

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

**BIOPROSPECTION OF ANTAGONISTIC YEASTS FOR BIOCONTROLLING
POSTHARVEST PATHOGENIC FUNGI AND PHYSICOCHEMICAL
CHARACTERIZATION OF A YEAST EXOPOLYSACCHARIDE**



Ph.D. THESIS

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Department of Food Engineering

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

**PATOJENİK KÜFLERİN HASAT SONRASI BİYOLOJİK KONTROLÜNDE
ANTAGONİSTİK MAYALARIN BİYOPROSPEKSİYONU VE BİR MAYA
EKZOPOLİSAKKARİTİNİN FİZİKOKİMYASAL KARAKTERİZASYONU**

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Sebahat ÖZTEKİN, a Ph.D. student of ITU Graduate School student ID 506142509 successfully defended the thesis/dissertation entitled “BIOPROSPECTION OF ANTAGONISTIC YEASTS FOR BIOCONTROLLING POSTHARVEST PATHOGENIC FUNGI AND PHYSICOCHEMICAL CHARACTERIZATION OF A YEAST EXOPOLYSACCHARIDE”, which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my beloved family,



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Sebahat ÖZTEKİN
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ABBREVIATIONS

ABTS	: 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt)
ANOVA	: Analysis of variance
ATCC	: American Type Culture Collection
BCA	: Biocontrol agent
BLAST	: Basic Local Alignment Search Tool
CFU	: Colony Forming Unit
CMC	: Carboxymethylcellulose sodium salt
CUPRAC	: Cupric ion reducing antioxidant capacity
DNA	: Deoxyribonucleic Acid
DPPH	: 2,2-diphenyl-1-picrylhydrazyl
DSC	: Differential scanning calorimeter
DSMZ	: German Collection of Microorganisms and Cell Cultures GmbH
EPS	: Exopolysaccharide
EU	: European Union
FDA	: Food and Drug Administration
FeCl₃	: Iron (III) Chloride
EFSA	: European Food Safety Authority
FAO	: Food and Agriculture Organization
FT-IR	: Fourier-transform infrared spectroscopy
GRAS	: Generally Recognized as Safe
HCl	: Hydrochloric Acid
HPLC	: High Performance Liquid Chromatography
ITS	: Internal Transcribed Spacer
LC	: Liquid Chromatography
MBA	: Methylene Blue Agar
MEGA	: Molecular Evolutionary Genetics Analysis
MS	: Mass Spectrometry
NaCl	: Sodium Chloride
NaOCl	: Sodium Hypochlorite

NCBI	: National Center for Biotechnology Information
NCYC	: National Collection of Yeast Cultures
NL1, NL4	: Primer pairs
NMR	: Nuclear Magnetic Resonance
OD	: Optical Density
OH	: Hydroxyl
PBS	: Phosphate-buffered saline
PCR	: Polymerase Chain Reaction
PDA	: Potato Dextrose Agar
PPM	: Parts Per Million
RAPD	: Random Amplified Polymorphic DNA
SPSS	: Statistical Package for the Social Sciences
QS	: Quorum Sensing
RASSF	: Rapid Alert System for Food and Feed
RH	: Relative Humidity
RNA	: Ribonucleic Acid
ROS	: Reactive Oxygen Species
RPM	: Rotation Per Minute
SDG	: Sustainable Development Goal
SDW	: Sterile Distilled Water
SEM	: Scanning Electron Microscopy
TCA	: Trichloroacetic acid
UN	: United Nations
UPGMA	: Unweighted Pair Group Method with Arithmetic Average
UV	: Ultraviolet
VOCs	: Volatile Organic Compounds
YEA	: Yeast Extract Glucose Agar
YNB	: Yeast Nitrogen Base
YEPD	: Yeast Extract Peptone Dextrose
WHC	: Water holding capacity
WSI	: Water solubility index
XRD	: X-ray diffraction

SYMBOLS

α	: alpha
β	: beta
bp	: base pair
°C	: centigrade degree
d	: day
Da	: Dalton
g	: gram
h	: hour
kDa	: kilodalton
M	: molarity
m	: milli (10^{-3})
mg	: milligram
min	: minute
mL	: millilitre
mM	: millimolar
mm	: millimetre
L	: Litre
log	: logarithmic
s	: second
t	: time
v	: volume
w	: weight
μ	: micro (10^{-6})
μg	: microgram
μL	: microliter



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BIOPROSPECTION OF ANTAGONISTIC YEASTS FOR BIOCONTROLLING POSTHARVEST PATHOGENIC FUNGI AND PHYSICOCHEMICAL CHARACTERIZATION OF A YEAST EXOPOLYSACCHARIDE

SUMMARY

The leading cause of food loss or fruit and vegetable waste was associated with fungi-induced postharvest diseases. Since fruit is rich in water, sugar, and essential nutrients, phytopathogenic and/or mycotoxigenic fungi can easily spread and find an ideal habitat, especially in damaged fruits. At this point, synthetic fungicides appear to be the first way of protection. Although these fungicides are efficient and economical, they can develop resistance in fungi, and their toxic residues may pose a health and environmental threat. The European Food Safety Authority (EFSA) has set tight regulations for the maximum pesticide residue levels in crops. It also encourages using low-risk or non-chemical fungicides for health and the environment. Recently, climate change has reduced crop yields and triggered multi-mycotoxin formation. Therefore, there is a need for non-toxic, natural, and efficient bio-fungicides to be used in agroecosystems.

Harnessing beneficial microorganisms, especially bacteria, yeasts, and moulds, has emerged as an alternative to synthetic fungicides. Among these microorganisms, antagonistic yeasts have a great potential for biopreservation with their GRAS (generally regarded as safe), biodegradable, non-toxic, genetically stable, and safety characteristics (non-pathogenic without producing antibiotics, mycotoxins, and allergenic spores). Additionally, antagonistic yeasts can use a variety of substances as carbon and energy sources, thereby limiting nutrients for spoilage fungi. They can also tightly attach to fruit and secrete hydrolytic enzymes, volatile organic compounds, mycocin, and biofilm. In line with the United Nations' Sustainable Development Goals of combating hunger and poverty, biocontrol yeasts have sparked much interest as a green and sustainable solution. The biocontrol yeasts can be a viable alternative to synthetic fungicides by bio-managing the notorious fungi and their mycotoxins, increasing agricultural productivity and food security. Regarding yeast metabolites, yeast-based exopolysaccharides (EPS) have received much attention due to their high yields, ease of extraction, and bioactive compounds.

Considering the above, a research strategy has been developed to investigate the biocontrol potential of fruit-isolated yeasts against selected fungi on lemons, mandarins, and grapes, along with their exopolysaccharide production and characterization. The objectives of this PhD dissertation were (i) bioprospecting *Metschnikowia* sp. isolates as biocontrol agents against postharvest fungal decays on lemons with their potential modes of action; (ii) evaluating the combinatorial effect of antagonistic yeasts (*Hanseniaspora uvarum*, *Meyerozyma guilliermondii*, and *Metschnikowia* aff. *pulcherrima* P01A016) on the bio-management of green mould disease on mandarin fruits; (iii) recruiting grape-isolated *Metschnikowia* aff. *fructicola*, *Metschnikowia pulcherrima*, and *Hanseniaspora uvarum* yeasts on the

biopreservation of Botrytized grapes; (iv) characterization of a novel exopolysaccharide produced by a cold-adapted yeast *Rhodotorula glutinis*.

Four research concepts (Chapters 3-6) were carried out to accomplish these objectives. Firstly, various fruits (grape, rosehip, hawthorn, blackberry, and cornelian cherry) and leaves were employed for yeast isolation. These yeasts were screened for their *in vitro* antifungal activity against various fungi. Since the highest antagonism was observed on *Penicillium* spp., lemons were selected as a substrate for further experiments. Then, pectinase-free antagonistic yeasts were applied to wounded lemon fruits to test their biocontrolling efficacy (Chapter 3). The compatible combinations of three antagonistic yeasts were employed to manage green mould decay on wounded and intact mandarin fruits. Furthermore, the findings were compared to the effectiveness of the synthetic fungicide, imazalil (Chapter 4). Grape-isolated yeasts *Metschnikowia* aff. *fructicola*, *Metschnikowia pulcherrima*, and *Hanseniaspora uvarum* were used in the biopreservation of grapes against *Botrytis cinerea*, along with elucidating antifungal mechanisms of action (Chapter 5). Finally, yeast isolates were screened for their exopolysaccharide production capabilities. A blackberry isolate, *R. glutinis*, was the most promising one with its high yield, characterized by its structural, rheological, antioxidant, and antibiofilm properties (Chapter 6).

The research framework and objectives of this PhD thesis are introduced in Chapter 1. Following that, Chapter 2 provided a comprehensive review of the exploitation of antagonistic yeasts for sustainable bio-management of postharvest phytopathogenic and mycotoxigenic fungi in fruits. The potential use of antagonistic yeasts for biocontrol purposes was mentioned, along with explaining their antifungal mechanisms of action. The current literature was screened to determine how antagonistic yeasts could be used with other agents or processes for improved biocontrolling activity. Subsequently, biocontrol yeasts' principles, advantages, disadvantages, and applications for commercial use were discussed.

In Chapter 3, eleven distinct yeast cultures of *Metschnikowia* sp. belonging to six different species were identified using polymerase chain reaction (PCR) with sequence-based analysis of the D1/D2 domain of 26S rDNA. Yeast antagonism (1×10^8 cells/mL) against various fungi (*Fusarium oxysporum*, *Botrytis cinerea*, *Penicillium digitatum*, *Penicillium expansum*, and *Alternaria alternata*) was determined through a dual culture method on plates. The highest antagonism was obtained against *Penicillium digitatum* NRRL 1202 and *Penicillium expansum* DSM6284 (83.63-100 %). Chitinase activity was present in all of the examined yeasts, while others also exhibited protease, pectinase, cellulase, β -1-3 glucanase, and gelatinase activities. Due to the high pectin content in lemons, three pectinase-free cultures with strong *in vitro* antagonistic effects on *Penicillium* were chosen. The interaction of lytic enzyme secretion, biofilm formation, iron depletion, and volatile organic compound (VOC) formation determined the antifungal mechanism of action. Pectinase-free *Metschnikowia* sp. yeasts significantly reduced the disease incidences and lesion diameters on lemons. *M. aff. fructicola* had the best biocontrol efficacy against *Penicillium* on lemons.

In Chapter 4, the antagonistic yeasts belonging to genera *Hanseniaspora uvarum*, *Meyerozyma guilliermondii*, and *Metschnikowia* aff. *pulcherrima* P01A016 were employed to inhibit *P. digitatum* in wounded and whole mandarins. All yeast cultures (73.85% - 80.64%) and their combinations (1:1, v/v; 1:1:1, v/v/v, 1×10^8 cells/mL) (78.40% - 83.18%) reduced the mycelial growth of green mould *in vitro*. *M. aff.*

pulcherrima reduced the disease incidence and lesion diameters by 75.5% and 91.3%, respectively, demonstrating the highest biocontrolling activity alone. *M. guilliermondii* exhibited the highest biofilm formation ($OD\ 0.93 \pm 0.01$) and antifungal activity (71.13%) via volatile organic compounds (VOCs), whereas *H. uvarum* exhibited cell-wall degrading β -1,3 glucanase activity in addition to mycocinogenic and VOCs activity. *M. guilliermondii* and *M. aff. pulcherrima* (M-1) were the most compatible, while *M. aff. pulcherrima* and *H. uvarum* (M-3) had the least compatibility. Combining these three yeasts resulted in synergistic cooperation, demonstrating the highest biocontrolling efficacy *in vitro* and *in vivo*.

In Chapter 5, grape-isolated yeasts (*Metschnikowia aff. fructicola*, *Metschnikowia pulcherrima*, and *Hanseniaspora uvarum*) were proposed to control *B. cinerea*-caused grey mould disease in grapes, while also elucidating their potential inhibitory mechanisms. The antifungal mechanisms included iron depletion, secretion of hydrolytic enzymes, diffusible compounds, inhibition of conidial germination, biofilm formation, wound-site colonisation, and VOC emission. *Metschnikowia* yeasts yielded comparable *in vitro* results but in *in vivo* experiments *M. aff. fructicola* outperformed all other yeasts. Interestingly, VOCs from *H. uvarum* held considerable potential as an antifungal biofumigant. All yeasts are adapted well to their ecological niche to protect the grapes against grey mould.

In Chapter 6, a novel EPS from *R. glutinis* was characterized by its monosaccharide composition by high-performance liquid chromatography (HPLC), molecular characterization by Fourier transform infrared (FT-IR) spectroscopy, thermal stability by differential scanning calorimetry (DSC), morphological characterization by scanning electron microscopy (SEM), crystallographic characteristics by X-ray diffraction (XRD) analysis, structural characterization by nuclear magnetic resonance (NMR) spectroscopy, antioxidant activity by $\bullet OH$, DPPH $\bullet$, CUPRAC, and ABTS radical scavenging activities, and rheological characterization by a rheometer. The findings revealed that EPS was a heteropolysaccharide composed of glucose and galactose. NMR and FT-IR analyses confirmed that the EPS-BMD26 structure had glucose and galactose without mannose. Differential scanning calorimetry (DSC) analysis revealed its thermal stability up to 326.16 °C. Scanning electron microscopy (SEM) revealed the porous microstructure with fissures. X-ray diffraction (XRD) analysis revealed that it was semi-crystalline. It demonstrated moderate and concentration-dependent antioxidant potential through $\bullet OH$, DPPH $\bullet$, CUPRAC, and ABTS radical scavenging methods. EPS-BMD26 exhibited high water holding capacity of $190 \pm 0.22\%$ with a water solubility index of $60.6 \pm 8.3\%$. The EPS-BMD26 also inhibited biofilm formation by *Staphylococcus aureus* ATCC 25923 (79.5% inhibition at 1250 ppm). Rheological analysis revealed its shear thinning and pseudoplastic behaviour.

Finally, in Chapter 7, based on the main outcomes of the previous chapters, the general discussion, conclusion, and future prospects in the biocontrol yeasts and yeast-derived exopolysaccharides are presented. From a biocontrol perspective, further research is required to examine the relationships between yeast, fungal pathogens, fruit, and microbiome. Integrating antagonistic yeasts with different microorganisms, natural agents, and physical processes can open new opportunities for designing effective microbial consortia for a tailored biofungicide. Further research is required to develop novel yeast-based biofungicides and understand their precise mode of action in large-scale applications.



PATOJENİK KÜFLERİN HASAT SONRASI BİYOLOJİK KONTROLÜNDE ANTAGONİSTİK MAYALARIN BİYOPROSPEKSİYONU VE BİR MAYA EKZOPOLİSAKKARİTİNİN FİZİKOKİMYASAL KARAKTERİZASYONU

ÖZET

Meyve ve sebzelerde gıda kaybı ve israfının önde gelen nedeni, küflerin neden olduğu hasat sonrası hastalıklarla ilişkilendirilmiştir. Özellikle meyveler su, şeker ve esansiyel besinler açısından zengindir ve bu durum meyvelerde fitopatojenik ve/veya mikotoksijenik küflerin kolayca yayılması ile sonuçlanabilir. Küfler özellikle zarar görmüş meyvelerde ideal bir yaşam alanı bulabilir. Bu aşamada sentetik fungusitler bitki hastalıklarına karşı korunmanın ilk yolu olarak karşımıza çıkmaktadır. Sentetik fungusitler etkili ve ekonomik olmalarına rağmen küflerde direnç geliştirebilmekte ve toksik kalıntıları sağlığı ve çevreyi tehdit edebilmektedir. Son zamanlarda, daha katı düzenleme politikaları ile sentetik fungusitlerin kullanımı sınırlandırılmıştır. Aynı zamanda, artan tüketici talebi ile gıdalarda potansiyel olarak tehlikeli kimyasalların kullanımı azaltılmaya çalışılmaktadır. Avrupa Gıda Güvenliği Otoritesi (EFSA), gıdalardaki maksimum pestisit kalıntı seviyeleri için sıkı düzenlemeler getirmiştir. Ayrıca sağlık ve çevre için düşük riskli veya kimyasal olmayan fungusitlerin kullanılması teşvik edilmektedir. Son zamanlarda, iklim değişikliği ekinlerin verimini azaltmakta aynı zamanda çoklu mikotoksin oluşumunu tetiklemektedir. Bu nedenle sağlığı, çevreyi ve tarımsal ekosistemleri korumak için toksik olmayan, doğal ve etkili biyofungisitlere ihtiyaç vardır.

Yararlı mikroorganizmaların ve özellikle bakteri, maya ve küflerin hastalık yapıcı organizmalara karşı kullanılması, sentetik fungusitlere alternatif olarak ortaya çıkmış bir yaklaşımdır. Bu mikroorganizmalar arasında, antagonistik mayalar, GRAS (genelde güvenli olarak kabul edilir), biyolojik olarak parçalanabilir, toksik olmayan, genetik olarak kararlı ve güvenlik özellikleri (antibiyotikler, mikotoksinler ve alerjenik sporlar üretmeyen ve patojen olmayan) ile biyokoruma potansiyeline sahiptir. Ek olarak, antagonistik mayalar, karbon ve enerji kaynakları olarak çeşitli maddeleri kullanabilir, böylece bozulmaya neden olan küfler için besinleri sınırlar. Ayrıca, mayalar meyve yüzeyine sıkıca tutunabilir ve hidrolitik enzimler, uçucu organik bileşikler, mikosin ve biyofilm salgılayarak etki gösterebilirler. Birleşmiş Milletler'in açlık ve yoksullukla mücadelede Sürdürülebilir Kalkınma Hedefleri doğrultusunda, biyokontrol mayaları yeşil ve sürdürülebilir bir çözüm olarak büyük ilgi uyandırmıştır. İnsanlar faydalarının farkına vardıkça doğal ürünler daha popüler hale gelmektedir. Bu bağlamda, biyokontrol mayaları, fitopatojenik küfleri ve bunların mikotoksinlerini biyolojik olarak yöneterek, tarımsal verimliliği ve gıda güvenliğini artırarak sentetik fungusitlere bir alternatif olarak kullanılabilir. Ayrıca, maya bazlı ekzopolisakkaritler (EPS), yüksek verimleri, ekstraksiyon kolaylığı ve biyoaktif bileşikleri ile büyük ilgi uyandırmaktadır.

Bu kapsamda limon, mandalina ve üzümlerdeki fitopatojenik/mikotoksijenik küflere karşı meyvelerden izole edilmiş mayaların biyokontrol potansiyelinin yanı sıra

ekzopolisakarit üretimi ve karakterizasyonunun çalışılması için bir araştırma stratejisi geliştirilmiştir. Bu doktora tezinin hedefleri (i) *Metschnikowia* sp. izolatlarının, potansiyel etki mekanizmaları ile limonlar üzerindeki hasat sonrası küflere karşı biyokontrol ajanları olarak biyoprospektifi; (ii) mandalina meyvelerinde yeşil küf hastalığının biyolojik tedavisi için üç farklı antagonistik mayanın (*Hanseniaspora uvarum*, *Meyerozyma guilliermondii* ve *Metschnikowia* aff. *pulcherrima* P01A016) tekli, ikili ve üçlü kombinasyonlarının uygulanması; (iii) *Botrytis cinerea* küfü ile enfekte olmuş üzümün biyolojik olarak korunmasında üzümde izole edilmiş *Metschnikowia* aff. *fructicola*, *Metschnikowia pulcherrima*, ve *Hanseniaspora uvarum* mayaların kullanılması; (iv) soğuğa adapte maya *Rhodotorula glutinis* tarafından üretilen yeni bir ekzopolisakaritin karakterizasyonu olarak açıklanabilir.

