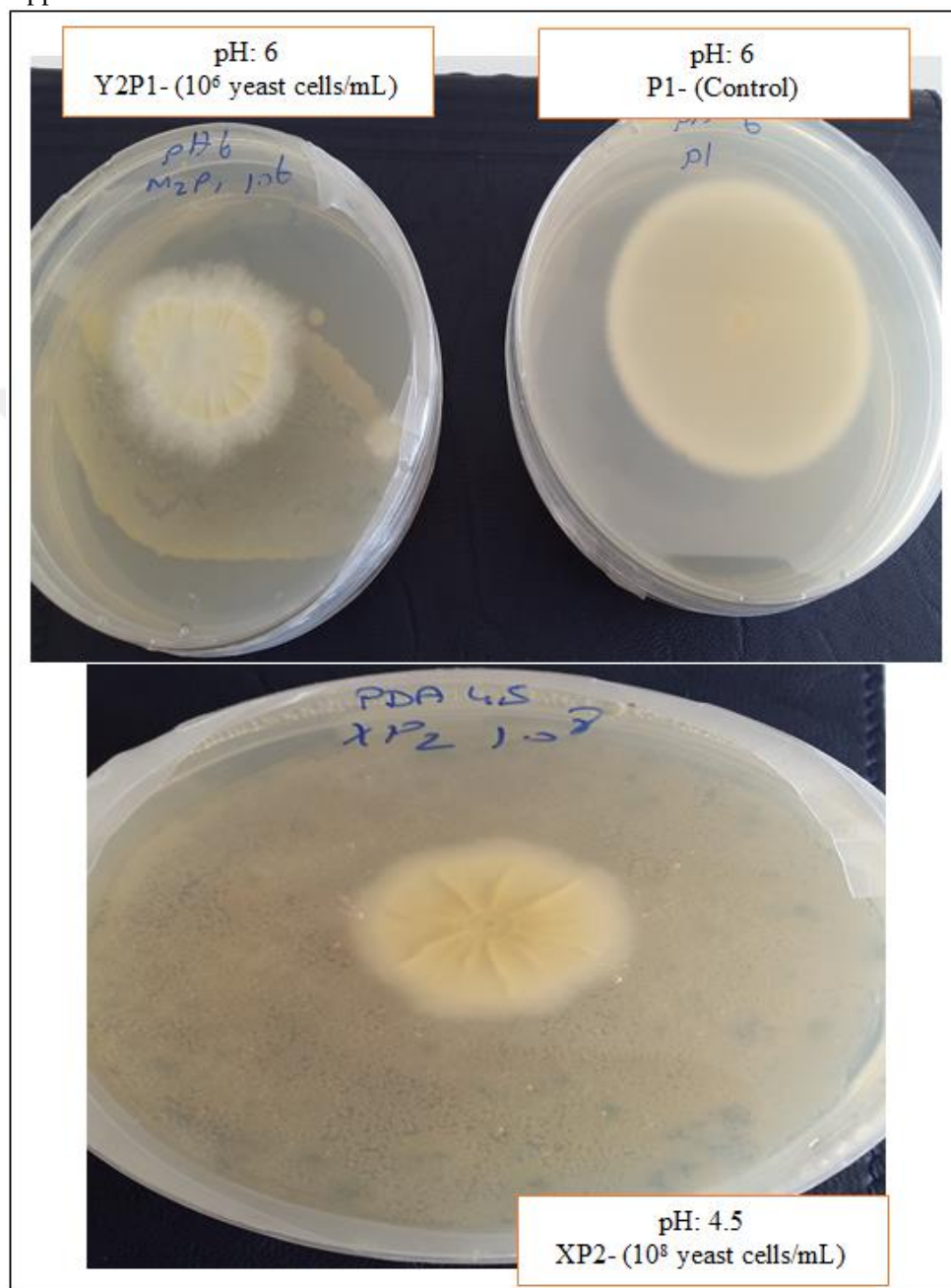
Appendix 4. Some photos during the evaluation of yeasts on spore germination of fungal pathogens