Tezde belirlenen hedeflere ulaşmak için çalışma dört farklı aşamada (Bölüm 3-6) yürütülmüştür. İlk olarak çeşitli meyveler (üzüm, kuşburnu, alıç, böğürtlen, kızılçık) ve yapraklarından maya izolasyonu gerçekleştirilmiştir. Bu mayalar, çeşitli küflere karşı *in vitro* antifungal aktiviteleri açısından taranmıştır. En yüksek antagonizm *Penicillium* spp.'a karşı gözlenmiş olup, sonraki deneyler limonlar üzerinde bu küfe karşı yapılmıştır. *In vitro* testlerin ardından, biyolojik kontrol etkinliklerini test etmek için yaralı limon meyvelerine pektinaz içermeyen antagonistik mayalar uygulanmıştır (Bölüm 3). Bunu takiben, yaralı ve bozulmamış mandalina meyveleri, yeşil küf çürümesinin biyokontrolü için üç farklı antagonistik maya izolatının uyumlu kombinasyonlarına tabi tutulmuştur. Ek olarak, mayaların biyokontrol etkinlikleri sentetik fungusit imazalil ile karşılaştırılmıştır (Bölüm 4). Üzümlerden izole edilen *Metschnikowia* aff. *fructicola*, *Metschnikowia pulcherrima*, ve *Hanseniaspora uvarum* mayaları üzümün *Botrytis cinerea*'ya karşı biyokorunmasında kullanılmıştır (Bölüm 5). Daha sonra, maya izolatları ekzopolisakarit üretim yetenekleri açısından taranmış ve bir böğürtlen izolatı olan *R. glutinis*'den yüksek verimde ekzopolisakarit (EPS) elde edilebildiği belirlenmiştir. Sonraki deneyler bu EPS'nin yapısal, reolojik, antioksidan ve antibiyofilm özelliklerini saptamak için yapılmıştır (Bölüm 6).

İlk bölümde bu doktora tezinin araştırma çerçevesi ve hedefleri tanıtılmıştır. Bunu takiben ikinci bölümde meyvelerde hasat sonrası fitopatojenik ve mikotoksijenik küflerin sürdürülebilir biyo-yönetimi için antagonistik mayaların kullanımına ilişkin kapsamlı bir derleme sunulmuştur. Öncelikle, antagonistik mayaların biyokontrol amaçlı potansiyel kullanımları ve antifungal etki mekanizmaları irdelenmiştir. İkinci olarak, hasat sonrası fungal patojenlerle mücadele için biyokontrol stratejileri ayrıntılı olarak sunulmuştur. Mevcut literatür, antagonistik mayaların yüksek biyokontrol aktivitesi için diğer ajanlar veya proseslerle nasıl kullanılabileceğini belirlemek için taranmıştır. Ardından, mayaların ticari uygulamalarından bahsedilmiş ve biyokontrol mayalarının prensipleri, avantajları, dezavantajları ve uygulamaları tartışılmıştır. Son olarak, sonuçlar ve gelecekteki araştırmalar için öngörüler sunulmuştur.

Üçüncü bölümde altı farklı türe ait *Metschnikowia* sp.'nin on bir farklı maya kültürü, 26S rDNA'nın D1/D2 bölgesinin dizi bazlı analizi ile polimeraz zincir reaksiyonu (PCR) kullanılarak tanımlandı. Çeşitli küflere (*Fusarium oxysporum*, *Botrytis cinerea*, *Penicillium digitatum*, *Penicillium expansum* ve *Alternaria alternata*) karşı maya antagonizması (1×10^8 hücre/mL) petrilerde ikili kültür yöntemiyle belirlenmiştir. En yüksek antagonizma *Penicillium digitatum* NRRL 1202 ve *Penicillium expansum* DSM6284'e (%83.63 - 100) karşı elde edilmiştir. İncelenen mayaların hepsinde kitinaz aktivitesi mevcutken, diğerlerin de proteaz, pektinaz, selüloz, β -1-3 glukanaz ve jelatinaz aktiviteleri gözlenmiştir. *Penicillium* üzerinde güçlü *in vitro* antagonistik etkileri olan pektinaz içermeyen üç maya kültürü seçilmiş ve test koşulları altında

limonlara uygulanmıştır. Limonlarda *P. digitatum* insidansı *P. expansum*'a göre daha yüksek bulunmuştur. *In vitro* ve *in vivo* çalışma bulgularının uyumlu olduğu görülmüştür. Antifungal etki mekanizması, litik enzim salgılanması, biyofilm oluşumu, demir bağlama yetenekleri ve uçucu organik bileşik (VOC) oluşumunun etkileşimi ile belirlenmiştir. Pektinaz içermeyen *Metschnikowia* sp. mayaları, limonlarda hastalık insidansını ve lezyon çaplarını önemli ölçüde azaltmıştır. *M. aff. fructicola*, limonların raf ömrünü uzatmış ve *Penicillium* küfüne karşı en iyi biyokorumayı sağlamıştır.

Dördüncü bölümde, *Hanseniaspora uvarum*, *Meyerozyma guilliermondii* ve *Metschnikowia aff. pulcherrima* P01A016 mayaları, yaralı ve bütün mandalinalarda *P. digitatum*'u inhibe etmede kullanılmıştır. Tüm maya kültürleri (%73.85 - 80.64) ve bunların kombinasyonları (1:1, v/v; 1:1:1, v/v/v, 1×10^8 hücre/mL) (%78.40 – 83.18) yeşil küfün *in vitro* misel büyümesini belli oranlarda azaltmıştır. *M. aff. pulcherrima*, hastalık insidansını ve lezyon çaplarını sırasıyla %75.5 ve %91.3 oranında azaltarak tek başına en yüksek biyokontrol aktiviteyi göstermiştir. *M. guilliermondii*, uçucu organik bileşikler (VOC'ler) aracılığıyla en yüksek biyofilm oluşumunu ($OD\ 0.93 \pm 0.01$) ve antifungal aktiviteyi (%71.13) sergilerken, *H. uvarum*, mikosinogenik aktivite göstermiş ve VOC'lere ek olarak hücre duvarı bozan β -1,3 glukanaaz aktivitesi sergilemiştir. Test edilen tüm mayalar, kitinaz, proteaz ve lösin arilamidaz aktivitesi açısından pozitif bulunmuştur. *M. guilliermondii* ve *M. aff. pulcherrima* (M-1) en uyumlu iken *M. aff. pulcherrima* ve *H. uvarum* (M-3) en az uyumu sergilemiştir. Bu üç mayanın kombinasyonu, *in vitro* ve *in vivo* olarak en yüksek biyokontrol etkinliğini göstererek sinerjistik işbirliği sağlanmıştır.

Beşinci bölümde, üzümelerde *B. cinerea*'nın neden olduğu gri küf hastalığını kontrol etmek için üzümünden izole edilen mayalar (*Metschnikowia aff. fructicola*, *Metschnikowia pulcherrima* ve *Hanseniaspora uvarum*) potansiyel inhibitör mekanizmaları da açıklanarak üzüm üzerinde denenmiştir. Bu mayalar, demir şelatlama, konidial çimlenmeyi inhibe etme, hidrolitik enzimler ve difüze metabolitler salgılama, biyofilm oluşturma, uçucu organik bileşikler salgılama ve meyvede kolonizasyon yetenekleri ile antifungal aktiviteye katkıda bulunmuştur. Ancak bu mekanizmaların bir arada bulunduğu bir mayaya rastlanmamıştır. *In vitro* deneylerde *Metschnikowia* mayalarında karşılaştırılabilir sonuçlar elde edilmiştir, ancak *in vivo* deneylerde *M. aff. fructicola* diğer mayalardan daha iyi performans sergilemiştir. Ayrıca, *H. uvarum*'un ürettiği uçucu organik bileşiklerin umut vaat eden antifungal biyofumigant olarak kullanım potansiyeline sahip olduğu not edilmiştir. Üzümleri gri küfe karşı korumak için tüm mayaların ekolojik nişlerine çok iyi adapte olduğu gözlenmiştir.

Altıncı bölümde, *R. glutinis*'in ürettiği yeni bir EPS'nin monomerik şeker kompozisyonu yüksek performanslı sıvı kromatografisi (HPLC) ile, moleküler karakterizasyonu Fourier dönüşümü kızılötesi (FT-IR) spektroskopisi ile, termal stabilitesi diferansiyel tarama kalorimetrisi (DSC) ile, morfolojik karakterizasyonu taramalı elektron mikroskobu (SEM) ile, kristalografik özellikleri X ışını kırınımı (XRD) analizi ile, yapısal karakterizasyonu nükleer manyetik rezonans (NMR) spektroskopisi ile, antioksidan aktivitesi •OH, DPPH•, CUPRAC ve ABTS radikal süpürme aktiviteleri ile ve reolojik karakterizasyon bir reometre ile incelenmiştir. Bulgular, EPS'nin glikoz ve galaktozdan oluşan bir heteropolisakkarit olduğunu ortaya koymuştur. NMR ve FT-IR analizleri, EPS-BMD26 yapısının mannoz içermeyip glikoz ve galaktozdan meydana geldiğini doğrulamıştır. DSC analizi, EPS-BMD26'nın 326.16 °C'ye kadar termal olarak kararlı olduğunu ortaya çıkarmıştır. EPS-BMD26'nın

gözenekli mikro yapısı, taramalı elektron mikroskobu (SEM) ile ortaya çıkarılmıştır. X-ışını kırınımı (XRD) analizi, bunun yarı kristal olduğunu ortaya koymuştur. EPS-BMD26, •OH, DPPH•, CUPRAC ve ABTS gibi çeşitli radikal yakalama yöntemlerini kullanarak orta düzeyde ve konsantrasyona bağlı antioksidan potansiyeli göstermiştir. EPS-BMD26, 60.6 ± 8.3 'lük suda çözünürlük indeksi ile 190 ± 0.22 'lik yüksek su tutma kapasitesi sergilemiştir. EPS-BMD26 ayrıca *Staphylococcus aureus* ATCC 25923 biyofilmini inhibe etmiştir (1250 ppm'de %79.5 inhibisyon). Reolojik analizler sonucunda EPS-BMD26 sulu çözeltisinin psödoplastik davranış sergilediği görülmüştür.

Özet olarak biyokontrol açısından bakıldığında, maya, fungal patojenler, meyve ve mikrobiyom arasındaki ilişkileri incelemek ve yeni maya bazlı biyofungusitlerin geliştirilmesi, büyük ölçekte uygulanması için daha fazla araştırmaya ihtiyaç vardır. Etkili bir mikrobiyal konsorsiyum tasarlamak için, antagonistik mayalar farklı mikroorganizmalar, doğal ajanlar ve fiziksel süreçler ile entegre edilebilir ve bu şekilde özelleştirilmiş bir biyofungusit üretilir.



1. INTRODUCTION

Yeasts are eukaryotic unicellular microorganisms that generally reproduce vegetatively by budding (Barnett et al., 2001). Yeasts can utilize sugars, organic acids, hydrocarbons, and alcohol as nutrition sources and grow at water activities (a_w) over 0.90, temperatures between 0 and 40 °C, and optimum pH values of 4.5–5.5. Yeasts can adapt to harsh environments and fruit surfaces more easily than bacteria because they can survive in extreme conditions, including high sugar and salt concentrations, low a_w , pH, and temperature. Unlike bacteria, yeasts can survive and disperse more readily in liquid substrates due to their high acid tolerance and ability to grow anaerobically (Pitt & Hocking, 2009).

Yeasts have been used to produce fermented foods to make bread, beer, and wine (Pérez-Torrado et al., 2015), or they can be consumed directly as a nutritional yeast (Jach et al., 2022). Besides, yeasts can produce antimicrobial and defensive compounds showing antagonism on different microorganisms (Mannazzu et al., 2019). In this regard, a growing body of research has been conducted on antagonistic yeasts employed for biocontrolling purposes (Hernandez-Montiel et al., 2021; Spadaro & Droby, 2016; H. Zhang et al., 2018). Yeasts, including *Saccharomyces*, *Metschnikowia*, *Candida*, *Hansenula*, *Hanseniaspora*, *Meyerozyma*, *Kluyveromyces*, *Pichia*, and *Rhodotorula*, can be used in this manner (Ahima et al., 2019; Izgü & Altinbay, 2004; Oztekin & Karbancioglu-Guler, 2021; Z. Wang, Li, Liu, Tian, et al., 2021; J. Zhang et al., 2017).

Antagonistic yeasts are GRAS (Generally Recognized As Safe) (FDA, 2021) and do not produce allergenic spores, antibiotics, or mycotoxins like other bacteria and fungi (Konsue et al., 2020; J. Liu et al., 2013). Although the mechanisms of action of biocontrol yeasts are yet unknown and need further research, Antagonistic yeasts were reported to function in different antifungal modes, such as competition for space and nutrients, rapid colonization and attachment, induction of host defense, iron depletion, mycoparasitism, and production of effective substances (lytic enzymes, biofilm, volatile organic compounds, and mycocin) (Agirman & Erten, 2020; da Cunha et al.,

2018; Di Francesco et al., 2016; Farbo et al., 2018; Y. Liu, Yao, et al., 2019; Oztekin & Karbancioglu-Guler, 2021). A biocontrol system is composed of four main elements: Host (fruit), pathogen (phytopathogenic/mycotoxigenic fungi), resident epiphytic flora (environment), and yeast (antagonistic yeast). Each antagonist's action method is determined by this quadruple relationship, which should be thoroughly explored to comprehend the mechanism (J. Liu et al., 2013; Spadaro & Droby, 2016).

On the other hand, antagonistic yeasts can perform inconsistent biocontrolling efficacy, which can be improved by combining them with other microorganisms (Panebianco et al., 2015), bio-agents (plant extracts and essential oils (Matrose et al., 2021; Papoutsis et al., 2019), β -glucan (D. Wang et al., 2018; Y. Zhao et al., 2020), methyl jasmonate (J. Guo et al., 2014; He et al., 2020), phytic acid (Mahunu et al., 2016), chitosan (Godana et al., 2020; T. Yu et al., 2012), salicylic acid (Qin et al., 2015), and gamma-aminobutyric acid (Xiao et al., 2022)) or physical treatments (hot air (Wei et al., 2016), microwave (D. Guo et al., 2016), ultraviolet light (Nie et al., 2020; Ou et al., 2016), and controlled atmosphere (de Paiva et al., 2017)). It should be noted that the methods mentioned above should have no effect on yeast viability at the treatment dose (J. Liu et al., 2013). These integrated disease management approaches may increase biocontrolling performance synergistically or additively. In some cases, antagonistic yeasts showing high antagonism in culture conditions (*in vitro*) may not work as well when applied to fruits (*in vivo*) (A. S. Dukare et al., 2019; Hernandez-Montiel et al., 2021). As a result, a thorough understanding of their antifungal mechanisms at various concentration levels and conditions is required to develop commercial formulations and successful on-field trials (A. Dukare et al., 2021). Further research is needed on antagonistic yeasts by testing them on various fruits and food matrices under specified conditions. This way, re-formulating existing biofungicides or discovering novel ones would be possible with large-scale applications. Despite extensive research has been conducted on antagonistic yeasts (da Cunha et al., 2018; Feng et al., 2021; Y. Liu et al., 2017; Parafati et al., 2016, 2017; Perez et al., 2017), there has been no study on considering pectinase activity of yeasts in fruit applications.

Furthermore, secondary metabolites of yeasts, such as killer toxin (mycocin) (Izgü et al., 2006) and exopolysaccharides (EPS) (Kheyrandish et al., 2022) need to be investigated as well. Mycocins are yeast extracellular protein toxins, mostly in the

form of glycoproteins or glycolipids, that are lethal to sensitive microbial cells (Izgü et al., 2006). Numerous yeasts were reported to produce mycocins, including *Saccharomyces*, *Metschnikowia*, *Hanseniaspora*, *Pichia*, *Debaryomyces*, *Candida*, *Zygosaccharomyces*, *Cryptococcus*, *Hansenula*, *Ustilago*, *Williopsis*, and *Kluyveromyces* (Bedir & Kuleaşan, 2021; Izgü et al., 2006; Öztekin & Karbancioglu-Guler, 2023). Mycocin-producing yeasts can be used in the biocontrolling of fruits and vegetables after harvest (Çorbacı & Uçar, 2018; Perez et al., 2016). They can also be employed to protect the fermentation from unwanted spoilage yeasts. Furthermore, purified mycocins can be classified as novel antimicrobials or antibiotics for use in medicine (Mannazzu et al., 2019). In addition, EPSs are produced as secondary metabolites of yeasts to defend themselves against environmental changes and pathogens (Kheyrandish et al., 2022). In this regard, *Rhodotorula* (Cho et al., 2001; Hamidi et al., 2020), *Lipomyces* (W. Li et al., 2021; Ragavan & Das, 2019), *Cryptococcus* (Pavlova et al., 2009), *Saccharomyces* (L. Liu et al., 2022), *Sporobolomyces* (Poli et al., 2010), *Hanseniaspora* and *Metschnikowia* (Yildiran et al., 2019) were classified among the EPS-producing yeasts.

Most postharvest fungal diseases in fruits are caused by fungi belonging to *Botrytis* spp., *Penicillium* spp., *Fusarium* spp., *Aspergillus* spp., *Alternaria* spp., and *Monilinia* spp. (Fernandez-San Millan et al., 2021; Y. Liu, Yao, et al., 2019; Oztekin & Karbancioglu-Guler, 2021; Parafati, Vitale, Restuccia, et al., 2015; Prendes et al., 2021; Ruiz-Moyano et al., 2016). Postharvest diseases cause massive food loss and waste, adversely affecting public health, the environment, and the economy (Tahir et al., 2022). Chemical fungicides currently provide effective and affordable solutions; however, their use causes several issues, including toxic residues and the development of fungicide-resistant phytopathogens (Bhatta, 2022; J. Zhang et al., 2021). As pesticide contamination is regarded as environmental pollution, the United Nations Sustainable Development Goals (SDG 2) aim to reduce or eliminate the use of pesticides for sustainable agriculture (UN, 2022). Increasing consumer demand and stricter regulations towards pesticide-free foods spotlighted the need for safe, non-toxic, efficient, and eco-friendly biocontrol alternatives (Hernández et al., 2022). Therefore, the trend is turning toward using biocontrol microorganisms (yeast, bacteria, and yeast-like fungi) to combat fungi-induced postharvest diseases (Bhatta, 2022; Wisniewski & Droby, 2019).

1.1 Purpose of Thesis

The main objectives of this thesis were to develop a green and sustainable solution for controlling postharvest fungal diseases with antagonistic yeasts and to characterize additional yeast features. To achieve this aim, the following objectives were followed;

1. To isolate and identify yeasts from different fruits (grape, rosehip, hawthorn, blackberry, and cornelian cherry) and leaves;
2. To investigate the antagonistic activity of yeast isolates against some phytopathogenic fungi (*Alternaria alternata*, *Botrytis cinerea*, *Fusarium oxysporum*, *Penicillium digitatum*, and *Penicillium expansum*) through *in vitro* mycelial growth inhibition;
3. To elucidate potential modes of action of antagonistic yeasts while evaluating their potential for use as natural fungicides instead of synthetic ones in the biological control of postharvest diseases in fruits;
4. To test *in vivo* the application of antagonistic yeasts on a variety of fruits, such as lemons, mandarins, and grapes, to improve quality, safety, and shelf life;
5. To determine the biocontrol efficacies of compatible antagonistic yeast combinations on green mould and their comparison with synthetic fungicides;
6. To investigate the exopolysaccharide production potential of yeast isolates, as well as to scrutinize and characterize the exopolysaccharide produced.

2. LITERATURE REVIEW¹

2.1 Antagonistic Yeasts and Their Antifungal Mechanisms of Action

2.1.1 Antagonistic yeasts

The ability of a microorganism to interfere with or inhibit the activity, growth, or reproduction of phytopathogens is called antagonism (A. S. Dukare et al., 2019) and utilizing pathogen and disease-controlling antagonistic microorganisms in agricultural products known as biological control (Heydari & Pessarakli, 2010). In this sense, a strain of *Bacillus subtilis* was the first reported antagonistic microorganism, which was used in the biocontrol of brown rot on peaches caused by *Monilia fructicola* (Pusey & Wilson, 1984).

Although antagonistic effect studies were first started with bacteria as a biological control method, it has been suggested that using antagonistic yeasts is more advantageous in preventing postharvest diseases (Palmieri et al., 2022). The important superiorities of yeast antagonists over bacteria or mycelial fungi are i) yeasts are more resistant to stress factors, even to UV, ii) yeasts can develop adequately in fruits despite their high sugar content and acidic structure, iii) yeasts can easily colonize even dry surfaces by the usage of inexpensive and simple substrates, iv) yeasts do not produce toxic secondary metabolites or allergenic spores (J. Liu et al., 2013; Muccilli & Restuccia, 2015; Perez et al., 2017; Spadaro & Droby, 2016). Although mostly the naturally occurring yeasts on the surfaces of fruits are used in the biocontrol of postharvest diseases (J. Liu et al., 2013), there are also antagonistic yeasts isolated from animal ecosystems (Armando et al., 2013), blue-veined Rokpol cheese (Grzegorzczuk, Żarowska, et al., 2017), water samples collected from Antarctica (Arrarte et al., 2017), soil from King George Island and (Antarctica) (Arrarte et al.,

¹ Öztekin, S., Dikmetaş, D. N., Devecioğlu, D., Acar, E. G., & Karbancıoğlu-Güler, F. (2023). Recent insights into the use of antagonistic yeasts for sustainable bio-management of postharvest pathogenic and mycotoxigenic fungi in fruits with their prevention strategies against mycotoxins, Journal of Agricultural and Food Chemistry

2023), terrestrial of King George Island (Martinez et al., 2016). The growth characteristics may change according to the environment in which the yeast is isolated. For instance, the resistance to extreme conditions such as high osmotic pressure and abiotic stress is higher in yeasts isolated from marine rather than from fruit surfaces (Hernández-Montiel et al., 2010).

Metschnikowia, *Debaryomyces*, *Aureobasidium*, *Saccharomyces*, *Candida*, *Pichia*, and *Meyerozyma* are the most commonly reported yeasts with antagonistic activity (Freimoser et al., 2019; Pawlikowska et al., 2019; Q. Yang et al., 2022). Factors such as growth conditions, nutrient requirements, simplicity, activity range, effectiveness and safety should be considered when deciding which antagonistic yeast to use from a wide range, such as the source of isolation and the genera it belongs to (Droby et al., 2009; X. Zhang et al., 2020). According to Wilson and Wisniewski (1989), antagonistic yeasts should be genetically stable, effective at low concentrations and against various pathogens, resistant to the chemicals used, able to tolerate extreme conditions, easy to dispense, adaptable to processing techniques, not harmful to humans and have long shelf-life. Additionally, the microorganisms used in food must be on the GRAS list approved by the FDA because of the necessary toxicity tests (FDA, 2018). GRAS reports of various yeast genera declare that there is no health risk for the consumption of food that contacts these microorganisms. Yeasts that can be used in the biocontrol of the diseases caused by the fungal pathogens in fruits should be selected considering these criteria.

Antagonistic yeasts can follow different pathways while inhibiting the growth and pathogenicity of fungal pathogens. It is critical to choose the optimum antagonistic yeast and ambient conditions to prevent postharvest disease in fruits and understand the mechanism that underlies the antagonistic effect to provide effective biological control. There is a continuous interaction between the antagonistic yeast, the fungal pathogen, the host fruit, and the natural microbiota of the host fruit. It is observed that yeasts in the pseudohyphal form may damage the tissues and accelerate the deterioration of any host fruit, while it has a successful antagonistic effect on another fruit (Maserti et al., 2015). Therefore, yeast's antagonistic properties should be tested for each host fruit. This complex relationship is highly influential on the mechanism of action of each antagonist, and the situation should be thoroughly studied to understand the mechanism (J. Liu et al., 2013; Spadaro & Droby, 2016).

2.1.2 Antagonistic yeasts' antifungal mechanisms of action

The main antifungal mechanisms of antagonistic yeasts are competition for nutrients and space, secretion of antimicrobial compounds, enzyme-related mechanisms, mycoparasitism, and induction of host defense (Di Francesco et al., 2016). The main antifungal mechanisms of action and factors are summarized in Figure 2.1. Antagonistic modes of action can be both direct and indirect. Direct mechanisms are highly selective for fungal pathogens; indirect mechanisms are not specific to the pathogen. While the competition for nutrients and space, secretion of antimicrobial compounds, and mycoparasitism are direct mechanisms, induction of host defense is an indirect antagonistic mechanism (Heydari & Pessarakli, 2010; Lahlali et al., 2022). *In vitro* and *in vivo* recent studies on the mechanisms of antagonistic yeasts against fungal pathogens are summarized in Table 2.1 and Table 2.2.

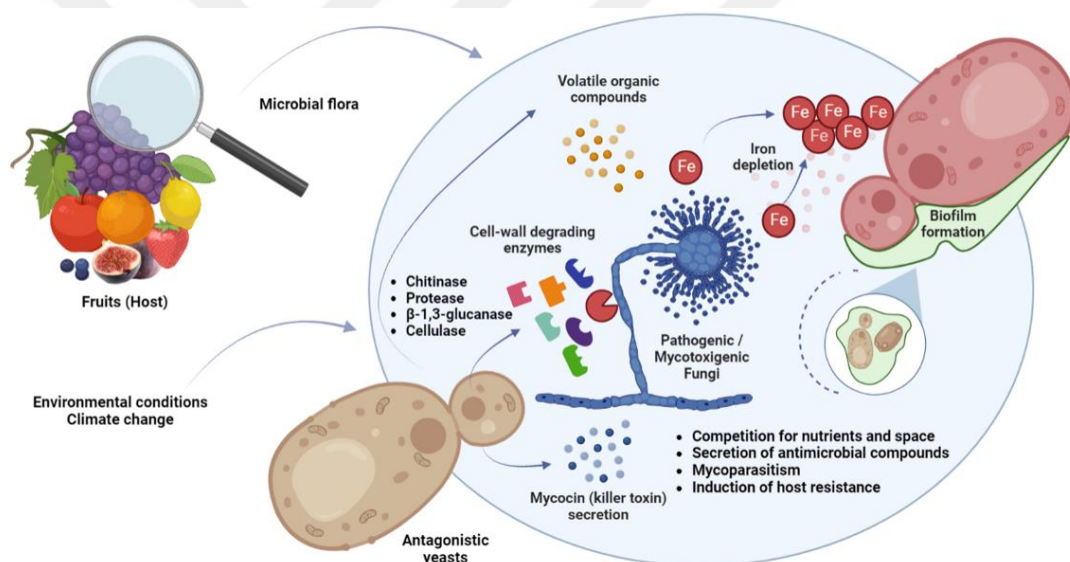


Figure 2.1 : Antifungal mechanisms of action of antagonistic yeasts.

2.1.2.1 Competition for nutrients and space

Competition for nutrients and space is the main mechanism of action since all antagonists can exert this mechanism to some extent (Ghorbanpour et al., 2018), and the antagonists invade host fruits by growing in wounds from the very first contact and causing depletion by consuming nutrients (X. Zhang et al., 2020). For the competition for space, antagonistic yeasts should be able to colonize on the host fruit surface, and biofilm formation can enhance the colonization (Hernández et al., 2022). Therefore, for the antagonistic yeasts to act with this mechanism, their superiority is their ability to fast-growing and biofilm formation through the wounded fruit surface (Spadaro &

Droby, 2016). These properties can be caused by the type and natural microbiota of the host fruit and the concentration of antagonistic yeast (L. Yu et al., 2020). Yeasts communicate about their cell density and express genes by quorum sensing for biofilm formation (J. Li & Zhao, 2020). Biofilms are formed by the aggregation and adhesion of yeast cells to themselves and the surface, the formation of an extracellular matrix composed of DNA, exopolysaccharides, and proteins, and proliferation (Miranda et al., 2022). Antagonistic yeasts can tolerate stress conditions better in the form of biofilm (Gouka et al., 2022; Miranda et al., 2022). One study observed that the transition of *Pichia kudriavzevii* from the yeast-like form to the biofilm form increased its tolerance to heat and oxidative stress and significantly reduced the lesion diameters and disease incidence caused by *Botrytis cinerea* and *Colletotrichum gloeosporioides* in pear (Chi et al., 2015).

For the competition for nutrients, antagonistic yeasts consume the nutrients in host fruits, including carbon and nitrogen, which are essential for the viability of both yeasts and fungi, causing pathogenic fungi to be unable to reach essentials and grow (Lahlali et al., 2022). It is stated that when sufficient micronutrients, nitrogen and carbon sources are provided, a significant reduction in the inhibition rate of *Penicillium digitatum* germination by the antagonistic species of *Pichia kudriavzevii* (Delali et al., 2021). Another component, iron, can act as a limiting factor for fungal growth since it is present in various proteins and enzyme structures. Antagonistic yeasts can produce iron chelators and siderophores to compete for iron, cause iron depletion, and inhibit fungi's conidial germination and pathogenesis (Spadaro & Droby, 2016). Some antagonistic yeasts secrete pulcherriminic acid, which reacts with Fe^{3+} and produces an iron chelate called pulcherrimin (Sipiczki, 2020). Pulcherrimin is an insoluble red pigment whose intensity increases with the amount of depleting iron in the medium and is correlated with the antagonistic activity of *Metschnikowia pulcherrima* (Figure 2.2.) (Saravanakumar et al., 2008; Sipiczki, 2006).

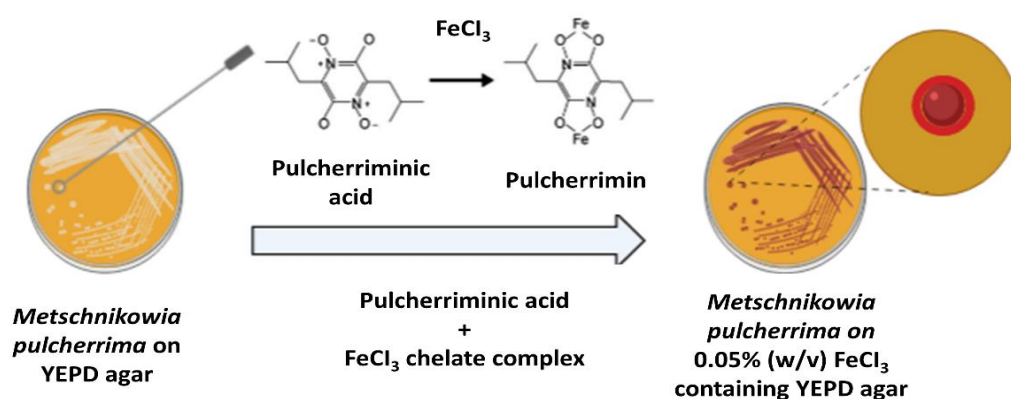


Figure 2.2 : Schematic explanation of *in vitro* pulcherrimin formation.

2.1.2.2 Secretion of antimicrobial compounds

Antagonistic yeasts can produce antimicrobial molecules as secondary metabolites, which are organic molecules that inhibit other microorganisms, except for the producer yeast due to its toxicity (Lahlali et al., 2022). The possibility of developing resistance in pathogens to antimicrobials produced by antagonists and health concerns should be considered (Spadaro & Droby, 2016). Killer toxins and VOCs are antagonistic yeasts' main antifungal secondary metabolites. Killer toxins, also called mycocins, are protein, glycoprotein, or glycolipids with a molecular weight of 10–30 kDa (Gil-Rodríguez & Garcia-Gutierrez, 2021), produced for competition with other environmental microorganisms and increasement of stress resistance (Parafati et al., 2022). Since the killer toxin secreted by the antagonistic yeast does not affect itself, it only has a lethal effect on other yeasts and moulds; toxin-producer yeast can compete for nutrients and space in the host fruit in this way (Gouka et al., 2022; Mannazzu et al., 2019). The action mechanisms of killer toxins are disrupting the cell membrane, inhibiting cell wall and DNA synthesis, and blocking calcium uptake (Gil-Rodríguez & Garcia-Gutierrez, 2021; Mannazzu et al., 2019; Shruthi et al., 2022). Mycocins are advantageous biocontrol mechanisms due to their non-toxicity to mammals, increased resistance to stress conditions, environmentally friendly and the possibility of pathogen resistance is not high (X. Zhang et al., 2020). A study showed that the killer toxins of *Debaryomyces hansenii* strains were highly stable at pH 2.5–5.5 and 5–37°C and could inhibit *Alternaria brassicicola*, *Alternaria citri*, *Aspergillus niger* and *Rhizopus stolonifer* in fruits (Çorbacı & Uçar, 2018). Grzegorzczuk et al. (Grzegorzczuk, Żarowska, et al., 2017) stated that the killer toxin activities of two different strains might vary since there will be changes in protein structure and enzymatic activity.

Other antifungal secondary metabolites, volatile organic compounds (VOCs), are highly water-soluble molecules with high vapor pressure and smaller than 300 Da, which may include aldehydes, ketones, hydrocarbons, alcohols, thioalcohols, cyclohexanes, heterocyclic compounds, phenols, etc.(Freimoser et al., 2019). A study conducted with *Wickerhamomyces anomalus* BS91, *Metschnikowia pulcherrima* MPR3, *Aureobasidium pullulans* PI1, and *Saccharomyces cerevisiae* BCA61, the main VOCs produced by antagonistic yeasts were found to be ethyl alcohol, ethyl acetate, isoamyl alcohol, isoamyl acetate and phenethyl alcohol (Contarino et al., 2019). The composition of VOCs may vary according to the producer antagonistic yeast, fungal pathogen, and the ecological niche (Parafati et al., 2017), and thus can be defined as “strain and target-dependent (Sipiczki, 2020).” When the *in vitro* effect of VOCs of four different yeasts (*Pichia kudriavzevii* KKP 3005, *Pichia occidentalis* KKP 3004, *Meyerozyma guilliermondii*, *Meyerozyma caribbica* KKP 3003) against five different fungal pathogens belonging to *Penicillium*, *Fusarium*, *Aspergillus*, *Mucor* and *Botrytis* was examined, it was observed that the composition of the VOCs secreted by the same yeast species changed in the headspace in contact with different fungal pathogens (Choińska et al., 2020). Similarly, when the VOCs of three different isolates, all belonging to *Saccharomyces cerevisiae*, were examined, it was seen that the gas composition changed, even though the most abundant molecules were the same (Natarajan et al., 2022). Its action mechanism is based on the modification of amino acid, protein and nucleus synthesis and inhibition of the fungal pathogen (Shruthi et al., 2022). Studies on VOCs produced by antagonists have recently intensified besides using antagonistic yeasts in biocontrol. Outstanding advantages such as being biodegradable, being effective even in small amounts (Z. Wang, Zhong, Chen, Yang, et al., 2021) and not requiring direct contact between antagonistic yeast and pathogen, and being able to spread rapidly by gas diffusion in an inhomogeneous medium consisting of solid, liquid and gas, where the target is far away, have made the VOC mechanism very promising and popular (Choińska et al., 2020). The most important issue to be considered when working with VOCs is that the VOCs' composition and fungal inhibition rates may vary with the *in vitro* results and in the natural conditions with the host fruit (Spadaro & Droby, 2016).

2.1.2.3 Mycoparasitism

Mycoparasitism is the mechanism of action in which antagonistic yeast is attached to the hyphae of the fungal pathogen and secretes cell wall-degrading enzymes that cause fungal lysis or destruction (Ghorbanpour et al., 2018). While glucan, the main structural polysaccharide that acts as a filling material, makes up 50-60% of the cell wall, the other 40-50% consists of half chitin, the backbone of the cell wall, and half of the protein (Seidl, 2008; Spadaro & Droby, 2016). Since yeasts disintegrate fungal cell walls to reach carbon sources and amino acids for their viability (Hernandez-Montiel et al., 2018a), mostly, activities of β -1,3-glucanase (GLU), chitinase (CHT), and proteases are strongly associated with the antagonistic activity (Di Francesco et al., 2016; Podgórska-Kryszczuk et al., 2022). Similar to other metabolites, enzyme profiles of antagonistic yeasts may also vary. When Oztekin and Karbancioglu-Guler (2021) examined the protease, pectinase, cellulase, GLU, and gelatinase enzyme activities of different isolates belonging to *Metschnikowia pulcherrima*, they reported that an isolate produced all enzymes. In contrast, one isolate did not produce only pectinase, and one isolate did not produce only protease.

The first evidence of mycoparasitism was the attachment of *Pichia guilliermondii* to fungal hyphae and the secretion of GLU against *B. Cinerea* (Wisniewski et al., 1991). More recently, the adhesion of *Meyerozyma caribbica* to the hyphae of the fungal pathogen *Colletotrichum gloeosporioides* on mango evidenced the action mechanism of mycoparasitism (Aguirre-Güitrón et al., 2019).

2.1.2.4 Induction of host defense

Unlike the mechanisms described above, the induction of host defense is an indirect mechanism; that is, the antagonistic yeast does not directly affect the pathogenic fungi but increases the resistance of the host fruit of the fungi against this pathogen (Lahlali et al., 2022). Pathogens and environmental factors also affect how antagonistic yeasts induce resistance (X. Zhang et al., 2020), and the induction of host resistance may occur by different mechanisms, but briefly, antagonistic yeasts stimulate the defense signals through elicitor, the expression of antioxidant genes and defense-related enzymes, and the production of reactive oxygen species (ROS), especially H₂O₂, in the host fruit (C. Li et al., 2014; Y. Liu et al., 2018; X. Xu et al., 2008). The secretion of both pathogenesis-related (PR) proteins (GLU and CHT) and defense-related or

antioxidant enzymes called phenylalanine ammonia-lyase (PAL), peroxidase (POD), polyphenol oxidase (PPO), and catalase (CAT), etc. by antagonistic yeasts may induce resistance in the host fruit (Di Francesco et al., 2016; Hassan et al., 2021).

Cheng et al. (2019) showed that *Hanseniaspora uvarum* induces host defense of kiwifruit by activating defense-related genes and enzymes, CHT and GLU. Another antagonistic yeast, *Metschnikowia pulcherrima* E1, could induce host resistance in loquat fruit to *Pestalotiopsis vismiae* by enhancing the activities of defense-related enzymes of PAL, POD, PPO, CAT, and ascorbate peroxidase (APX) (H. Yang et al., 2021). The application of *Rhodotorula mucilaginosa* on strawberries has delayed the senescence and induced the host resistance by promoting both the defense-related enzymes and PR proteins against *Rhizopus stolonifer* and *Botrytis cinerea* (H. Zhang et al., 2014). It has also been reported that the induction of host defense by the biosynthesis of phenylpropanoid is the action mechanism of *Pichia galeiformis* against *Penicillium digitatum* on citrus fruit (O. Chen et al., 2021). Proteomic studies should be increased to understand this mechanism better, explore the plant's affected metabolic pathways, and find the most effective antagonist against fungi.

Table 2.1 : Representative *in vitro* antifungal mechanisms of action of antagonistic yeasts used in recent years.

Action mechanism	Antagonistic yeast	Origin	Fungal pathogen	Reference
Competition for nutrient and space	<i>Candida stellimalicola</i> ACBL-07	Citrus leaves	<i>Penicillium italicum</i>	(da Cunha et al., 2018)
	<i>Debaryomyces hansenii</i> ECP4	Surface of papaya	<i>Colletotrichum gloeosporioides</i>	(Hernandez-Montiel et al., 2018b)
	<i>Debaryomyces nepalensis</i>	Soil of unsprayed mango orchards	<i>Colletotrichum gloeosporioides</i>	(Zhou et al., 2018)
	<i>Pichia kudriavzevii</i> S ₂ C	Kimchi	<i>Penicillium digitatum</i>	(Delali et al., 2021)
	<i>Metschnikowia citriensis</i> FL01	Citrus leaves	<i>Geotrichum citri-aurantii</i>	(S. Wang et al., 2021)
	<i>Metschnikowia pulcherrima</i> 26-BMD	Blackberry	<i>Penicillium digitatum</i> , <i>Penicillium expansum</i>	(Oztekin & Karbancioglu-Guler, 2021)o
Biofilm formation	<i>Candida oleophila</i> L12, <i>Debaryomyces hansenii</i> L16	Fruits of several citrus varieties	N/A ¹	(Hammami et al., 2022)
	<i>Cryptococcus laurentii</i> FRUC DJ1	Grapefruit		(Deng et al., 2021)
	<i>Debaryomyces nepalensis</i>	Soil of unsprayed mango orchards		(Zhou et al., 2018)
	<i>Metschnikowia</i> aff. <i>fruticola</i> 1-UDM, <i>Metschnikowia pulcherrima</i> 26-BMD	Green grapes Blackberry		(Oztekin & Karbancioglu-Guler, 2021)
	<i>Pichia kudriavzevii</i> S ₂ C	Kimchi		(Delali et al., 2021)
	<i>Torulaspota indica</i> DMKU-RP31	Leaves of economic plants		(Konsue et al., 2020)
	<i>Vishniacozyma victoriae</i> EPL4.5 and EPL29	Apple		(Sepúlveda et al., 2022)
Volatile Organic Compounds (VOCs)	<i>Aureobasidium pullulans</i> GE17, <i>Meyerozyma guilliermondii</i> KL3	Golden delicious apple Kutdiken lemon	- ²	(Agirman & Erten, 2020)
	<i>Candida oleophila</i> L12, <i>Debaryomyces hansenii</i> L16	Fruits of several citrus varieties		(Hammami et al., 2022)
	<i>Candida sake</i> 41E	Soil and water samples collected in Antarctica	<i>Penicillium expansum</i>	(Arrarte et al., 2017)
	<i>Debaryomyces hansenii</i> ECP4	Surface of papaya	<i>Colletotrichum gloeosporioides</i>	(Hernandez-Montiel et al., 2018b)
	<i>Galactomyces geotrichum</i> JYC549	Cherry tomato	<i>Fusarium proliferatum</i>	(R. Y. Chen et al., 2022)
	<i>Metschnikowia pulcherrima</i>	Soil of a mango orchard	<i>Colletotrichum gloeosporioides</i>	(Tian, Li, Jiang, Jing, & Shao, 2018)
	<i>Metschnikowia pulcherrima</i> 34-UEM	Red grapes	<i>Penicillium digitatum</i> , <i>Penicillium expansum</i>	(Oztekin & Karbancioglu-Guler, 2021)

¹: Not applicable. ²: It is not specified.