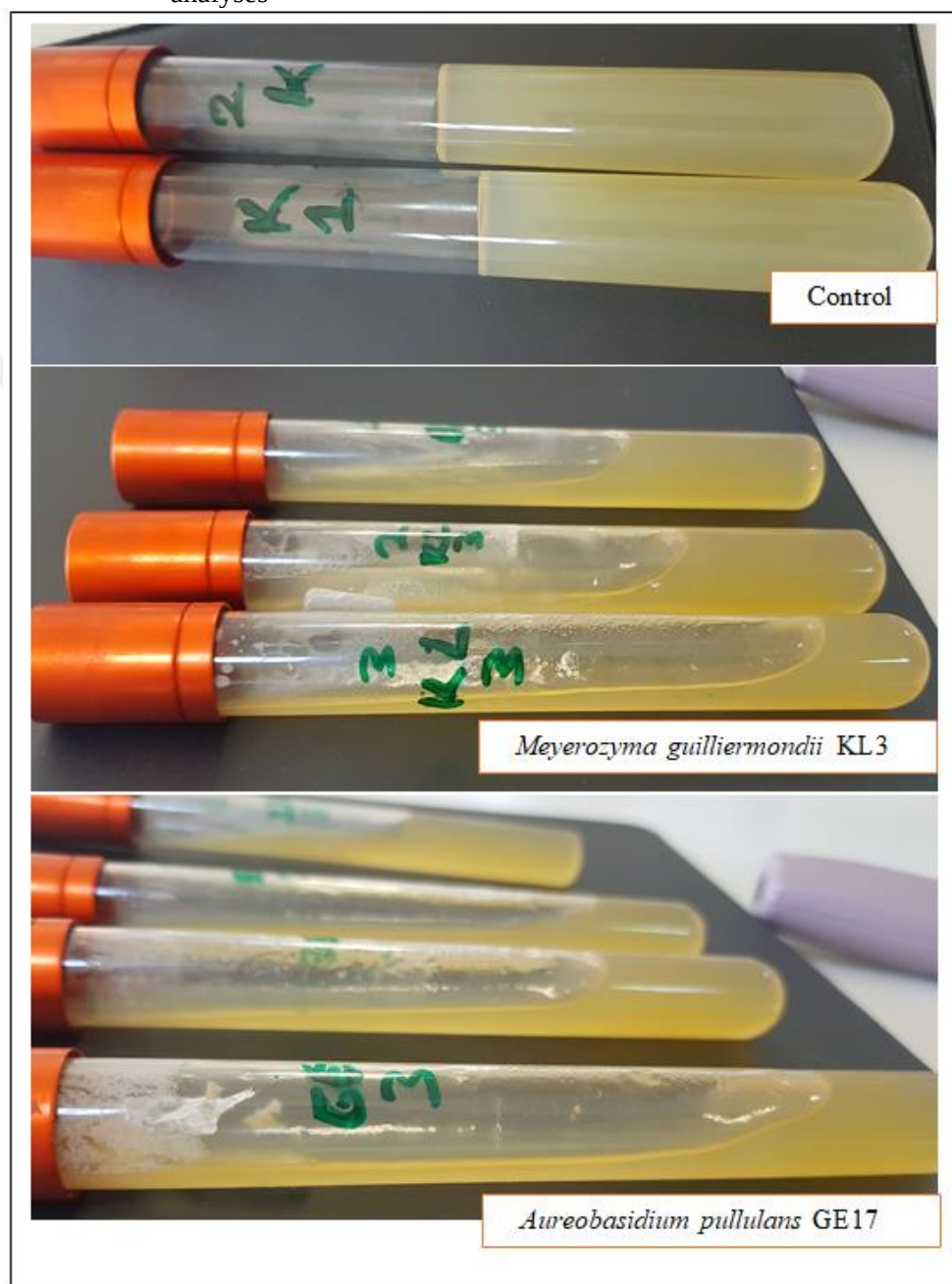
Appendix 5. Some photos during *in vitro* VOCs analyses