Table 2.1 (continued) : Representative *in vitro* antifungal mechanisms of action of antagonistic yeasts used in recent years.

Action mechanism	Antagonistic yeast	Origin	Fungal pathogen	Reference
Volatile Organic Compounds (VOCs)	<i>Meyerozyma guilliermondii</i> /M. caribbica, <i>Pichia occidentalis</i> , <i>Pichia kudriavzevii</i> /Issatchenkia orientalis	Organic grape	<i>Penicillium chrysogenum</i> , <i>Fusarium poae</i> , <i>Aspergillus fumigatus</i> , <i>Mucor</i> sp., <i>Botrytis cinerea</i>	(Choińska et al., 2020)
	<i>Pichia galeiformis</i> BAF03	Lemon	<i>Penicillium digitatum</i>	(O. Chen et al., 2020)
	<i>Wickerhamomyces anomalus</i> BS91	Naturally fermented olive brine	<i>Monilinia fructigena</i> and <i>Monilinia fructicola</i>	(Grzegorzczuk, Żarowska, et al., 2017)
	<i>Candida stellimalicola</i> ACBL-07	Citrus leaves	<i>Penicillium italicum</i>	(da Cunha et al., 2018)
Killer toxin	<i>Debaryomyces hansenii</i> MI1a and KI2a	Blue-veined Rokpol cheese	-	(Grzegorzczuk, Żarowska, et al., 2017)
Secretion of lytic enzyme	<i>Aureobasidium pullulans</i> GE17, <i>Meyerozyma guilliermondii</i> KL3	Golden delicious apple Kutdiken lemon	N/A ¹	(Agirman & Erten, 2020)
	<i>Candida oleophila</i> L12, <i>Debaryomyces hansenii</i> L16	Fruits of several citrus varieties		(Hammami et al., 2022)
	<i>Debaryomyces hansenii</i> ECP4	Surface of papaya		(Hernandez-Montiel et al., 2018b)
	<i>Debaryomyces hansenii</i> MI1a and KI2a	Blue-veined Rokpol cheese		(Grzegorzczuk, Żarowska, et al., 2017)
	<i>Pichia galeiformis</i> BAF03	Lemon		(O. Chen et al., 2020)
	<i>Saccharomyces cerevisiae</i> ACBL-11	Citrus flower		(da Cunha et al., 2018)
Adhesion to fungal hyphae (mycoparasitism)	<i>Debaryomyces nepalensis</i>	Soil of unsprayed mango orchards	<i>Colletotrichum gloeosporioides</i>	(Zhou et al., 2018)
	<i>Metschnikowia pulcherrima</i> L672	Fig	<i>Cladosporium cladosporioides</i>	(Ruiz-Moyano et al., 2016)
	<i>Pichia kudriavzevii</i> S2C, <i>Yarrowia lipolytica</i> S4A	Kimchi	<i>Penicillium digitatum</i>	(Delali et al., 2021)

¹: Not applicable.

Table 2.2 : Representative *in vivo* antifungal mechanisms of action of antagonistic yeasts used in recent years.

Action Mechanism	Antagonistic Yeast	Origin	Fungal Pathogen	Host Fruit	Reference
Competition for and nutrient space	<i>Hanseniaspora opuntiae</i> M479, <i>Metschnikowia pulcherrima</i> L672	Fig	<i>Penicillium expansum</i> , <i>Botrytis cinerea</i> , <i>Cladosporium cladosporioides</i>	Apple	(Ruiz-Moyano et al., 2016)
	<i>Metschnikowia pulcherrima</i>	Soil of a mango orchard	<i>Colletotrichum gloeosporioides</i>	Mango	(Tian, Li, Jiang, Jing, & Shao, 2018)
	<i>Meyerozyma guilliermondii</i>	Surface of rotten pears	<i>Penicillium expansum</i>	Pear	(Y. Yan, Zhang, Zheng, Apaliya, et al., 2018)
	<i>Metschnikowia</i> aff. <i>fruticola</i> 1-UDM	Green grapes	<i>Penicillium digitatum</i> , <i>Penicillium expansum</i>	Lemon	(Oztekin & Karbancioglu-Guler, 2021)
Wound colonization site	<i>Candida oleophila</i> P316	The surface of pear fruit	N/A ¹	Kiwifruit	(Gao et al., 2021)
	<i>Meyerozyma guilliermondii</i>	Surface of rotten pears		Pear	(Y. Yan, Zhang, Zheng, Apaliya, et al., 2018)
	<i>Meyerozyma guilliermondii</i> Y-1	Grape		Apple	(Y. Huang et al., 2021; C. Sun et al., 2021)
	<i>Pichia galeiformis</i> BAF03	Lemon		Citrus fruit	(O. Chen et al., 2020)
	<i>Wickerhamomyces anomalus</i> BS91	Naturally fermented olive brine		Peach and plum	(Grzegorzczuk, Żarowska, et al., 2017)
Adhesion to fungal hyphae (mycoparasitism)	<i>Cryptococcus laurentii</i> FRUC DJ1	Grapefruit	<i>Penicillium digitatum</i>	Grapefruit	(Deng et al., 2021)
	<i>Debaryomyces nepalensis</i>	Soil of unsprayed mango orchards	<i>Colletotrichum gloeosporioides</i>	Mango	(Zhou et al., 2018)
	<i>Meyerozyma caribbica</i>	²	<i>Colletotrichum gloeosporioides</i>	Mango	(Aguirre-Gutiérrez et al., 2019)
Volatile Organic Compounds (VOCs)	<i>Candida sake</i> 41E	Soil and water samples collected in Antarctica	<i>Penicillium expansum</i>	Apple	(Arrarte et al., 2017)
	<i>Hanseniaspora uvarum</i> 793	Fig	<i>Botrytis cinerea</i>	Strawberries and cherries	(Ruiz-Moyano et al., 2020)

¹: Not applicable. ²: It is not specified.

Table 2.2 (continued) : Representative *in vivo* antifungal mechanisms of action of antagonistic yeasts used in recent years.

Action Mechanism	Antagonistic Yeast	Origin	Fungal Pathogen	Host Fruit	Reference
Volatile Organic Compounds (VOCs)	<i>Wickerhamomyces anomalus</i> , <i>Metschnikowia pulcherrima</i> , <i>Saccharomyces cerevisiae</i>	-	<i>Botrytis cinerea</i> , <i>Monilinia fructicola</i> , <i>Alternaria alternata</i> , <i>Aspergillus carbonarius</i> , <i>Penicillium digitatum</i> , <i>Cladosporium</i> spp., <i>Colletotrichum</i> spp.,	Strawberry	(Oro et al., 2018)
	<i>Torulaspora indica</i> DMKU-RP31	Leaves of economic plants	<i>Lasiodiplodia theobromae</i>	Mango	(Konsue et al., 2020)
Secretion of defense-related enzyme	<i>Candida oleophila</i> P316	The surface of pear fruit	-	Kiwifruit	(Gao et al., 2021)
	<i>Cryptococcus laurentii</i> FRUC DJ1	Grapefruit	-	Grapefruit	(Deng et al., 2021)
	<i>Debaryomyces hansenii</i> AII4b	Blue-veined Rokpol cheese	<i>Monilinia fructicola</i>	Apple	(Czarnecka et al., 2019)
	<i>Debaryomyces nepalensis</i>	Soil of unsprayed mango orchards	<i>Colletotrichum gloeosporioides</i>	Mango	(Zhou et al., 2018)
	<i>Kluyveromyces marxianus</i> XZ1	Apple	-	Apple	(Zheng et al., 2023)
	<i>Metschnikowia pulcherrima</i>	Soil of a mango orchard	<i>Colletotrichum gloeosporioides</i>	Mango	(Tian, Li, Jiang, Jing, & Shao, 2018)
	<i>Meyerozyma guilliermondii</i>	Surface of rotten pears	-	Pear	(Y. Yan, Zhang, Zheng, Apaliya, et al., 2018)
	<i>Meyerozyma guilliermondii</i> Y-1	Grape	-	Apple	(Y. Huang et al., 2021; C. Sun et al., 2021)
	<i>Pichia guilliermondii</i>	-	-	Peach	(Y. Zhao et al., 2020)
	<i>Candida oleophila</i> P316	The surface of pear fruit	N/A	Kiwifruit	(Gao et al., 2021)
Induction of host defense	<i>Kluyveromyces marxianus</i> XZ1	Apple		Apple	(Zheng et al., 2023)
	<i>Meyerozyma guilliermondii</i>	Surface of rotten pears		Pear	(Y. Yan, Zhang, Zheng, Apaliya, et al., 2018)
	<i>Meyerozyma guilliermondii</i> Y-1	Grape		Apple	(Y. Huang et al., 2021; C. Sun et al., 2021)
	<i>Pichia guilliermondii</i>	N/A		Peach	(Y. Zhao et al., 2020)

2.2 Antagonistic Yeasts as Biocontrol Agents Against Fruit Diseases

In developed countries, the predicted postharvest losses reached 25% of total production, while in underdeveloped countries, they were greater than 50% (Tahir et al., 2022). The Food and Agriculture Organization reported that plant diseases cost the worldwide economy some \$220 billion annually (Hulot & Hiler, 2021). About 85% of postharvest diseases in fruit have been caused by fungi (Shi et al., 2021). It is well documented that most fruit loss at the postharvest stage is highly caused by fungal pathogens such as *Alternaria*, *Aspergillus*, *Botrytis*, *Colletotrichum*, *Diplodia*, *Monilinia*, *Penicillium*, *Phomopsis*, *Rhizopus*, *Mucor* and *Sclerotinia* (Matrose et al., 2021). Postharvest diseases by fungal pathogens have been summarized as brown rot, blue mould, green mould, gray mould, and anthracnose caused by *Monilinia* sp., *Penicillium expansum*, *Penicillium digitatum*, *Botrytis cinerea*, *Colletotrichum musae*, respectively (Carmona-Hernandez et al., 2019).

In addition to economic concerns, diseased fruits may pose a health risk. Several fungal species, including *Penicillium*, *Alternaria*, and *Fusarium*, produce mycotoxins that are toxic to living organisms (J. Liu et al., 2013). Numerous studies have been reported as biological control methods, eco-friendly alternatives for controlling postharvest fruit losses against synthetic fungicides with several bacteria, yeasts and filamentous fungi (C. Chen et al., 2020; J. Liu et al., 2013; Sharma, 2021; S. Wang et al., 2021; H. Zhang et al., 2014; Zhong et al., 2021). Most postharvest decay in fresh fruit is caused by the fungi *Penicillium* and *Botrytis* (Matrose et al., 2021). Due to their wide host range, multiple attack strategies, and capacity for both their asexual and sexual phases to live in favorable or unfavorable environments, these pathogens continue to be challenging to manage (Hua et al., 2018). Besides, *Botrytis cinerea* is one of the most extensively studied necrotrophic fungal pathogens and causes gray mould rot in more than 500 plant species (Hua et al., 2018). *Colletotrichum* sp. causes anthracnose in banana fruit, and anthracnose is especially controlled by synthetic fungicides (Hartati et al., 2015; V.Y. Zhimo, 2016). The fungus *Cladosporium cladosporioides* is the causative agent of grape disease with a significant economic impact on global grape and vine output (Solairaj et al., 2020).

A potential option for fruit protection against phytopathogens at the postharvest stage is presently provided by biocontrol strategies, which protect plants against fungal

infections (Carmona-Hernandez et al., 2019). Developing, registering, and commercializing biocontrol agents to control important postharvest diseases such as *Penicillium expansum*, *Penicillium digitatum*, *Penicillium italicum*, *Fusarium sambucinum*, *Rhizopus stolonifer*, and *Botrytis cinerea* have advanced significantly in the last decade (Spadaro & Droby, 2016). Various yeast species have been tested for their ability to prevent postharvest fruit diseases with *in vitro* and *in vivo* studies (Table 2.3 and Table 2.4).

In addition to these studies, the antifungal activity of yeast VOCs was investigated and suggested as a potential biological control technique against *Botrytis cinerea*, *Penicillium expansum*, *Penicillium digitatum*, *Penicillium italicum*, and *Monilinia* sp. with several studies (Di Francesco et al., 2017, 2020; Grzegorzczak, Zarowska, et al., 2017). Furthermore, due to the small sizes of these molecules and their diffusion through the environment and soil, yeast VOCs could play a key part in their antagonistic activities (Lemos Junior et al., 2020). The antifungal activity of the VOCs of *W. anomalus*, *M. pulcherrima*, and *S. cerevisiae* has been observed in *in vitro* and *in vivo* studies on grapes against *B. cinerea* (Parafati, Vitale, Restuccia, et al., 2015). In a recent study by Sansone et al. (2018), preventive and curative effects of yeast culture on apples were investigated. *Rhodosporidium fluviale* severity was reduced to 55 and 75% at 5 and 10 antagonistic yeasts, respectively, on preventive effect. However, on the curative effect, the reduction in decay was 48% that is lower than the preventive effect. To conclude, when yeast was applied before infection (preventive effect), *Botrytis cinerea* control was more successful than when infection was already present (curative effect). Similar to these studies, higher efficiency has been obtained in preventive than curative activity (Y. Liu et al., 2017; Tang et al., 2015; Vilaplana et al., 2020). As mentioned above, antagonistic yeasts were more effective if they were applied before fungal contamination.

The biocontrol activity of antagonistic yeast was increased with higher concentrations, which was also proved by several researchers (G. Li et al., 2016; Türkoğlu et al., 2022). For example, 9 log *Cryptococcus albidus* was applied to control *Penicillium expansum* infection on fuji fruit; the spoilage area of the samples reduced to 90.54 or 91.39% at 95% relative humidity; however, there was no significant impact with the application of 6 logs of yeast (Türkoğlu et al., 2022).

In another study, Wang et al.(2020) the surface colonization and interaction between *Metschnikowia citriensis* and *Geotrichum citri-aurantii* with SEM studies. As a result, fungal pathogens have been surrounded by yeast species without deformation. So far, the yeast did not cause any damage to the spores and hyphae but could inhibit the spore germination of *Geotrichum citri-aurantii*. Also, several studies have indicated that spore germination rates have significantly decreased with the yeast treatment, in addition to the reduction of germ tube length (Delali et al., 2021; Lin et al., 2022; S. Wang et al., 2020).

When using biocontrol yeasts, the most critical issue is the harmful effect of the yeast and any fermentation in fruit wounds. More than likely, no negative impacts were observed in any fruit when the biocontrol yeasts were used. Several researchers have previously reported that biocontrol yeasts did not affect quality criteria such as fruit firmness, total soluble solids, and titratable acidity during storage (X. Li et al., 2017; Qin et al., 2015; Solairaj et al., 2020; Vilaplana et al., 2020). Habiba et al.(2019) found that the firmness of kinnow fruit has been significantly decreased with the extension of the storage period for both non-treated and yeast-treated samples. Additionally, the least rise in total soluble solids and the least drop of ascorbic acid concentration were reported compared to an untreated kinnow fruit set. . For instance, black rot in red pitahaya was reduced to 20.5% and 7.4% by *Candida inconspicua* and *Pichia kluyveri*, respectively, after 21 d antagonistic yeasts of storage. In comparison, imazalil-treated fruit diseases have decreased to 47.6% (Vilaplana et al., 2020). The outcomes of these studies demonstrated that a biological agent's effects depend on storage conditions and the type of fruit.

Also, Czarnecka et al.(2019) reported that POD activity significantly increased in the wounded tissue of apples, while CAT activity decreased in the control of *Monilinia fructicola* with *Debaryomyces hansenii* and *Wickerhamomyces anomalus* treatment. However, Li et al.(2017) reported that *Sporidiobolus pararoseus* treated table grapes enzymatic activities including PPO, CAT, PAL and APX have been increased. Sun et al.(2018) reported that autoclaved yeast *Rhodospiridium paludigenum* effectively controlled *Penicillium expansum* in pear fruits. Furthermore, the use of autoclaved yeast has the potential to evolve into an antifungal biopesticide for managing postharvest fungal rot in pear fruit since it is non-toxic, economical, and environmentally safe. To conclude, yeasts have been identified as possible biocontrol

agents because of their minimal nutritional requirements, ability to colonize the surface of fruits quickly, and great stability during storage.

Recent studies also investigated the physicochemical quality of yeast-treated fruits by weight loss, fruit firmness, total soluble solids, titratable acidity, and pH analysis (Lu et al., 2019; Vilaplana et al., 2020). These studies demonstrated that yeast may stop fruit from dehydrating because antagonists decrease deterioration and offer an additional barrier to water diffusion (Mahunu et al., 2016; Vilaplana et al., 2020).

Large volumes of yeast biomass are required to scale up the experiments. This biomass must be generated in bioreactors using affordable growth conditions to get high yields while maintaining the candidate bioagent's antagonistic action. For this reason, postharvest decay of pear by *Penicillium expansum* and *Botrytis cinerea* tried to control by *Pichia membranifaciens* NPCC 1250 and *Vishniacozyma victoriae* NPCC 1263 in two different packing houses and showed a higher reduction of incidence (Lutz et al., 2020). In other field trial studies, yeast treatments' biocontrol efficiency has been reported to be much more successful than the artificially contaminated and damaged control when mechanical damage was performed (Ponsone et al., 2016).

Table 2.3 : Evaluation of the antifungal activity of the yeast through *in vitro* studies.

Yeast	Origin	Target fungi	Antagonistic assays	Optimum concentration of biocontrol agent	Inhibition of mycelial growth (%)	References
<i>Aeorobasidium pullulans</i>	Grape berries	<i>Aspergillus tubingensis</i>	Dual culture method	- ¹	12.97-95.69	(Pantelides et al., 2015)
<i>Candida catenulata</i>	Fruits and leaves of citrus plants	<i>Penicillium digitatum</i>	Dual culture method	-	<15	(Perez et al., 2017)
<i>Candida tropicalis</i>	Mandarin orange	<i>Colletotrichum musae</i>	Dual culture method	1 × 10 ⁸ cells/mL	31.3	(Zhimo et al., 2017)
<i>Debaryomyces hansenii</i>	Citrus varieties	<i>Penicillium digitatum</i> <i>Penicillium italicum</i>	Dual culture method	-	41 6	(Hammami et al., 2022)
<i>Debaryomyces hansenii</i>	Marine environment	<i>Mucor circinelloides</i> , <i>Aspergillus</i> sp., <i>Fusarium proliferatum</i> and <i>Fusarium subglutinans</i>	Agar plate inhibition	1 × 10 ⁸ cells/mL	97.2-98.3	(Medina-Córdova et al., 2018)
<i>Debaryomyces hansenii</i>	Blue-veined Rokpol cheese	<i>Monilinia fructicola</i>	Dual culture method	1 × 10 ⁸ yeast cells/mL	69.53	(Czarnecka et al., 2019)
<i>Hannaella pagnoccae</i> , <i>Hannaella luteola</i> , <i>Vishniacozyma carnescens</i> , <i>Hannaella</i> spp. and <i>Rhodotorula paludigena</i>	Cacao pods and leaves	<i>Moniliophthora roreri</i>	Dual culture method	-	> 71	(Estrela Junior et al., 2022)
<i>Hanseniaspora opuntiae</i>	-	<i>Penicillium expansum</i> <i>Botrytis cinerea</i>	Dual culture method	1 × 10 ⁶ cells/mL	45.2 53.4	(Ruiz-Moyano et al., 2016)
<i>M. aff. pulcherrima</i>	Hawthorn	<i>Penicillium expansum</i> <i>Penicillium digitatum</i>	Dual culture method	1 × 10 ⁸ cells/mL	84.2 95.3	(Oztekin & Karbancioglu-Guler, 2021)
<i>Pichia fermentans</i>	Fruits and leaves of citrus plants	<i>Penicillium digitatum</i>	Dual culture method	-	16- 39	(Perez et al., 2017)
<i>Pichia fermentans</i>	Grapes	<i>Penicillium expansum</i> <i>Botrytis cinerea</i>	Dual culture method	1 × 10 ⁶ cells/mL	0-25	(Fernandez-San Millan et al., 2021)
<i>Pichia kluyveri</i>	Marula juice	<i>Botrytis cinerea</i> <i>Monilinia laxa</i>	Agar plate inhibition	1 × 10 ⁸ cells/mL	44.5 54.6	(Mewa-Ngongang et al., 2021)
<i>Rhodotorula mucilaginosa</i>	Black olives	<i>Aspergillus carbonarius</i>	Disc diffusion method	1 × 10 ⁵	75.8	(Tryfinopoulou et al., 2020)
<i>Rhodotorula glutinis</i>	Apple	<i>Monilinia fructigena</i>	Dual culture method	-	18	(Madbouly et al., 2020)

Table 2.3 (continued) : Evaluation of the antifungal activity of the yeast through *in vitro* studies.