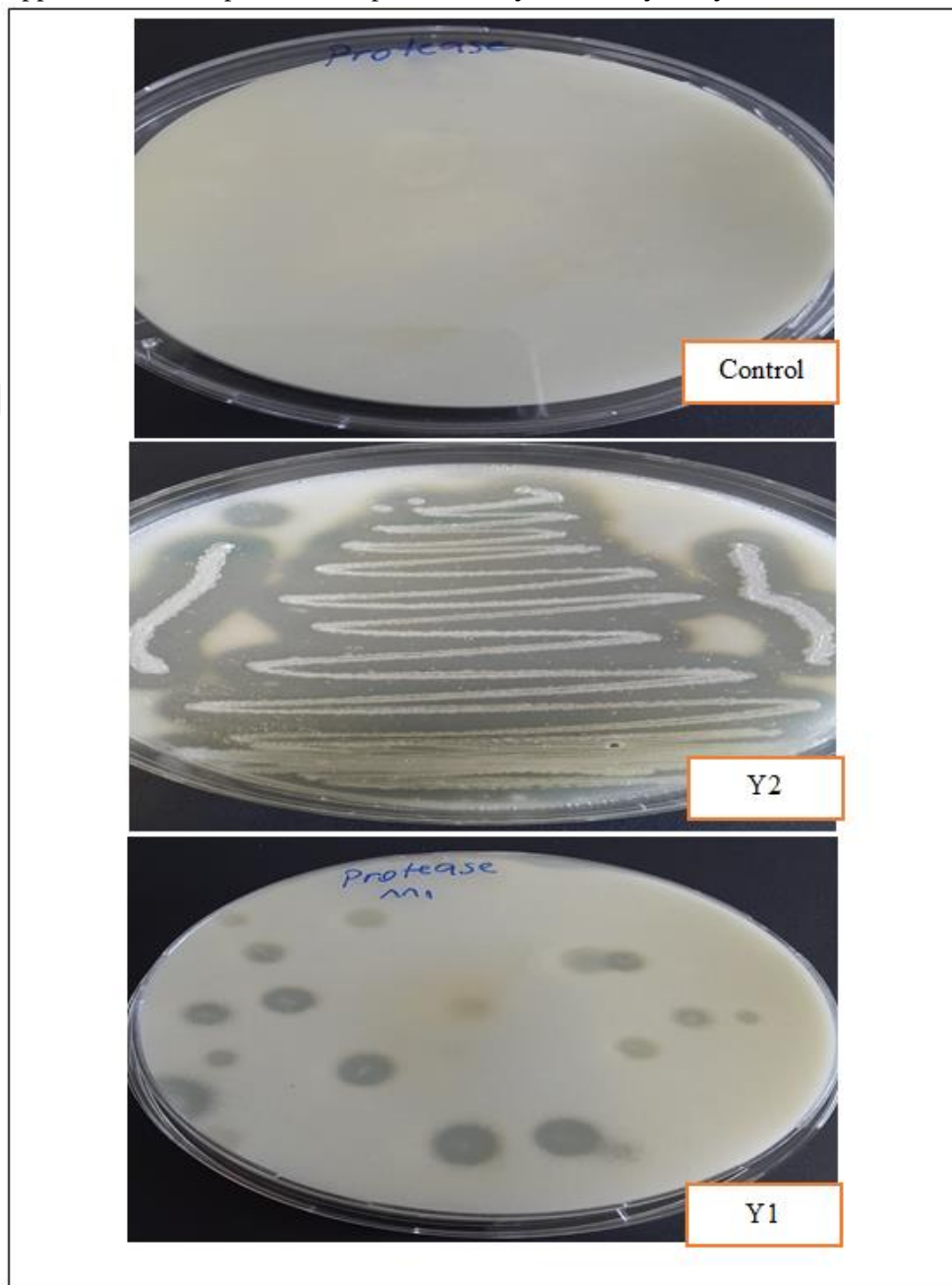
Appendix 5. Continued



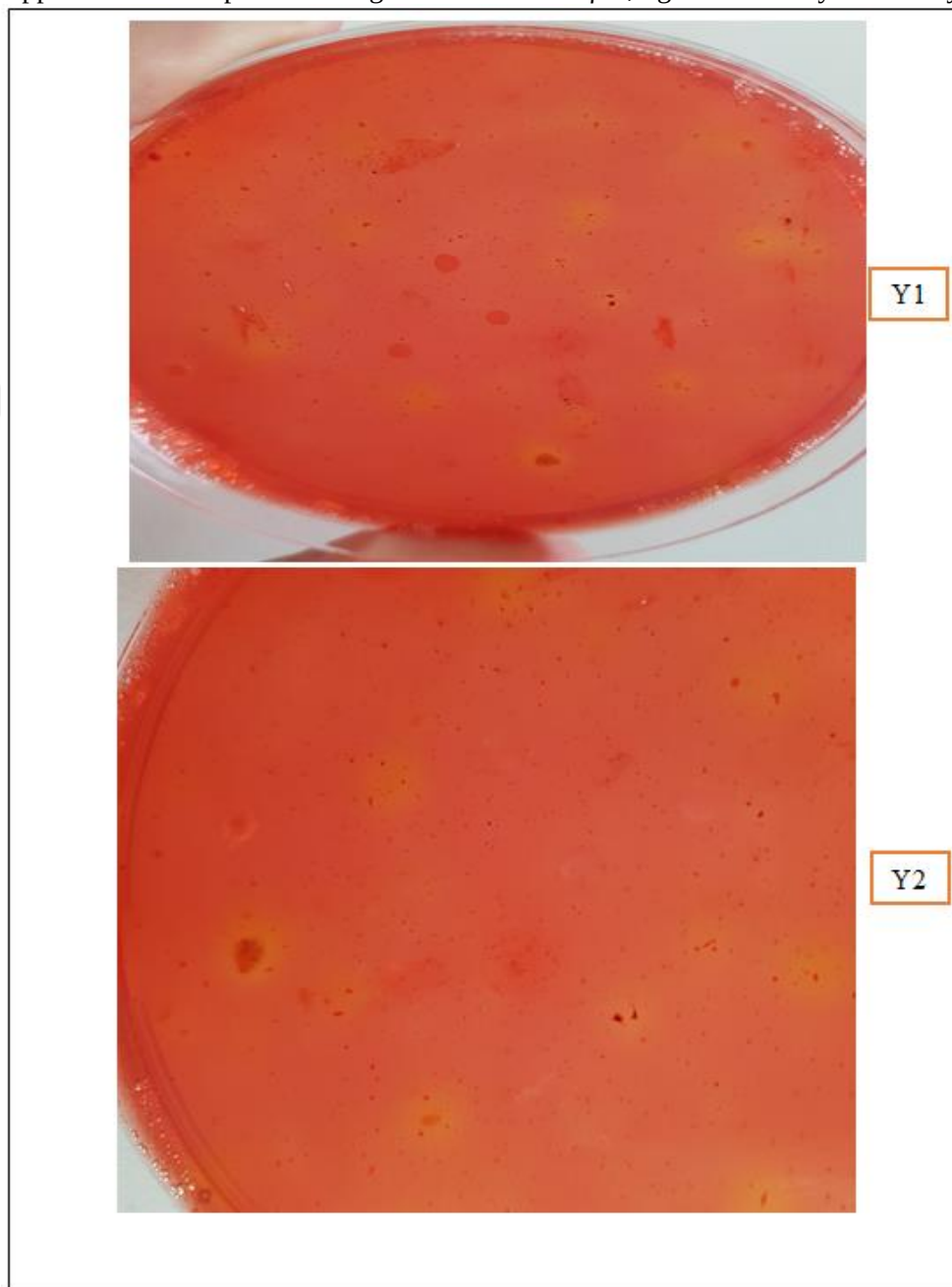
Appendix 6. Some photos during determination of gelatinase enzyme activity analyses



Appendix 7. Some photos from protease enzyme activity analyses



Appendix 8. Some photos during determination of β -1,3 glucanase enzyme activity



Appendix 9. Some images from the analysis of wound size colonization on apples



Appendix 10. Some images from the analysis of wound size colonization on lemons



Appendix 11. Some photos from *in vivo* VOCs analyses of apples



Appendix 12. Some photos from *in vivo* VOCs analyses of lemon



Appendix 13. Some images from experimental design of apples for storage at ambient condition



Appendix 13. Continued



Appendix 13. Continued



Appendix 14. Some images from experimental design of lemons for storage at ambient condition