Yeast	Origin	Target fungi	Antagonistic assays	Optimum concentration of biocontrol agent	Inhibition of mycelial growth (%)	References
<i>Rhodotorula paludigena</i>	Cacao pods and leaves	<i>Moniliophthora roreri</i>	Dual culture method	-	> 71	(Estrela Junior et al., 2022)
<i>Saccharomyces cerevisiae</i>	Fruits and leaves of citrus plants	<i>Penicillium digitatum</i>	Dual culture method	-	16-39	(Perez et al., 2017)
<i>Saccharomyces cerevisiae</i>	Black olives	<i>Aspergillus carbonarius</i>	Disc diffusion method	1×10^5	66.1	(Tryfinopoulou et al., 2020)
<i>Saccharomyces cerevisiae</i>	Citrus	<i>Penicillium italicum</i>	Pour plate method	1×10^7 cells/mL	> 80	(da Cunha et al., 2018)
<i>Saccharomyces cerevisiae</i>	Mango	<i>Colletotrichum musae</i>	Dual culture method	1×10^8 cells/mL	24.3	(Zhimo et al., 2017)
<i>Saturnispora diversa</i>	Loquat	<i>Colletotrichum gloeosporioides</i>	-	1×10^7 cells/mL	74-85.7	(F. Yan et al., 2022)
<i>Wickerhamomyces anomalus</i>	Grapes	<i>Penicillium expansum</i> <i>Botrytis cinerea</i>	Dual culture method	1×10^6 cells/mL	76-100	(Fernandez-San Millan et al., 2021)
<i>Wickerhamomyces anomalus</i>	Blue-veined Rokpol cheese	<i>Monilinia fructicola</i>	Dual culture method	1×10^8 yeast cells/mL	66.08	(Czarnecka et al., 2019)
<i>Wickerhamomyces anomalus</i>	Peach	<i>Monilinia fructigena</i> <i>Monilinia fructicola</i>	Dual culture method	-	44.12 23.53-37.40	(Grzegorzczuk, Żarowska, et al., 2017)

Table 2.4 : Efficacy of biocontrol strategies for the management of postharvest pathogens in fruits through antagonistic yeasts.

Fungal microorganism	Fruit	Antagonistic yeast	Optimum concentration of biocontrol agent	Disease incidence (%)	References
<i>Alternaria alternata</i>	Pitahaya	<i>Candida inconspicua</i> , <i>Pichia kluyveri</i>	1×10^8 cells/mL	7.4-20.5	(Vilaplana et al., 2020)
<i>Aspergillus carbonarius</i>	Grape berries	<i>Saccharomyces cerevisiae</i>	1×10^6 cells/mL	80	(C. Zhu et al., 2015)
<i>Aspergillus niger</i>	Table grape	<i>Sporidiobolus pararoseus</i>	1×10^9 cells/mL	75	(Q. Li et al., 2017)
<i>Aspergillus ochraceus</i>	Grape berries	<i>Saccharomyces cerevisiae</i>	1×10^6 cells/mL	40	(C. Zhu et al., 2015)
<i>Aspergillus tubingensis</i>	Grape	<i>Aureobasidium pullulans</i>	1×10^7 cells/mL	92.6	(Pantelides et al., 2015)
<i>Botrytis cinerea</i>	Apple	<i>Hanseniaspora opuntiae</i> <i>Metschnikowia pulcherrima</i>	1×10^7 cells/mL	14.2 6	(Ruiz-Moyano et al., 2016)
<i>Botrytis cinerea</i>	Strawberry	<i>Sporidiobolus pararoseus</i>	1×10^8 cells/mL	20	(Shen et al., 2019)
<i>Botrytis cinerea</i>	Grape	<i>Hanseniaspora uvarum</i>	1×10^8 cells/mL	51.8	(Qin et al., 2015)
<i>Cladosporium cladosporioides</i>	Table grape berries	<i>Saccharomyces cerevisiae</i>	1×10^8 cells/mL	51.11	(Solairaj et al., 2020)
		<i>Rhodosporidium paludigenum</i>		37.78	
		<i>Rhodosporidium fluviale</i>		26.67	
		<i>Wickerhamomyces anomalus</i>		42.22	
<i>Colletotrichum gloeosporioides</i>	Ripe olive fruit	<i>Wickerhamomyces anomalus</i>	1×10^8 cells/mL	10-40	(Nally et al., 2015)
<i>Colletotrichum gloeosporioides</i>	Mango	<i>Pseudozyma hubeiensis</i>	1×10^8 cells/mL	49.8	(Konsue et al., 2020)
<i>Colletotrichum musae</i>	Banana	<i>Saccharomyces cerevisiae</i>	1×10^8 cells/mL	25	(Zhimo et al., 2017)
		<i>Candida tropicalis</i>		25	
<i>Fusarium oxysporum</i>	Tomato	<i>Wickerhamomyces anomalus</i>	1×10^6 cells/mL	<50	(Fernandez-San Millan et al., 2021)
<i>Geotrichum citri-aurantii</i>	Orange	<i>Saccharomyces cerevisiae</i>	1×10^7 cells/mL	38	(Ferraz et al., 2016)
		<i>azyma</i>		32	
		<i>Rhodotorula mucilaginosa</i>		28	
<i>Geotrichum citri-aurantii</i>	Citrus	<i>Metschnikowia citriensis</i>	1×10^8 cells/mL	30-50	(S. Wang et al., 2021)
<i>Monilinia fructicola</i>	Apple	<i>Debaryomyces hansenii</i> ,	1×10^8 yeast cells/mL	70.02-92.56	(Czarnecka et al., 2019)
		<i>Wickerhamomyces anomalus</i>			
<i>Monilinia fructicola</i>	Plums	<i>Pichia membranaefaciens</i> , <i>Kloeckera apiculata</i>	5×10^8 cells/mL	19.7	(J. Zhang et al., 2017)
<i>Monilinia fructigena</i>	Apple	<i>Rhodotorula glutinis</i> .	3×10^7 cells/mL	8.9	(Madbouly et al., 2020)
		<i>hansenii</i>		10.6	
<i>Penicillium digitatum</i>	Citrus	<i>Kluyveromyces marxianus</i>	1×10^8 cells/mL	28	(Delali et al., 2021)
		<i>lipolytica</i>		18	
<i>Penicillium digitatum</i>	Mandarin	<i>Yarrowia lipolytica</i>	1×10^7 , 1×10^8 cells/mL	16.67, 19.44	(H. Zhu et al., 2019)

Table 2.4 (continued) : Efficacy of biocontrol strategies for the management of postharvest pathogens in fruits through antagonistic yeasts.

Fungal microorganism	Fruit	Antagonistic yeast	Optimum concentration of biocontrol agent	Disease incidence (%)	References
<i>Penicillium digitatum</i>	Chongqing orangery	<i>Candida fermentati</i> <i>Metschnikowia</i> sp., <i>Hanseniaspora uvarum</i>	1×10^8 cells/mL	6.67 16.67 66.67	(Y. Liu et al., 2017)
<i>Penicillium digitatum</i>	Lemons	<i>Metschnikowia pulcherrima</i>	1×10^8 cells/mL	33.3	(Oztekin & Karbancioglu-Guler, 2021)
<i>Penicillium digitatum</i>	Oranges	<i>Rhodotorula mucilaginosa</i>	1×10^8 cells/mL	31.25	(Ahima et al., 2020)
<i>Penicillium expansum</i>	Apple	<i>Meyerozyma guilliermondii</i>	1×10^8 yeast cells/mL	42.4	(He et al., 2020)
<i>Penicillium expansum</i>	Table grape	<i>Aureobasidium pullulans</i> <i>Cryptococcus magnus</i> <i>Metschnikowia</i> <i>Rhodotorula glutinis</i>	1×10^6 cells mL	33.33-50 6.67-50 10-50 43.33-50	(Rodriguez Assaf et al., 2020)
<i>Penicillium expansum</i>	Apple	<i>Kluyveromyces marxianus</i>	1×10^8 yeast cells/mL	44	(Zheng et al., 2023)
<i>Penicillium expansum</i>	Pear	<i>Wickerhamomyces anomalus</i>	1×10^8 yeast cells/mL	5.56	(Q. Zhang et al., 2019)
<i>Penicillium expansum</i>	Apple	<i>Hanseniaspora opuntiae</i> <i>Metschnikowia pulcherrima</i>	1×10^7 cells/mL	42.1-59.4 42.1	(Ruiz-Moyano et al., 2016)
<i>Penicillium expansum</i>	Table grape berries	<i>Saccharomyces cerevisiae</i> <i>Rhodosporidium paludigenum</i> <i>Rhodosporidium fluviale</i> <i>Wickerhamomyces anomalus</i>	1×10^8 cells/mL	83.33 73.61 77.78 90.28	(Solairaj et al., 2020)
<i>Penicillium italicum</i>	Mandarin	<i>Meyerozyma guilliermondii</i>	1×10^7 yeast cells/mL	57.5	(Z. Wang, Li, Liu, Tian, et al., 2021)
<i>Penicillium expansum</i>	Apple	<i>Meyerozyma guilliermondii</i>	1×10^8 yeast cells/mL	42.4	(He et al., 2020)
<i>Penicillium italicum</i>	‘Valência’ sweet orange	<i>Saccharomyces cerevisiae</i>	1×10^7 cells/mL	75-92	(da Cunha et al., 2018)
<i>Penicillium italicum</i>	Mandarin	<i>Meyerozyma guilliermondii</i>	1×10^7 cells/mL	57.5	(Z. Wang, Li, Liu, Tian, et al., 2021)
<i>Penicillium italicum</i>	Mandarin	<i>Yarrowia lipolytica</i>	1×10^7 cells/mL 10^8 cells/mL	16.67 11.11	(H. Zhu et al., 2019)
<i>Penicillium italicum</i>	Leaves, flowers, fruits, and soils	<i>Saccharomyces cerevisiae</i>	1×10^7 cells/mL	>80	(da Cunha et al., 2018)
<i>Pestalotiopsis vismia</i>	Loquat fruit	<i>Metschnikowia pulcherrima</i>	1×10^8 cells/mL	43.68	(H. Yang et al., 2021)
<i>Rhizopus stolonifer</i>	Peach	<i>Pichia guilliermondii</i>	1×10^8 cells/mL	12.6	(Y. Li et al., 2023)

2.3 Combined Application of Antagonistic Yeasts For Enhanced Biocontrol Efficacy

Studies have also been conducted to see if the combinations of antagonistic yeasts with different agents or processes enhance their performance since inconsistent efficiency of antagonistic yeasts remains one of the challenging issues that need to be addressed. While studies indicate that their biocontrol efficiency may be increased, there are limited studies examining the combination effect on antimycotoxigenic characteristics of yeasts. When these compounds are combined with antagonistic yeasts, their biocontrolling activity against phytopathogenic/mycotoxigenic fungi is enhanced, potentially inhibiting mycotoxins while having no negative effects on yeast viability at the same concentrations. Numerous different agents, such as β -glucan,(D. Wang et al., 2018; Y. Zhao et al., 2020) phytic acid,(Mahunu et al., 2016) methyl jasmonate,(J. Guo et al., 2014; He et al., 2020) melatonin,(C. Sun et al., 2021) N-acetylglucosamine,(Wu et al., 2022) gamma-aminobutyric acid,(Xiao et al., 2022) cinnamic acid,(J. Li et al., 2019) glycine betaine,(Abdelhai, Awad, et al., 2019; J. Liu, Wisniewski, Droby, Vero, et al., 2011; X. Zhang et al., 2017) chitosan,(Godana et al., 2020; T. Yu et al., 2012) and arginine(S. Wang et al., 2022) were combined with yeasts. Apart from the combination with an agent, the efficiency of the antagonistic yeast was also investigated by combining it with different technologies, including hot air,(Wei et al., 2016) microwave,(D. Guo et al., 2016) UV-C irradiation(K. Huang et al., 2015; Nie et al., 2020; Ou et al., 2016; C. Zhang et al., 2013) and heat shock (J. Liu, Wisniewski, Droby, Tian, et al., 2011).

A natural polysaccharide called β -glucan, which is found in the cell walls of many cereals and microorganisms, played an important role in the yeast cell wall by increasing yeast resistance to stress and thereby contributing to the enhancement of antagonistic activity (L. Zhao et al., 2020). In the study of Wang et al.(2018) the application of antagonistic yeast with β -glucan on apple wounds resulted in the induction of enzymes involved in the development of disease resistance and the reduction of oxidative damage. Phytic acid was also combined with yeast and applied to apples by Mahunu et al.(2016) and it was observed that phytic acid had a significant effect on antagonistic activity (Table 2.7).

Table 2.5 : Agents and processes to enhance the biocontrol efficiency of antagonistic yeasts.

Combination		Yeast	Pathogen	Fruit	Storage conditions		Decay incidence (%)			Reference
							Control ¹	Yeast	Combination	
Agent	Compound class									
Adansonia digitata L. (Baobab)	Plant extract	<i>Sporidiobolus pararoseus</i> Y16	<i>Penicillium expansum</i>	Apple	20°C, 95% RH ²		100%	~360%	~40%	(Abdelhai, Tahir, et al., 2019)
Alginate oligosaccharide	Bioactive compound	<i>Meyerozyma guilliermondii</i>	<i>Penicillium italicum</i>	Mandarin	25°C, 4 d		100%	88.3%	36.7%	(Z. Wang, Li, Liu, Tian, et al., 2021)
		<i>Debaryomyces hansenii</i>	<i>Penicillium expansum</i>	Apple	20°C, 95% RH, 5 d		100%	~35-40%	15.28%	(Xi et al., 2022)
					20°C, 5 d		100%	~25%	10.37%	(Q. Yang et al., 2020)
Ascorbic acid	Water soluble vitamin	<i>Pichia caribbica</i>	<i>Penicillium expansum</i>	Apple	25°C, 95% RH, 15 d		100%	38.9%	19.4%	(C. Li et al., 2014)
Beta-aminobutyric acid	Non-protein amino acid	<i>Hanseniaspora uvarum</i>	<i>Botrytis cinerea</i> , <i>Alternaria alternata</i>	Kiwifruit	25°C, 4 d		100%	45-53%	~30-35%	(L. Cheng et al., 2019)
β-glucan	Polysaccharide	<i>Cryptococcus podzolicus</i>	<i>Penicillium expansum</i>	Pear	20°C, 95% RH, 4 d		100%	52.77%	30.53%	(L. Zhao et al., 2020)
		<i>Cryptococcus podzolicus</i>	<i>Penicillium expansum</i>	Apple	20°C, 5 d		100%	~70%	40.88%	(Y. Wang et al., 2018)
Calcium chloride	Inorganic compound	<i>Meyerozyma guilliermondii</i> YS-1, <i>Meyerozyma caribbica</i> YS-3, <i>Cryptococcus albidus</i> YS-4, and <i>Cryptococcus</i> sp. YS-5	<i>Penicillium expansum</i>	Apple	21°C, 7 d		100%	100%	75-80%	(Tournas & Katsoudas, 2019)
Carboxymethyl chitosan	Chitosan derivative, polysaccharide	<i>Cryptococcus laurentii</i>	<i>Penicillium italicum</i>	Grapefruit	22°C, 85-95%, 4 d		73.33%	~45-50%	36.66%	(F. Wang et al., 2019)

Table 2.5 (continued) : Agents and processes to enhance the biocontrol efficiency of antagonistic yeasts.

Combination	Yeast	Pathogen	Fruit	Storage conditions	Decay incidence (%)			Reference	
					Control ¹	Yeast	Combination		
<i>Agent</i>	<i>Compound class</i>								
Chitosan	Polysaccharide	<i>Pichia anomala</i>	<i>Penicillium expansum</i>	Grape	20°C, 95% RH, 5 d	100%	~23%	~15%	(Abdelhai, Tahir, et al., 2019)
Cinnamic acid	Organic compound	<i>Cryptococcus laurentii</i>	<i>Penicillium italicum</i>	Mandarin	25°C, 95% RH, 4 d	100%	~50%	~20%	(Z. Wang, Li, Liu, Tian, et al., 2021))
Methyl jasmonate	Volatile organic compound	<i>Cryptococcus laurentii</i>	<i>Penicillium digitatum</i>	Mandarin	20°C, 4 d	100%	~80%	40.3%	(J. Guo et al., 2014)
		<i>Meyerozyma guilliermondii</i>	<i>Penicillium expansum</i>	Apple	20°C, 95% RH	100%	42.4%	21.6%	(He et al., 2020)
Gamma-aminobutyric acid	Non-protein amino acid	<i>Sporidiobolus pararoseus</i> Y16	<i>Aspergillus tubingensis</i>	Grape	20°C, 70-75% RH, 5 d	100%	52.78%	16.67%	(Xiao et al., 2022)
Glycine betaine	Organic osmolytes	<i>Pichia caribbica</i>	<i>Penicillium expansum</i>	Apple	25°C, 95% RH, 15 d	91.66%	48.81%	32.14%	(X. Zhang et al., 2017)
		<i>Sporidiobolus pararoseus</i> Y16	<i>Penicillium expansum</i>	Apple	20°C, 90% RH, 7 d	100%	- ⁴	11.1%	(Abdelhai, Awad, et al., 2019)
Phosphatidylcholine	Soybean extract	<i>Hanseniaspora uvarum</i> Y3	<i>Penicillium digitatum</i>	Orange	20°C, 95% RH, 7 d	~100%	~30-35%	~5-6%	(W. Li et al., 2016)(W. Li et al., 2016)
Phytic acid	Antioxidant	<i>Pichia caribbica</i>	<i>Penicillium expansum</i>	Apple	20°C, 95% RH, 10 d	100%	~75%	~30%	(Mahunu et al., 2016)
		<i>Rhodotorula mucilaginosa</i>	<i>Botrytis cinerea</i>	Strawberry	20°C, 95% RH, 3 d	100%	~50%	~25-55%	(C. Zhang et al., 2013)
Salicylic acid	Phenolic compound	<i>Pichia membranaefaciens</i>	<i>Penicillium digitatum</i> <i>Penicillium italicum</i>	<i>Citrus sinensis</i> L.	20°C, 85-90% RH, 6 d	100%	88.89%	77.56%	(Zhou, Ming, et al., 2014)
							83.33%	72.22%	
Sodium bicarbonate	Chemical compound	<i>Kluyveromyces marxianus</i>	<i>Penicillium digitatum</i>	Mandarin	3-6 d	100%	41.67-70%	18.33-58.33%	(Geng et al., 2011)
Trehalose	Glucose derivative	<i>Sporidiobolus pararoseus</i> Y16	<i>Penicillium expansum</i>	Grape	20°C, 6 d	100%	~25-30%	~15-20%	(Dhanasekaran et al., 2021)

Table 2.5 (continued) : Agents and processes to enhance the biocontrol efficiency of antagonistic yeasts.

Combination	Yeast	Pathogen	Fruit	Storage conditions	Decay incidence (%)			Reference	
					Control ¹	Yeast	Combination		
<i>Agent</i>	<i>Compound class</i>								
Oligochitosan	Oligosaccharide	<i>Pichia caribbica</i>	<i>Alternaria alternata</i>	Tomato fruit	20°C, 90% RH, 4 d	~85%	~20%	~5-20%	(X. Zhang, Zhou, et al., 2022)
Oligogalacturonide	Oligosaccharide	<i>Candida oleophila</i>	<i>Botrytis cinerea</i> , <i>Alternaria alternata</i>	Kiwifruit	25°C, 4 d	100%	51%	33%	(Gao et al., 2021)
<i>Process</i>	<i>Condition</i>								
Hot water treatment	45°C, 5 min	<i>Aureobasidium pullulans</i> L1, L8 and <i>Trichoderma harzianum</i> Th1	<i>Neofabraea vagabunda</i> ID02	Apple in two ripening class	20°C, 21 d	Untreated ~60-80%	~5-10%	Complete inhibition	(Di Francesco et al., 2022)
	53°C, 2 min	<i>Pichia membranaefaciens</i>	<i>Penicillium digitatum</i> <i>Penicillium italicum</i>	<i>Citrus sinensis</i> L.	20°C, 8 d	Untreated ~100%	~100%	88.89% 83.33%	(Zhou, Deng, et al., 2014)
Microwave	2450 MHz, 2 min	<i>Metschnikowia pulcherrima</i>	<i>Penicillium citrinum</i>	Jujube	25°C, 5 d	100%	36%	21%	(D. Guo et al., 2016)
UV-C irradiation	4 kJ/m ² , 12 min	<i>Cryptococcus laurentii</i>	<i>Botrytis cinerea</i> , <i>Alternaria alternata</i>	Tomato fruit	20°C, 4 d	Untreated 100%	~40-50%	~24-30%	(C. Zhang et al., 2013)(C. Zhang et al., 2013)
	5 kJ/m ² , 15 min	<i>Metschnikowia pulcherrima</i>	<i>Alternaria alternata</i>	Jujube	22°C, 7 d	100%	~20-30%	~15-20%	(D. Guo et al., 2015)(D. Guo et al., 2015)
	4 kJ/m ² , 12 min	<i>Pichia cecembensis</i>	<i>Fusarium oxysporum</i> , <i>Alternaria alternata</i>	Melon	25°C, 5 d	Untreated 100%	~40-50%	~20-25%	(K. Huang et al., 2015)

¹: sterile distilled water, ²: relative humidity, ³: about, ⁴: unexamined

Zhang et al.(2013) showed that spoilage was reduced when phytic acid was applied to strawberries together with antagonistic yeast, and this activity was also related to the concentration of the agent. In the range of 2-10 $\mu\text{mol/mL}$ phytic acid concentrations tested, 4 $\mu\text{mol/mL}$ was found to be the most effective, followed by 6 $\mu\text{mol/mL}$. It is also known that various extracts of natural origin also have antimicrobial effects. For this reason, its use as a biocontrol agent with yeasts has also been the subject of studies. In one study, *Adansonia digitata* L. (Baobab) extract was applied together with *Sporidiobolus pararoseus* Y16 to increase the yeast's activity against *Penicillium expansum*. There was no correlation between the disease incidence value and the concentration of the agent used with the yeast. In the concentration range of 25-200 mg/mL of extract analysed, the most effective concentration was 100 mg/mL with a disease incidence value of approximately 40% (Abdelhai, Tahir, et al., 2019). In another study, the activity of *Wickerhamomyces anomalus* BS91 was increased by using cardoon leaf extracts, which have been shown to have antimicrobial activity in different studies (Restuccia et al., 2020). The antimicrobial activity of plants was associated with the various components in their composition; they can be effective against pathogens by taking part in the mechanism against pathogens either when considered alone or in combination with antagonistic yeasts. One of them is salicylic acid, a phenolic component found in plants and emerged as an alternative to manage fungal diseases (Lyoufsi et al., 2021). In addition, yeast combination studies also included salicylic acid, and its significant effect has been shown (Coqueiro et al., 2015). There is a gap in the literature that needs to be filled due to the lack of studies exploring the causes of the increase in efficacy. Coqueiro et al.(2015) observed the molecular and genetic changes caused by the applied agents in food. Salicylic acid was thought to be involved in the defense mechanisms based on the application results on sweet oranges. With the salicylic acid application, Ahima et al.(2020) detected an increase in lignin, one of the most important mechanisms against fungal infections. Zhou et al.(2014) discovered that when salicylic acid is applied to citrus fruit alone, it has no effect but has an antagonistic impact when combined with antagonistic yeasts. Qin et al.(2015) observed an increase in enzyme activity when salicylic acid was combined with *Hanseniaspora uvarum*. In another study investigating the mechanism, the lowest decay incidence result was obtained at 250 $\mu\text{g/mL}$ of ascorbic acid in *Pichia caribbica* yeast combination application at concentrations ranging from 150-350 $\mu\text{g/mL}$ of ascorbic acid. When the expressed proteins were analyzed, some decreased

while others increased. In particular, the expression of genes associated with some enzymes increased, and it was stated that these genes were involved in the biocontrol of antagonistic yeasts (Q. Yang et al., 2020). Similarly, the combination of 250 µg/mL ascorbic acid with *Pichia caribbica* was more effective and improved oxidative stress tolerance and antioxidant enzyme activities (C. Li et al., 2014).

In addition to these agents, gamma and beta aminobutyric acid compounds, which are classified as different non-proteinogenic amino acids, were applied to the fruits in combination with yeasts (L. Cheng et al., 2019; Xiao et al., 2022). While it is important to examine the increase of antagonistic activity at the disease incidence level, the yeast population is expected to increase as yeast adaptation to the environment increases. According to Cheng et al.(2019), since no adverse effects were observed in the yeast population, the beta aminobutyric acid and yeast combination can be employed to manage postharvest diseases.

In the studies, almost all of the agents used for combination were preferred because of their protective role on foods, inducing various enzyme activities or indirectly affecting nutrients. For instance, methyl jasmonate, a plant-derived volatile compound known as endogenous phytohormone (H. Wang et al., 2020), increased the activity of enzymes (POD, PPO, PAL, and CAT) that are important in the treatment of blue mould disease (He et al., 2020). In addition to the effect on enzyme, the effect on antioxidant activity was also shown for Chinese bayberries (K. Wang et al., 2010). Besides, Guo et al.(2014) emphasized that the application of methyl jasmonate alone was not as effective as its combination with antagonistic yeast. Additionally, enzymes responsible for defencing in *Kluyveromyces marxianus* were found to be improved when combined with N-acetylglucosamine (Wu et al., 2022).

On the other hand, the antagonistic activities of yeasts have been associated with compounds such as pulcherrimin. Therefore, promoting pulcherrimin production will lead to increased antagonistic activity, as reported by Wang et al.(2022). They used arginine with *Metschnikowia citriensis* yeast against *Geotrichum citri-aurantii* and found that it effectively enhanced the yeast's efficiency. However, as in other publications, although various mechanisms have been examined, further studies on the causes of inducing the mechanism are required.

Nowadays, consumers tend to use natural products; therefore, combining antagonistic yeasts with natural and safe agents appears to be an important parameter in biocontrol (T. Yu et al., 2012). Blue mould disease in fruits can cause significant food loss; therefore, Godano et al.(2020) studied and showed enhanced inhibition of blue mould disease in grapes when *Pichia anomala* was supplemented with chitosan. For this reason, chitosan has been indicated as an alternative polymer among the components to be combined. Zhang et al.(2022) reported that oligochitosan combined with *Pichia caribbica* positively affected the ROS mechanism and the enzymes active in this mechanism. The implementation of combined treatment increased fruit resistance by decreasing the disease-causing components in the fruit. The natural ingredients include different plant extracts and bioactive plant components. For example, it was determined that the combination of the key antagonistic yeast *Pichia caribbica* with bamboo leaf flavonoid prevented the growth of *Penicillium expansum* and reduced the amount of PAT in *in vitro* analyses (Mahunu et al., 2018). Calcium chloride is a compound used in combination with yeasts and has been shown to increase the antifungal activity of *Vishniacozyma victoriae* and *Pichia membranifaciens* yeasts (Gramisci et al., 2018).

Additionally, the storage temperature may also have a significant impact on the efficiency of antagonistic yeasts. For example, while the decay incidence was found to be in the range of 75%-80% when the yeast and CaCl₂-treated apples were stored at 21 °C, it was observed between 30% and 60% at 3 °C (Tournas & Katsoudas, 2019). The effectiveness of the agent used may vary depending on the product and the target microorganism. For example, calcium chloride alone has no effect on *Colletotrichum musae*, the cause of crown rot on bananas, whereas the antagonistic activity of yeast significantly increased the use of calcium chloride (Bastiaanse et al., 2010).

Different physical methods may be applied to extend the shelf life of fruits and vegetables and to reduce losses due to postharvest mould-related diseases. As with the agents, the processes can be combined with yeasts to provide various advantages by increasing their antagonistic activity (H. Zhang et al., 2017). UV-C irradiation is one of the methods applied to fruit and has also been tested for preventing postharvest fungal diseases (Sánchez et al., 2021). According to a recent study, it helps to improve the postharvest quality of fruit by boosting the activity of antioxidant enzymes and reducing mycotoxins (T. Sun et al., 2022). In addition to demonstrating the method's

efficacy when applied alone, combination studies with yeasts were also available. Likewise, the study of Zhang et al.(2013) reported that enzyme production was enhanced, and UV-C irradiation had no effect on the growth of *Cryptococcus laurentii* yeast applied for biocontrol purposes. *Pichia cecembensis*, one of the antagonistic yeasts used for biocontrol, significantly contributed to the control of postharvest decay in melon when combined with UV-C (K. Huang et al., 2015). Increased enzyme activity and improved antioxidant properties contributed to the effectiveness of the combination of UV-C irradiation and *Candida tropicalis* against fungal decay in pineapple (Ou et al., 2016). Besides, it was aimed to prevent the undesirable changes caused by *Penicillium citrinum* in jujube fruit by combining *Metschnikowia pulcherrima* and microwave application (D. Guo et al., 2016). As in other studies, this combination produced more effective results than the applications conducted alone. Terao et al.(2017) combined hot water brushing and ultraviolet C irradiation methods with *Candida membranifaciens* CMAA-1112 antagonistic yeast as postharvest treatment. It was observed that the combination of process and yeast had no adverse effect and showed an additive effect to control green mould on orange. In UV-C and yeast treatment, as in other studies, the change in enzyme activities was also analysed since it is an important criterion. Activity changes of PAL, GLU, and CHT enzymes were analyzed, and UV-C treatment with *Candida tropicalis* was presented as an alternative to extend the shelf life of pineapple (Ou et al., 2016). The modified atmosphere packaging (MAP) method, which is one of the important food preservation methods, controlled fungal growth in apples by applying antagonistic yeasts depending on the selected gas composition (Sadeghi et al., 2021). Another study found that combining MAP and yeasts on sweet cherries increased the product's shelf life and delayed the appearance of fungal disease effects (de Paiva et al., 2017). Zhao et al.(2009) treated cherry tomatoes with hot air at 38 °C in combination with *Pichia guilliermondii*, and obtained more effective results contrary to single applications. There may be different mechanisms behind this increased effect. For example, in the study of Wei et al. (2017), it was shown that hot air treatment affected defense mechanisms in cherry tomato fruit, especially phenylpropanoid metabolisms, which have an impact on phenolics and flavonoids. In addition, thermal shock treatment has also been shown to increase the antagonistic activity of yeasts. Cheng et al.(2016) documented that thermal shock treatment increased the antagonistic activity of *Rhodotorula mucilaginosa* and improved its stress tolerance, comparable results were

also obtained in a study with *Metschnikowia fructicola* (J. Liu, Wisniewski, Droby, Tian, et al., 2011). Ultrasound and ohmic heating were also mentioned as alternative physical application methods (H. Zhang et al., 2017). Furthermore, different antagonistic yeasts combined with hot water treatment showed a remarkable effect by completely inhibiting the growth of *Neofabraea vagabunda* in apples (Di Francesco et al., 2022). Similarly, Zhou et al. (2014) showed the effectiveness of hot water treatment in combination with antagonistic yeast to manage green and blue mould disease. While the treatment did not affect the growth of antagonistic yeast at different storage temperatures, it was interpreted that the hot water treatment had an effect, especially on nutrient and space competition, and the higher efficiency of the combination was attributed to this mechanism. Additionally, the activities of different enzymes, especially those responsible for the defense mechanism, were increased. However, it has been shown that the biocontrol activities of antagonistic yeasts change in the presence of temperature stress (Dai et al., 2021). Therefore, when antagonistic yeasts are combined with hot applications, it is important how the procedure is carried out. Another application method of antagonistic yeasts is the production of fruit-coating material containing biocontrol agents. Apple coating material integrated with *Metschnikowia pulcherrima* was produced and observations made during storage showed that the coating with the biocontrol agent increased the antagonistic yeast's capacity to colonize and was more effective in terms of antifungal activity. In addition, it was shown that the application also has an antimycotoxigenic effect (Settier-Ramírez et al., 2022). As a result, combinations with antagonistic yeasts may be used to reduce fungi-induced postharvest diseases in fruits to the maximum extent possible. In this way, it will be possible to reduce the use of synthetic fungicides (Wei et al., 2016).

2.4 Commercial Application of Yeast-Based Biocontrol Products

Scientists have been driven to develop novel antifungal compounds as bio-preservatives due to the increased public desire for pesticide-free foods (Hernández et al., 2022). Despite extensive research, developing efficient biocontrol products and transferring knowledge from the lab to the commercial level remains challenging (Hernández et al., 2022; Wisniewski et al., 2016). These difficulties can be attributed to a number of things, such as a lack of mechanistic understanding, poor marketing,

yeast cell viability, inconsistent efficacy issues, challenging and costly registration issues, and low commercial potential of these products (Freimoser et al., 2019).

Some yeast-based biofungicides have already been commercialized from *Candida oleophila*, *Aureobasidium pullulans*, *Metschnikowia fructicola*, *Metschnikowia pulcherrima*, *Cryptococcus albidus*, and *Saccharomyces cerevisiae* (Table 2.6) (A. Dukare et al., 2021; Spadaro & Droby, 2016; Wisniewski et al., 2016). The first-generation yeast-based biofungicide derived from *Candida oleophila* I-182 strain and marketed under the brand name Aspire[®]. However, it is no longer available due to poor marketing and inconsistent efficacy (Wisniewski et al., 2016). Then, Nexy[®] was produced from *C. oleophila* O and approved to be used in Europe as a plant protection agent (Freimoser et al., 2019). Recently, *Metschnikowia fructicola* was approved in Europe and Israel to be used on stone fruits, strawberries, and grapes (EFSA, 2017). It was first marketed as Shemer[®] by Bayer Company (Germany) and more recently as Noli[®] by Koppert Biological Systems (Netherlands) (Piombo et al., 2018; Spadaro & Droby, 2016).

Table 2.6 : Commercially available yeast-based biofungicides.

Product	Antagonistic yeast	Country/Company	Fruit	Target pathogens	In use
Aspire	<i>Candida oleophila</i>	Ecogen/USA	Pome, citrus, strawberry, stone fruit	<i>Botrytis</i> , <i>Penicillium</i> , <i>Monilinia</i>	No
Nexy	<i>Candida oleophila</i>	Lesaffre/Belgium	Pome	<i>Botrytis</i> , <i>Penicillium</i>	Yes
Candifruit	<i>Candida sake</i>	IRTA/Sipcam-Inagra, Spain	Pome	<i>Botrytis</i> , <i>Penicillium</i> , <i>Rhizopus</i>	No
Yield Plus	<i>Cryptococcus albidus</i>	Lallemand/South Africa	Pome, citrus	<i>Botrytis</i> , <i>Penicillium</i> , <i>Mucor</i>	No
Noli	<i>Metschnikowia fructicola</i>	Koppert/Netherlands	Table grape, pome, strawberry, stone fruit, sweet potato	<i>Botrytis</i> , <i>Penicillium</i> , <i>Rhizopus</i> , <i>Aspergillus</i>	Yes
Boni Protect	<i>Aureobasidium pullulans</i>	Bio-ferm/Austria	Pome	<i>Penicillium</i> , <i>Botrytis</i> , <i>Monilinia</i>	Yes
Excellence B-Nature	<i>Metschnikowia pulcherrima</i> <i>Torulaspora delbrueckii</i>	Lamothe-Abiet/France	Grapes	To control ecological niche	Yes
Zymaflore Egide	+ <i>M.pulcherrima</i>	Laffort, France	Grapes	To inhibit the growth of indigenous flora.	Yes

In order to form novel yeast-based formulations, it is necessary to comprehend the biocontrol mechanisms of action. Optimizing parameters that maintain yeast viability and consistent biocontrolling activity is also required to produce effective commercial biopesticides. For this reason, yeasts are subjected to various stressful environmental conditions, including high temperatures, freeze/spray drying (desiccation), and

oxidative stress. Furthermore, the application method should be considered during commercialization because there are several typical ways to utilize fungicides, including spraying, dipping, drenching, and coating (J. Liu et al., 2013).

Commercial biofungicides should have an adequate shelf life not to lose their efficacy, which can be produced in dry and liquid formulations. Compared to liquid formulations, dry formulations have advantages such as longer shelf life, simpler transportation, and distribution options without the need for refrigeration during storage. However, the need for spray/freeze drying facilities and filler addition raises manufacturing costs. In contrast, liquid formulations are simple to produce without needing rehydration. Sugar protectants (5-10% trehalose or galactose) or oxidative stability agents (L-ascorbic acid) can be added to these formulations to maintain cell viability and biocontrol efficacy (A. Dukare et al., 2021; J. Liu et al., 2013). For example, Bühlmann et al.(2021) investigated dry and liquid *Metschnikowia pulcherrima* formulations for biocontrol applications against apple postharvest diseases. When *M. pulcherrima* was combined with 10% skim milk powder and 10% sucrose during freeze-drying, cell viability climbed to 72.1% with 3.6×10^{12} viable cells. The cell viability was stable for the first 10 weeks at both 4 and 22 °C, further, it declined at 22 °C and remained stable 4 °C for eight months. Furthermore, *M. pulcherrima* survived for several months in water at 22 °C without the use of any protectant. Additional research is required to find new yeast isolates and develop novel yeast-based green formulations to replace them with synthetic fungicides.

2.5 Conclusions and Future Prospects

In conclusion, growing public awareness and tighter regulations have led to trends toward bio-safe, ecological and cost-effective strategies to inhibit post-harvest diseases in fruits and fruit-based products caused by pathogenic and/or mycotoxigenic fungi. Here, the current literature findings on the use of antagonistic yeasts in the postharvest treatments of fruit-related were discussed. Additively, several applications for enhancing the activity of antagonistic yeasts were evaluated to ensure the highest performance under the best conditions.

Antagonistic yeasts may exhibit inconsistent biocontrolling efficacy. They can be used in collaboration with other yeasts, bacteria, bio-agents, and physical processes to overcome this issue. However, before applying these combinations, yeasts must be

checked to verify if their viability is maintained at the applying concentration. In this way, these integrated disease management approaches may act synergistically or additively to increase biocontrolling activity. Understanding the mechanisms of biological control action is critical for the development of rational combinations of selected fungi or antagonistic consortia. However, there are limited studies regarding how tailored microbial consortia will prevent fungi proliferation and decompose mycotoxins, in which food matrix, at what level, and the conditions. As a result, future approaches may rely on different combinations of microorganisms to provide additional benefits in mycotoxin degradation and adsorption.

Furthermore, climate change has reduced crop yields and triggered the proliferation of phytopathogenic and mycotoxigenic fungi. Thus, Good Agricultural Practice and Good Manufacturing Practice applications should be implemented in this regard during the postharvest stage. The main challenge is adapting these strategies from the laboratory to large-scale commercial applications.