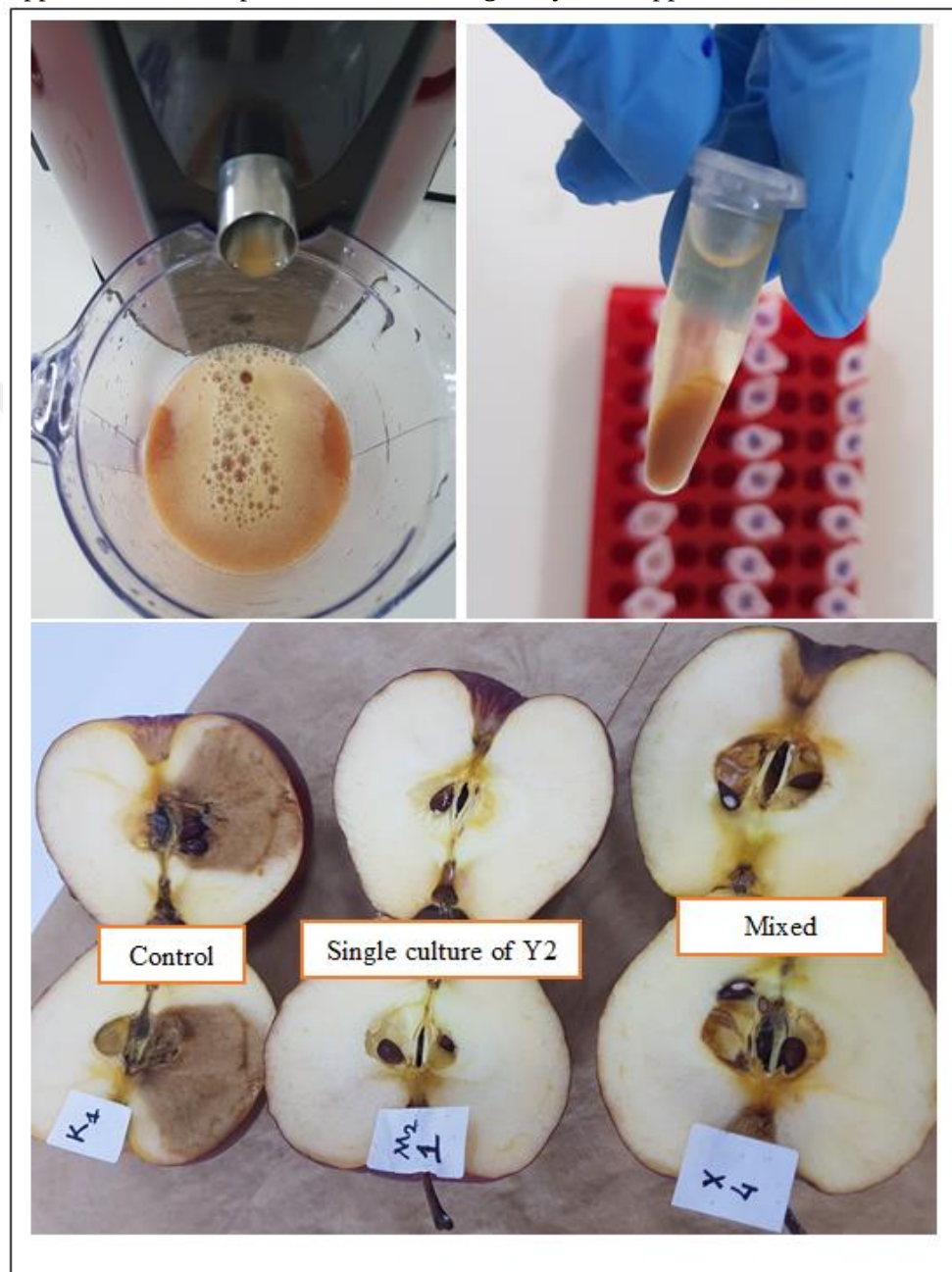
Appendix 14. Continued



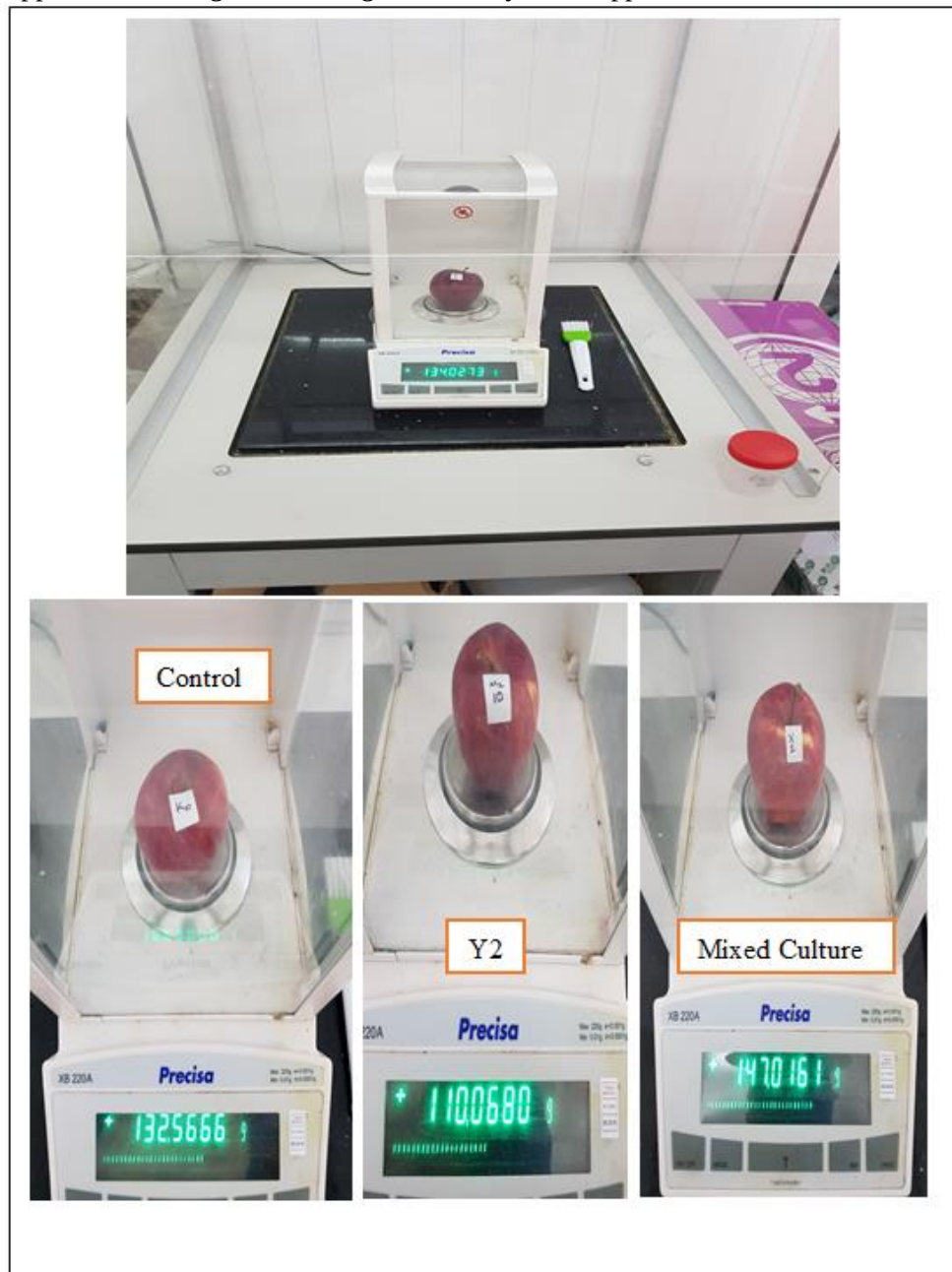
Appendix 14. Continued



Appendix 15. Some photos from browning analyses of apples



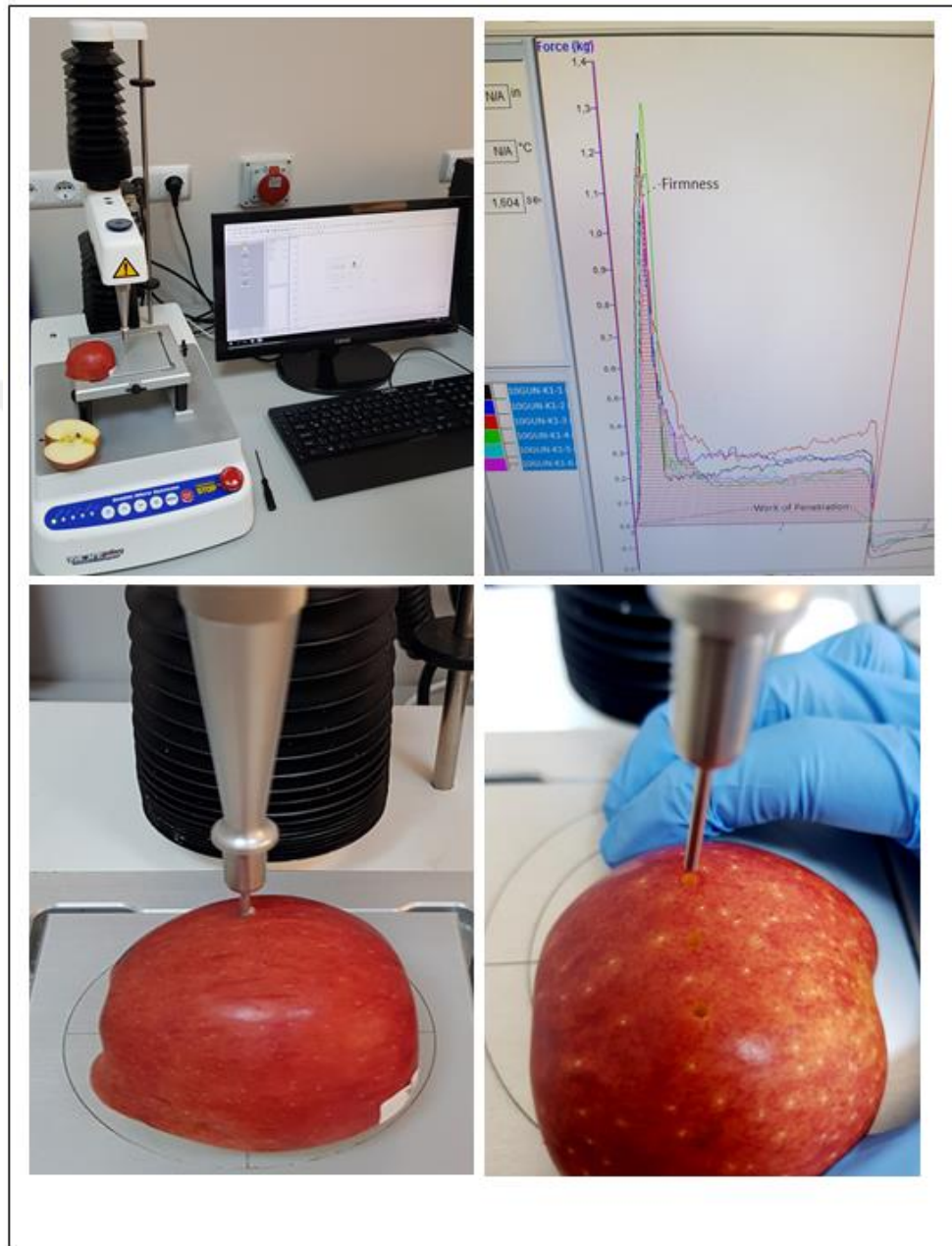