3. BIOPROSPECTION OF *METSCHNIKOWIA* SP. ISOLATES AS BIOCONTROL AGENTS AGAINST POSTHARVEST FUNGAL DECAYS ON LEMONS WITH THEIR POTENTIAL MODES OF ACTION²

3.1 Abstract

In this study, various fruit were employed as biocontrol yeast reservoirs, and eleven distinct yeast cultures of *Metschnikowia* sp. belonging to 6 different species were identified with sequence-based analysis of the D1/D2 domain of 26S rDNA. For initial screening, *Metschnikowia* isolates were tested on *Fusarium oxysporum*, *Botrytis cinerea*, *Penicillium digitatum*, *Penicillium expansum*, and *Alternaria alternata*. The highest antagonism was obtained on green and blue *Penicillium* (83.63-100 %). All tested yeasts showed chitinase activity, while some had protease, pectinase, cellulase, β -1-3 glucanase, and gelatinase activities. Since lemons have high pectin content, three pectinase-free cultures at tested conditions with high *in vitro* antagonism on *Penicillium* were selected and used on lemons. The activities of the *in vitro* antifungal studies were found to be compatible with those of the *in vivo*, and *P. digitatum*'s incidence was found to be higher than that of *P. expansum* on lemons. All tested pectinase-free *Metschnikowia* sp. lead to a significant reduction in the disease incidences and lesions at varying levels. The combined effect of lytic enzyme secretion, iron depletion, and volatile organic compounds (VOCs) production determined the antifungal mechanism of action. *M. aff. fructicola* demonstrated the highest biocontrol efficacy against *Penicillium* on lemons with an increasing shelf-life. The use of tentatively pectinase-negative *Metschnikowia* sp. as an antifungal biocontrol agent on lemons was considered for the first time. The findings will shed light on the effective use of *Metschnikowia* sp. as a potential biofungicide against the growth of postharvest fungal pathogens.

² This chapter is based on the paper “Oztekin, S., & Karbancioglu-Guler, F. (2021). Bioprospection of *Metschnikowia* sp. isolates as biocontrol agents against postharvest fungal decays on lemons with their potential modes of action. *Postharvest Biology and Technology*, 181, 111634.”

Keywords: Biocontrol yeast, *Metschnikowia* sp., biofungicide, *Penicillium*, postharvest disease, pulcherrimin

3.2 Introduction

Nearly 33 % of food is wasted worldwide, accounting for around 1.3 billion tons per year (FAO, 2014). Fungal spoilage-induced postharvest diseases rate first in creating large quantities of food waste. As fruit contain a high amount of water, sugar, and essential nutrients, fungi can quickly proliferate and find optimal habitat, particularly in wounded fruit (Nunes, 2012; Spadaro & Gullino, 2004). Moreover, some fungi may produce toxic secondary metabolites called mycotoxins, threatening public health (Y. Chen et al., 2017).

Postharvest decay of fresh fruits and vegetables was estimated to be 35 % in developed countries and up to 50 % in developing countries due to inadequate storage and transportation facilities (Ahmad & Siddiqui, 2015; Bordoh et al., 2020). Postharvest diseases are mostly associated with fungi, including *Penicillium*, *Aspergillus*, *Botrytis*, *Geotrichum*, *Fusarium*, *Alternaria*, *Mucor*, *Monilinia*, *Rhizopus*, and *Gloeosporium* (Barkai-Golan, 2001).

Citrus fruits (orange, lemon, mandarin grapefruit and lime) are highly prone to different phytopathogens such as fungi, bacteria and viruses (Z. Wang et al., 2020). Common names of postharvest diseases in citrus fruit are based on the symptoms they produce (Zacarias et al., 2020). These postharvest diseases, including green and blue mould, sour rot, *Rhizopus* rot, diplodia stem-end rot, phomopsis stem-end rot and brown rot, significantly affect the fresh fruit quality and cause economic losses (Cerioni et al., 2013; Zacarias et al., 2020). Among them, green and blue mould disease are the most important and the primary cause for green and blue mould diseases in citrus fruit was mostly associated with *Penicillium digitatum* (Perez et al., 2017) and *Penicillium expansum* (Vilanova et al., 2012), respectively. Additionally, *P. expansum* can produce patulin (Frisch & Niessen, 2019) and cause carcinogenic, mutagenic, and teratogenic effects (Spadaro et al., 2013).

Harnessing of beneficial microbes during the postharvest period was more effective than preharvest treatments for fungal diseases (A. S. Dukare et al., 2019). Many postharvest diseases can be overcome with synthetic fungicides, but pesticide residues

and the development of resistant fungi may lead to additional issues (Jensen et al., 2016). Therefore, natural and eco-friendly biofungicides are desperately needed as promising biological control agents from beneficial microorganisms (Droby et al., 2009; Vilaplana et al., 2020). Furthermore, the European Food Safety Authority (EFSA) urges using low-risk or non-chemical pesticides for health and the environment and set strict regulations for maximum pesticide residue levels in crops (EFSA et al., 2020; Sarrocco & Vannacci, 2018).

Low toxicity chemicals (Palou et al., 2016) such as organic and inorganic salts (Cerioni et al., 2013), essential oils (Oliveira Filho et al., 2021), plant extracts (Gatto et al., 2016), antifungal peptides (Luz et al., 2017), antifungal coatings (Shao et al., 2015) and biological control methods using different microorganisms (P.-H. Chen et al., 2018; da Cunha et al., 2018; Y. Liu, Yao, et al., 2019; Perez et al., 2016; Z. Wang et al., 2020; Z. Wang, Zhong, Chen, Du, et al., 2021) were evaluated as alternative methods to conventional fungicides. The trend towards employing antagonistic yeasts for biological control purposes has recently grown exponentially with yeasts from the phyllosphere, roots, and soil (A. S. Dukare et al., 2019). The fruit surface serves as a natural reservoir for antagonistic microorganisms corresponding to additive-free and safe biocontrol yeasts (Collazo et al., 2017). The most widely used antagonistic microorganisms for biocontrol purposes refer to bacteria and yeasts. Moreover, the yeasts come to the fore with their unique characteristics such as rapid colonization on fruit surfaces, genetic stability, growing potential without special nutrients, being safe to human health, being effective against pathogenic fungi, not producing secondary metabolites, and toxic pesticides (J. Liu et al., 2013). Yeasts can antagonize with pathogenic bacteria and spoilage yeasts/moulds, making them potential biocontrol agents (Hatoum et al., 2012). Yeasts have been utilized in agricultural applications due to their GRAS (Generally Recognized As Safe) nature (Naga Padma et al., 2011). Overall, antagonistic yeasts can be recruited as biofungicides to minimize or prevent the use of chemicals as green alternatives to conventional fungicides (Choińska et al., 2020; Vilaplana et al., 2020; H. Zhang et al., 2016).

Metschnikowia sp. holds great potential to be natural and successful biocontrol tools, capable of producing a maroon-red pigment called pulcherrimin, and the color intensity was proportional to antimicrobial activity, or vice versa (Sipiczki, 2006, 2020). Hydrophilic pulcherriminic acid can form a complex with ferric ions to produce

hydrophobic pulcherrimin with high antifungal activity (Saravanakumar et al., 2008). Some yeast-based products are commercially available against *Penicillium*, *Aspergillus*, *Rhizopus*, and *Botrytis* (A. S. Dukare et al., 2019). For example, European Union approved the biofungicide Shemer (from *M. fructicola* NRRL Y-27328) to be used in various fruit such as stone fruit, strawberries, and grapes (EFSA et al., 2017; Kurtzman & Droby, 2001; Piombo et al., 2018). It may be possible to re-formulate existing products or discover novel yeast-based products for postharvest disease control by elucidating the modes of action of yeasts (X. Zhang et al., 2020). Nutrient and space competition, induced fruit resistance, mycoparasitism, and antibiosis are thought to be key mechanisms of antagonistic yeasts. Furthermore, volatile organic compounds (VOCs), siderophores, extracellular lytic enzymes, reactive oxygen species (ROS), and biofilm formation capabilities can contribute to the inhibition mechanism (Di Francesco et al., 2016; Spadaro & Droby, 2016). VOCs, biofilm formation, enzyme production have an important role in the inhibition mechanism of yeasts. VOCs, unlike other mechanisms, do not require contact, affect by damaging or hydrolyzing the cell wall, inhibiting the DNA synthesis and inducing systemic host resistance (Choińska et al., 2020; X. Zhang et al., 2020). The firm attachment of the yeast cells to fungal hyphae and the partial degradation of fungal mycelia have been associated with hydrolytic enzyme production by yeasts (Bar-Shimon et al., 2004; Parafati, Vitale, Restuccia, et al., 2015). The lytic activity of one group of enzymes may stimulate that of the second such as α - and β -glucosidase influence the activity of β -glucanases (Pretscher et al., 2018). The biofilm formation ability of yeasts was also directly associated with its adherence capacity to surfaces. Biofilms on injured tissues of the plant surface can provide a physical barrier against the expansion of the hyphae of the pathogenic fungi (Sipiczki, 2020). The colonization capacity and pulcherrimin production were also accompanied by increased biofilm formation (Y. Liu, Yi, et al., 2019; Parafati, Vitale, Restuccia, et al., 2015).

Growing awareness has increased the trend of natural products that have led us to test the efficacy of fruit-based *Metschnikowia* yeasts against postharvest fungal pathogens along with their potential mode of action (extracellular lytic enzymes, enzymatic profiles, and iron depletion). Secondly, the selected pectinase-free yeasts were tested against *Penicillium* sp. through *in vitro* (biofilm formation and VOCs production) and *in vivo* analysis on lemons. In this study, the use of specifically selected pectinase-

negative *Metschnikowia* yeasts for biocontrol purposes on lemons was considered for the first time.

3.3 Material and Methods

3.3.1 Mould cultures

Fusarium oxysporum and *Alternaria alternata* were obtained from Bayburt University Food Microbiology Laboratory (Purutoğlu et al., 2020), and *Botrytis cinerea* was taken from the Department of Plant Protection, Selçuk University, Konya, Turkey. *Penicillium digitatum* NRRL 1202 and *Penicillium expansum* DSM6284 were purchased from the ARS Culture Collection (Peoria, IL, USA) and DSMZ (German Collection of Microorganisms and Cell Cultures GmbH), respectively. All mould cultures were cultivated on Potato Dextrose Agar (PDA) at 25 °C, and stock solutions were prepared in 20 % (v/v) glycerol and kept at -80 °C for further analysis.

3.3.2 Fruit

Lemons (*Citrus limon* L.) were obtained from a local organic store by random selection based on their uniform size, color, maturity, and defect-freeness. The fruit were superficially disinfected with 2 % (w/v) of sodium hypochlorite (NaOCl) solution for 2 min, and then rinsed twice with pure water and air-dried at 25 °C.

3.3.3 Sampling and yeast isolation

The yeasts belonging to *Metschnikowia* sp. genera were isolated from various fruit (grape, rosehip, hawthorn, blackberry, and cornelian cherry) collected about 500 g from organic bazaars in Turkey between April and October 2018. After labeling, the samples were stored in sterile specimen tubes and delivered aseptically. Approximately 10 g of relevant fruit samples were weighed and homogenized in 90 mL of 0.85 % sterile saline solution. Once decimal dilutions prepared, 100 µL of serial dilutions were spread over yeast extract peptone dextrose agar (YEPA agar) plates supplemented with chloramphenicol and 0.05 % (w/v) FeCl₃ (YEPA-10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 20 g/L agar, 0.1 g/L chloramphenicol). All chemicals used in this study were supplied from Merck (Darmstadt, Germany). Following the incubation at 25 °C for 72 h, the reddish colonies were randomly selected for their pulcherrimin production characteristics and examined for cell

morphology under a light microscope. Single-cell colonies of each culture were obtained by streak plate method on YEPD agar with 0.05 % (w/v) FeCl₃ and incubated under the same conditions as previously mentioned. Finally, each pure culture was preserved at 4 °C in slants and 20 % glycerol stock at -80 °C for further use.

3.3.4 Yeast Identification

Genotypic characterization and differentiation of yeasts were conducted by RAPD-PCR analysis using M13 primer (Dertli et al., 2016). The phenol-chloroform method was used for genomic DNA extraction. The reactions were conducted in a 25 µL reaction mix containing: 5 µL 5 × PCR buffer for Taq polymerase (Promega, Madison, USA), 2.5 mM of each dNTPs (Bioline, London, UK), 1.5 U Taq polymerase (Promega), 25 pMol of primer M13 (5' – GAGGGTGGCGGTTCT – 3') and 50-100 ng yeast DNA as a template. PCR was conducted using a thermal cycler (TC9639, Benchmark Scientific, New Jersey, USA). RAPD-PCR program consisted of: 94 °C, 2 min; 35 cycles: 94°C, 1 min; 42°C, 20 s; 72°C, 2 min; and 72°C, 10 min. The PCR products were electrophoresed on 1 % (w/v) agarose gels at 90 V for 1.5 h and photographed under UV illumination following ethidium bromide staining. The yeasts showing different RAPD-PCR patterns were selected for further genotypic identification.

Metschnikowia yeasts were identified by sequencing of the D1/D2 domain of the 26S rDNA using NL1 (5' – GCATATCAATAAGCGGAGGAAAAG – 3') and NL4 (5' – GGTCCGTGTTTCAAGACGG – 3') primers (O'Donnell K, 1993). Relevant yeast DNAs were used as templates in PCR reaction, as mentioned above. PCR program for the amplification of 600 bp region was: 95 °C, 10 min; 40 cycles: 94 °C, 1 min; 54 °C, 30 s; 72 °C, 1 min, and 72 °C, 7 min. The amplified gene was separated using 1 % agarose electrophoresis. The amplicons were sequenced using the Applied Biosystems 3500xL Genetic Analyzer at MedSanTek (İstanbul, Turkey). BioEdit 7.2 sequence alignment editor software was used to analyze and compare the NCBI GenBank database sequences via Basic Local Alignment Research Tool (BLAST) (URL-1).

3.3.5 Preparation of yeast cell and mould spore suspensions

Each yeast culture was inoculated (1 % v/v) into YEPD Broth (YEPD-10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) and incubated at 28 °C for 24 h at 200 rpm. After incubation, the turbidity of yeast cells was adjusted to 0.6 and 0.8 of the

McFarland (Den-1B McFarland BioSan Densitometer) standard by reading against control (YEPD Broth without yeast) which corresponds to approximately 1×10^7 and 1×10^8 cells/mL.

The mould spore suspensions (*Penicillium digitatum* NRRL 1202 and *Penicillium expansum* DSM6284) were prepared from one-week-old mould cultures and adjusted to the desired concentrations (1×10^4 , 1×10^6 spores /mL) in sterile distilled water with 0.1% (v/v) Tween 80 (Merck, Germany) using a hemocytometer (Marienfeld, Germany) (Y. Liu, Yao, et al., 2019).

3.3.6 In vitro assays

3.3.6.1 Yeast antagonism on culture media

A modified dual culture method was employed to test yeast antagonism (Pantelides et al., 2015). Firstly, yeast suspension (20 μ L, 1×10^8 cells/mL, 0.8 McFarland) was streaked on YEPD agar, 2 cm away from the plate's edge (90 mm diameter). After 48 h, spore suspensions (20 μ L, 1×10^4 /mL) of pathogenic fungi (6×6 mm²) from 7 d old fungal cultures were placed at 2 cm from the opposite edge of the dish and incubated at 25 °C for 7 d. The plates without yeasts were used as a control, and mycelial growth was measured with a caliper. The mycelial growth inhibition was calculated using the following equation (3.1).

$$I (\%) = \frac{(C - T)}{C} \times 100 \quad (3.1)$$

where I % represented the growth inhibition, C was radial growth of control (mm), and T was the radial growth of fungi with yeast treatment (mm). Each treatment received three replicates within each experiment, and experiments were repeated twice.

3.3.6.2 Lytic enzymes activity

The lytic enzymes such as chitinase, pectinase, β -1,3 glucanase, cellulase, protease, and gelatinase were screened in yeast isolates. Each yeast suspension (1×10^7 cells/mL) taken from 24 h of yeast culture was spot-inoculated (10 μ L) on a solid medium containing the specified substrate (Parafati, Vitale, Restuccia, et al., 2015). a) For chitinase activity, colloidal chitin (pH 7.0) was prepared from shrimp shell chitin (Sigma C7170, USA) based on the first Method explained by Souza et al. (2009).

Chitinase detection media (g/L) was prepared by adding K_2HPO_4 , 1.6; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; KH_2PO_4 , 0.2; NaCl, 0.1; $CaCl_2 \cdot 2H_2O$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.01; agar, 15.0 and 1.2 % (w/v) of colloidal chitin. Following incubation at 25 °C for 7 d, chitinase activity was observed by overlaying Lugol's solution for 3 min. The clear yellowish color was detected around the chitinase positive colonies, while the rest of the plate remained dark (Pretscher et al., 2018). b) Pectinase activity was observed using citrus pectin (Sigma P9135, Denmark) as a carbon source in mineral medium: (g/L) citric pectin, 2.0; yeast extract, 1.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.8; KH_2PO_4 , 0.2; $CaCl_2$, 0.05; $MnSO_4$, 0.05 and agar, 15.0 at pH 4.5. Following the incubation at 28 °C for 48 h, pectinase activity was determined by the addition of Lugol's solution with the formation of clear halos around pectinase positive colonies (Merín et al., 2011). c) β -1,3 glucanase activity was evaluated using 5 g/L laminarin (Sigma L9634, USA) in 6.7 g/L Yeast Nitrogen Base (BD, Franklin Lakes, NJ, USA) and 15 g/L agar (Lutz et al., 2013). Following the incubation at 25 °C for 72 h, 0.6 g/L Congo Red (Sigma C6277, USA) was poured onto the plates and waited for 60 min at room temperature and finally washed with 1M NaCl for 30 min. The formation of yellow-orange zones around β -1,3 glucanase positive colonies was noted (Y. Liu, Yao, et al., 2019). d) Cellulase activity was investigated on YEPD agar plates supplemented with 0.4 % carboxymethylcellulose sodium salt (CMC) (Sigma C4888, Denmark) by incubating at 30 °C for 5 d. After washing plates with SDW, the plates were applied to 0.1 % of Congo Red (Sigma C6277, USA) for 30 min. Then the plates were rinsed for 15 min with 1M NaCl solution. The positive CMCase activity was determined by the formation of a clear halo where the substrate was used, and the background of the plate remained orange-colored (Escribano et al., 2017; Pretscher et al., 2018). e) Protease activity was studied on solid media containing 10 % skim milk powder (LP0031, Oxoid, Basingstoke, Hampshire, UK) and 2% agar as described by Gardini et al. (2006) with some modifications. The yeasts were inoculated (20 μ L) into each well on the solidified media and incubated at 25 °C by checking daily throughout the week (Vidyasagar et al., 2006). The positive protease activity was indicated by a clear zone around the inoculated well. f) Gelatinase activity was detected on sterilized tubes containing gelatin (Gardini et al., 2006). The media was prepared with 5 g/L of glucose and 100 g/L of gelatin by transferring media to test tubes. Yeasts were inoculated (100 μ L) into each test tube, and gelatin liquefaction was tested for 3 weeks, while non-inoculated tubes were used as a control.

3.3.6.3 Enzymatic profiles

The API ZYM semi-quantitative commercial test (BioMérieux, Lyon, France) was employed to screen the hydrolytic enzyme profile of yeast isolates. Following the manufacturer's instructions, the API ZYM test containing 20 cupules with a specific substrate and buffer was employed to detect 19 different enzymes: alkaline phosphatase, esterase (C 4), esterase lipase (C 8), lipase (C 14), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase. Results were determined by the color intensity and evaluated in nanomoles (nmol) of the hydrolyzed substrate on a 5-step scale, ranging from 0 (no activity) to 5 (maximum activity), i.e., 1 = 5 nmol, 2 = 10 nmol, 3 = 20 nmol, 4 = 30 nmol and 5 $\geq$ 40 nmol of API ZYM substrate metabolized (Pini et al., 2017). Each test was checked dublicately. ClustVis (URL-2) web tool was used to create heat maps of the enzymatic profiles.

3.3.6.4 Iron depletion activity on culture media

PDA plates containing 5, 15, and 20 $\mu\text{g/mL}$ of FeCl_3 were used to check whether iron sequestration induced antagonism (Saravanakumar et al., 2008). The yeast isolates (1×10^8 cells/mL) were streaked on the plate's center and incubated for 48 h at 30 °C. The fungal plug (approximately 5-mm deep and 5-mm long) was removed from a 10 d PDA plate using a sterile puncher and positioned 25 mm away on both sides of the streaked yeast. Inhibition zone and halo width around the yeasts were recorded after 3 - 10 d of incubation at 25 °C. Tropolone, an iron-chelating agent, was used as a positive control (Sipiczki, 2006). PDA plates without iron were considered as a negative control. The experiment was performed three times for each iron level with double repetition.