Appendix 16. Images from weight loss analyses on apples



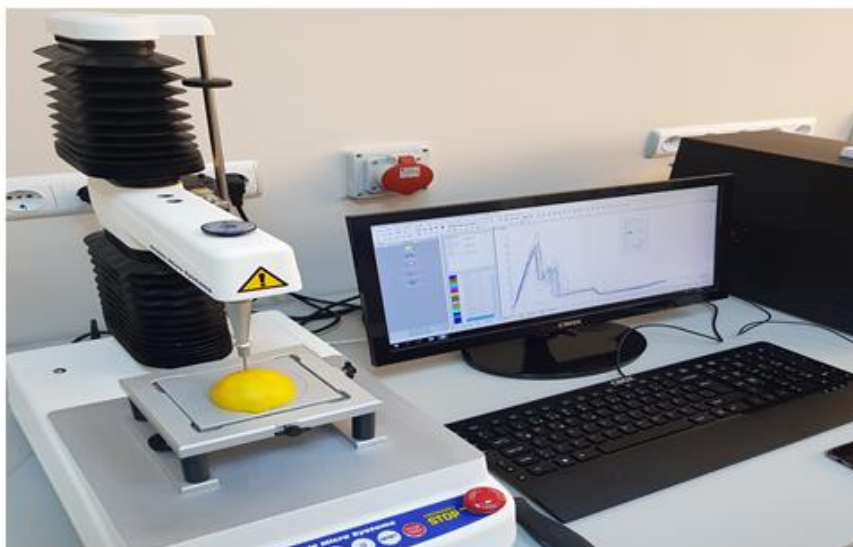
Appendix 17. Images from weight loss analyses on lemons



Appendix 18. Images during firmness analyses on cv. Starking Delicious apples



Appendix 19. Images during firmness analyses on cv. Kutdiken lemons



Appendix 20. Description of the cold room. a) humidity sensory, b) CO₂ sensor, c) ethylene sensor, d) Cezio humidifier device, e) Digital control panel f) overall view of cold room



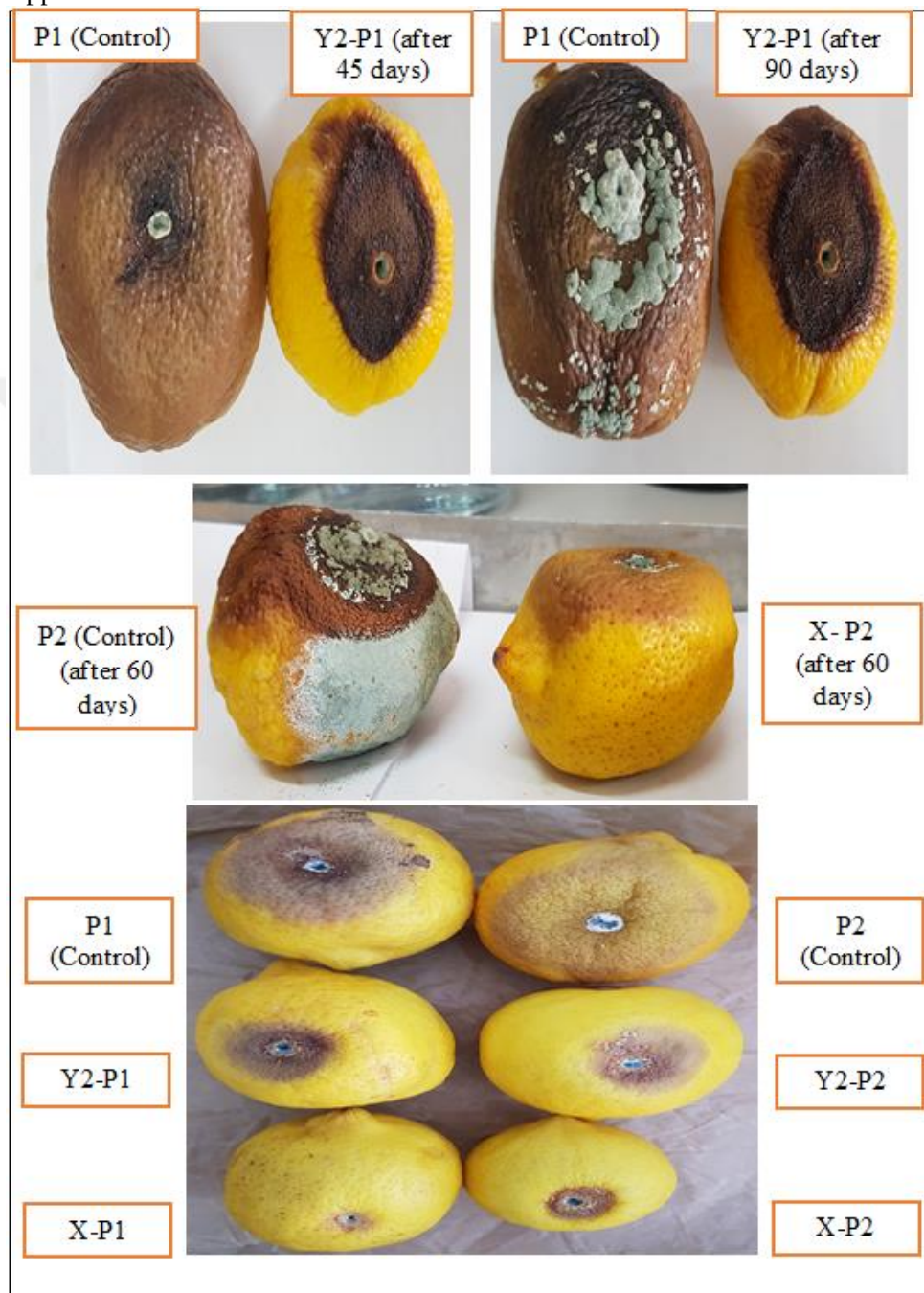
Appendix 21. Some images from cold room storage of cv. Starking Delicious apples



Appendix 22. Some images from cold room storage of cv. Kutdiken lemons



Appendix 22. Continued



Appendix 23. DAD spectrum of the patulin a) from working standard solution, b)
from sample