3.3.6.5 Biofilm formation

The antagonist tentatively pectinase-free yeasts were investigated for their biofilm production capabilities based on their interaction with polystyrene surface (Jin et al., 2003). The yeasts were grown in a yeast nitrogen base (YNB) medium containing glucose (100 mM) overnight at 28 °C. Following harvesting, remaining yeast cells were subjected to phosphate-buffered saline (PBS, pH 7.2). Using the same media

above, the 100 μL of yeast suspensions (1×10^7 cells/mL) were seeded triplicate into 96-well polystyrene plates and cultivated at 28 °C for 3 h with shaking at 75 rpm. The cells without yeast were used as control. Following the adhesion, 150 μL PBS was used for double-washing, and 100 μL of YNB was pipetted into the wells and incubated at 28 °C, 75 rpm for 72 h. The wells were loaded with fresh media daily throughout incubation. The wells were double-washed with PBS and stained with 100 μL of crystal violet (0.4 %) for 45 min. After a four-time wash with SDW, 200 μL of 95 % ethanol was added to the wells and waited for 45 min to remove the stain. Finally, 100 μL of this solution was pipetted into a new polystyrene 96-well plate, and crystal violet absorbance was read at OD_{590 nm}. All experiments were conducted twice. The net absorbance values were determined by the equation (3.2).

$$A_{\text{net}} = \text{OD}_{\text{control}} - \text{OD}_{\text{sample}} \quad (3.2)$$

where A_{net} is the net absorbance value, $\text{OD}_{\text{control}}$ is the absorbance value of control and $\text{OD}_{\text{sample}}$ is the absorbance value of sample.

3.3.6.6 Volatile organic compounds (VOCs)

The tentatively pectinase-free yeasts were investigated for their ability to secrete VOCs against *P. expansum* and *P. digitatum* according to Farbo et al. (2018) and Parafati et al. (2015) with slight modifications. Firstly, aliquots of 100 μL yeast cell suspensions (1×10^8 cells/mL) were spread on YEPD agar plates (pH 4.5 and pH 6.0) using a sterile spreader and incubated at 25 °C for 24 h. SDW was used instead of yeast as control. Following day, 20 μL aliquots of spore suspensions of *P. digitatum* NRRL 1202 and *P. expansum* DSM6284 were adjusted to 1×10^6 spores/mL and spotted at the center of YEPD. Then, agar and plates were allowed to dry at room temperature for 30 min. The plates' lids were removed, and two base plates were covered face-to-face using a double layer of Parafilm® plus a single layer of regular tape. Double-dishes were incubated at 25 °C for 9 d. The plates inoculated only with fungal spores were used as control. The percentage of growth inhibition of fungal mycelia by the yeasts was calculated using the equation (3.3). The experiment was conducted in triplicate and repeated twice.

$$I (\%) = \frac{(C - T)}{C} \times 100 \quad (3.3)$$

where I % represented the growth inhibition, C was radial growth of control (mm), and T was the radial growth of fungi with yeast treatment (mm).

3.3.7 *In vivo* assays

3.3.7.1 Antifungal activity of selected yeast cells against *P. expansum* and *P. digitatum* on lemons

The selected yeasts were tested for *in vivo* antagonism based on Liu et al.(2017). The prepared lemons (based on Section 3.3.2) were artificially wounded (2-mm deep and 3-mm wide) with a sterile needle in the equatorial area. Each wound was inoculated with 20 μ L of yeast suspension (1×10^8 cells/mL), then allowed to dry for 4 h, while SDW was used as a control. Then, fungal spore suspensions (1×10^4 cells /mL) were inoculated (10 μ L) into each wound and air-dried. Treated fruit were kept at 25 °C for 7 d in sealed plastic trays covered with wet paper towels to ensure high humidity. After the fifth day of incubation, the disease incidence (DI) and the lesion diameter (LD) were calculated using the equation (3.4).

$$DI (\%) = \frac{DF}{TW} \times 100 \quad (3.4)$$

where DF stands for decaying fruit count and TW for total wound count. LD was also calculated as the average measurement of two perpendicular diameters around the decay. The selected yeast isolates were tested in triplicate, using five lemons per replication with a double repeat. A total of 30 lemons were used in a completely randomized design with three replicates per treatment, five fruits in each replicate, and the experiment was repeated two times.

3.3.7.2 Iron depletion activity of selected yeast cells against *P. expansum* and *P. digitatum* on lemons

In vivo iron depletion tests were conducted using tentatively pectinase-free yeasts against *P. digitatum* and *P. expansum* on lemons. The fruit were prepared as described in section 3.3.2. Fruits were wounded artificially with a sterile needle at the equatorial region (approximately 2-mm deep and 3-mm wide). Each of the wounds was inoculated with 20 μ L of yeast cell suspensions (1×10^8 cells/mL) whereas SDW was used as a control. After 4h, 10 μ L of fungal spore suspensions (1×10^4 spores/mL) were added to the wounds. Following drying, 20 μ L of FeCl₃ (20 μ g/mL) was applied

to each wound, and SDW was used as a control. Inoculated fruit were incubated for 7 d at 25 °C, while DI and LD were assessed at the end of the incubation (Y. Liu, Yao, et al., 2019). Each treatment was performed in triplicate, and the experiment was repeated twice.

3.3.8 Statistical analysis

The phylogenetic relationship between yeast isolates was constructed with 1000 bootstrap replicates using The Molecular Evolutionary Genetics Analysis (MEGA Version 7.0.21) through the neighbor-joining (NJ) method (Saitou & Nei, 1987; Tamura et al., 2007). Clustering analysis was conducted using the UPGMA (unweighted pair group method with arithmetic mean) method for branch robustness (Martín et al., 2006).

The experimental data were analyzed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) by expressing mean $\pm$ standard deviation. Different treatments were compared with one-way analysis of variance (ANOVA) test. Duncan's multiple range test performed mean separations and differences were considered significant at $p < 0.05$.

3.4 Results

3.4.1 Screening and molecular identification of yeast isolates

Pulcherrimin-producing maroon red yeasts were screened using ferric ion supplemented YEPDC agar. The plate (C) shows Hu (*Hanseniaspora uvarum*) as a negative control, 32 (*M. aff. pulcherrima* P01A016 32-AMM) and 34 (*M. pulcherrima* 34-UEM) are pulcherrimin producers, while 36 (*Metschnikowia* sp. 36-KMM) is a weak pulcherrimin producer with a pale red colour (Figure 3.1). Phylogenetic analyses were performed with the D1/D2 domain with ≥ 98 % identity to 26S rDNA sequences for *Metschnikowia* clade (Figure 3.2). The isolation code, source, location, GenBank accession numbers, and percentage identities are shown in Table 3.1.

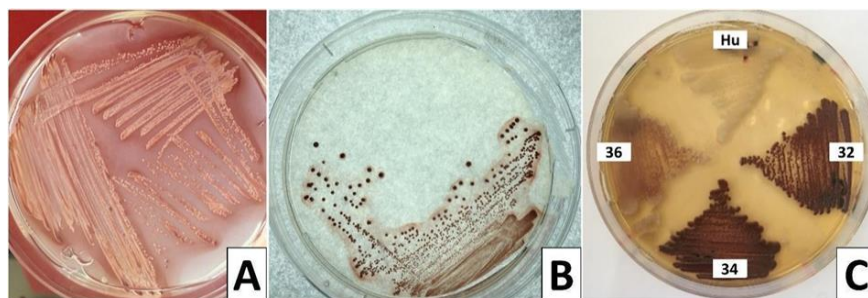


Figure 3.1 : Screening (A), isolation (B) and testing (C) pulcherrimin-producing yeasts on Fe³⁺ added YEGC agar.

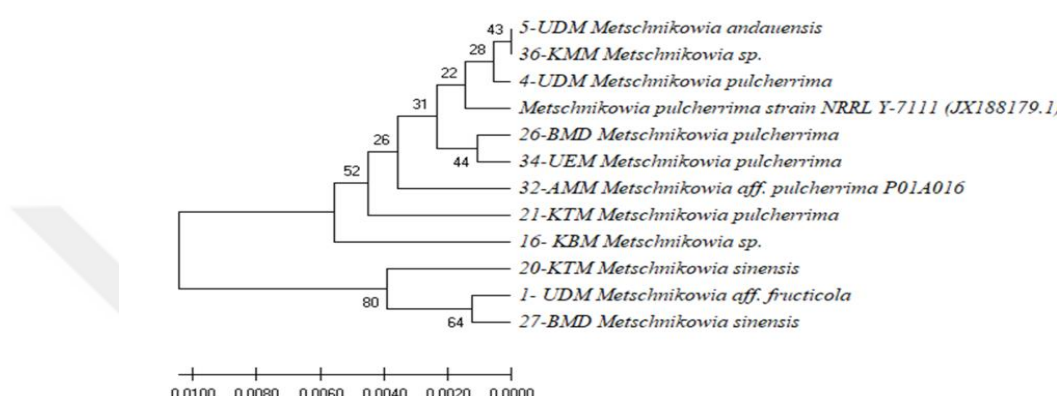


Figure 3.2 : Phylogenetic tree based on sequences of the D1/D2 domains of the large subunit 26S rDNA region of *Metschnikowia* sp.

Table 3.1 : Molecular identification of yeast isolates by comparing the genomic sequences in the GenBank database using the BLAST algorithm.

Isolate code	Source	Culture	Pulcherrimin production*	Accession Number	Identity (%)
1-UDM	Green grapes (<i>Vitis vinifera</i> L., Bozcaada Çavuşu)	<i>M. aff. fructicola</i>	H	KU862650.1	99.54
4-UDM	Green grapes (<i>Vitis vinifera</i> L., Bozcaada Çavuşu)	<i>M. pulcherrima</i>	H	MG210677.1	100
5-UDM	Green grapes (<i>Vitis vinifera</i> L., Bozcaada Çavuşu)	<i>M. andauensis</i>	M	MK612098.1	98.77
16-KBM	Rosehip-1 (Black, <i>Rosa pimpinellifolia</i> L.)	<i>Metschnikowia</i> sp.	W	KT922525.1	99.79
20-KTM	Rosehip-2 (Red, <i>Rosa canina</i> L.)	<i>M. sinensis</i>	H	MT032435.1	99.79
21-KTM	Rosehip-2 (<i>Rosa canina</i> L.)	<i>M. pulcherrima</i>	H	MN371967.1	99.36
26-BMD	Blackberry (Red, <i>Rubus fruticosus</i>)	<i>M. pulcherrima</i>	H	MT505669.1	99.57
27-BMD	Blackberry (<i>Rubus fruticosus</i>)	<i>M. sinensis</i>	H	MK612096.1	98.80
32-AMM	Hawthorn (<i>Crataegus pontica</i>)	<i>M. aff. pulcherrima</i> P01A016	H	JX188181.1	100
34-UEM	Red grapes (<i>Vitis vinifera</i> L., Erzincan Cimin)	<i>M. pulcherrima</i>	H	MN371975.1	99.79
36-KMM	Cornelian cherry (<i>Cornus mas</i> L.)	<i>Metschnikowia</i> sp.	W	KU350486.1	100

*Pulcherrimin production was indicated as high (H), medium (M) and weak (W).

3.4.2 *In vitro* assays

3.4.2.1 Yeast antagonism on culture media

A total of 11 *Metschnikowia* yeasts inhibited the mycelium growth of *F. oxysporum*, *B. cinerea*, *A. alternata*, *P. digitatum*, and *P. expansum* at varying levels. *Penicillium* and *Alternaria* were completely inhibited by some yeasts (Table 3.2). *P. expansum* and *P. digitatum* were selected due to their sensitivity for further tests. Figure A.1 shows images of *in vitro* mycelial growth inhibition.

Table 3.2 : In vitro mycelial growth inhibition of 11 yeast isolates against *F. oxysporum*, *B. cinerea*, *A. alternata*, *P. digitatum*, and *P. expansum*.

Isolate	Mycelial growth inhibition (%)					
	<i>F. oxysporum</i>	<i>B. cinerea</i>	<i>P. digitatum</i>	<i>P. expansum</i>	<i>A. alternata</i>	
1-UDM	74.78 ± 3.90 ^{cd*}	83.13 ± 3.18 ^b	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	81.30 ± 2.03 ^e	
4-UDM	79.90 ± 2.56 ^{ab}	86.80 ± 0.60 ^a	94.93 ± 0.23 ^d	85.61 ± 2.69 ^{de}	100.0 ± 0.0 ^a	
5-UDM	74.88 ± 1.56 ^{cd}	79.10 ± 0.51 ^{cd}	96.70 ± 0.52 ^c	91.83 ± 0.92 ^{bc}	89.10 ± 1.27 ^b	
16.-KBM	75.63 ± 1.45 ^{bcd}	82.93 ± 0.79 ^b	98.05 ± 0.42 ^b	85.09 ± 3.46 ^{de}	80.90 ± 1.19 ^e	
20-KTM	79.50 ± 1.22 ^{ab}	82.30 ± 0.76 ^{bc}	100.0 ± 0.0 ^a	88.65 ± 1.44 ^{cd}	97.24 ± 1.96 ^a	
21-KTM	79.14 ± 0.81 ^{abc}	81.70 ± 1.08 ^{bc}	100.0 ± 0.0 ^a	84.49 ± 1.15 ^e	88.54 ± 1.26 ^b	
26-BMD	79.10 ± 1.82 ^{abc}	82.30 ± 0.49 ^{bc}	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	86.51 ± 1.12 ^{bc}	
27-BMD	73.30 ± 0.73 ^d	78.20 ± 0.79 ^d	98.80 ± 0.86 ^b	87.26 ± 0.53 ^{de}	100.0 ± 0.0 ^a	
32-AMM	76.41 ± 2.40 ^{bcd}	81.50 ± 0.84 ^{bc}	95.30 ± 0.79 ^d	84.20 ± 2.10 ^e	85.1 ± 1.41 ^{cd}	
34-UEM	81.10 ± 1.73 ^a	82.10 ± 1.45 ^{bc}	100.0 ± 0.0 ^a	94.07 ± 0.30 ^b	89.50 ± 2.15 ^b	
36-KMM	66.80 ± 1.29 ^e	78.38 ± 2.27 ^d	91.31 ± 0.98 ^e	83.63 ± 1.40 ^e	82.78 ± 0.67 ^{de}	

*Different letters in each column are statistically different by Duncan's multiple range test ($p < 0.05$). Each value is the mean ± standard deviation of three replicates.

3.4.2.2 Extracellular lytic enzyme activity

Metschnikowia sp. can produce different enzymes such as chitinase, protease, pectinase, β -1,3 glucanase, and gelatinase at varying levels (Table 3.3). In addition to their antagonistic activity, the yeasts were selected based on their enzymatic activities.

Since pectinase causes a softening of the lemons through depolymerizing pectin (Jayani et al., 2005), tentatively pectinase-free isolates (1-UDM, 26-BMD, and 34-UEM) with higher mycelial growth inhibition abilities were selected as promising antagonistic candidates for further analysis. The other two pectinase-free yeasts (16-KBM and 36-KMM) exhibited relatively lower mycelial growth inhibition with pale-coloured pigmentation in the presence of ferric ions, indicating low pulcherrimin production. A representative image of the lytic enzyme activities is shown in Figure A.2. Each treatment had three replications and lytic enzyme activities were carried out twice. Although enzyme production capabilities of microorganisms were generally screened on solid medium, pectinase-free isolates should still be confirmed by LC-MS/MS.

Table 3.3 : Lytic enzyme activities of yeast isolates.

Isolate	Chitinase	Protease	Pectinase	Cellulase	β -1,3 glucanase	Gelatinase
1-UDM	+	+	-	+	+	+
4-UDM	+	-	+	+	+	+
5-UDM	+	+	+	+	+	+/-
16-KBM	+	-	-	+	+	+
20-KTM	+	-	+	-	+	+
21-KTM	+	+	+	+	+	+
26-BMD	+	-	-	+	-	+
27-BMD	+	-	+	+	+	+
32-AMM	+	+	+	+	+	+
34-UEM	+	+	-	+	+	+
36-KMM	+	-	-	+	+	+/-

*Presence (+), absence (-), or weak (+/-) of enzymes were indicated for the corresponding enzymes.

3.4.2.3 Enzymatic Profiles

All tested isolates showed high α -glucosidase and leucine arylamidase activity, while most of them showed significant enzymatic activity for valine arylamidase and β -glucosidase activities based on API ZYM test. The yeasts also showed intense enzymatic activities for esterases and weak activity for acid phosphatase, cysteine arylamidase, and naphthol-AS-BI-phosphohydrolase. However, the enzyme activities

were lacking for alkaline phosphatase, lipase, α -mannosidase, α -galactosidase, N-acetyl- β -glucosaminidase, β -galactosidase, β -glucuronidase, α -chymotrypsin, trypsin, and α -fucosidase activities. The yeast cultures and enzymes were shown on rows and columns, respectively (Figure 3.3). Distance measure: Euclidean. Clustering algorithm: Ward.

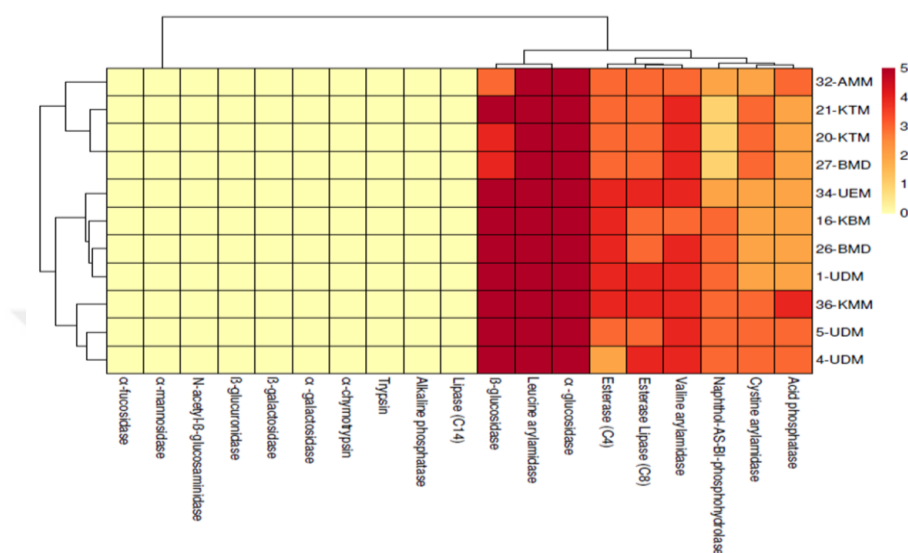


Figure 3.3 : Enzymatic profiles of *Metschnikowia* sp. Hierarchical clustering of positive (1–5) and negative (0) results.

3.4.2.4 *In vitro* iron depletion assay

Selected pectinase-free *Metschnikowia* yeasts exhibited antagonism against *P. digitatum* and *P. expansum* through iron depletion and elevated ferric ions (5, 15, and 20 $\mu\text{g/mL}$) in the media increased red color pigmentation by decreasing inhibition and pigmented halo zones, or vice versa (Figure 3.4). However, the strong pectinase activity of *Metschnikowia* sp. limited their use on lemons with high pectin content (Fidalgo et al., 2016). *M. pulcherrima* 26-BMD showed the highest *in vitro* iron depletion activity on *P. digitatum* and *P. expansum* in the absence (0 $\mu\text{g/mL}$) and presence (20 $\mu\text{g/mL}$) of FeCl_3 . Iron chelator agent tropolone confirmed the iron deprivation effect on *P. digitatum* and *P. expansum* by the formation of ferric tropolone precipitate, which could be reversed with the addition of FeCl_3 (Y. Liu, Yi, et al., 2019; Sipiczki, 2006). Following tropolone treatment, *P. digitatum* (13 mm) had higher inhibition zones than that of *P. expansum* (8 mm), suggesting that *P. digitatum* was more sensitive to iron deficiency (Figure A.3).

Our data indicated that the iron requirement of *P. digitatum* for fungal growth was higher than that of *P. expansum*. Since the inhibition zones of *P. digitatum* were not completely lost at excessive iron levels. When plates were supplemented with 20 $\mu\text{g/mL}$ FeCl_3 , *M. aff. fructicola* 1-UDM and *M. pulcherrima* 34-UEM depleted the iron in the media leading to loss of inhibition zones, while *M. pulcherrima* 26-BMD had 1 mm inhibition zone for only *P. digitatum*.

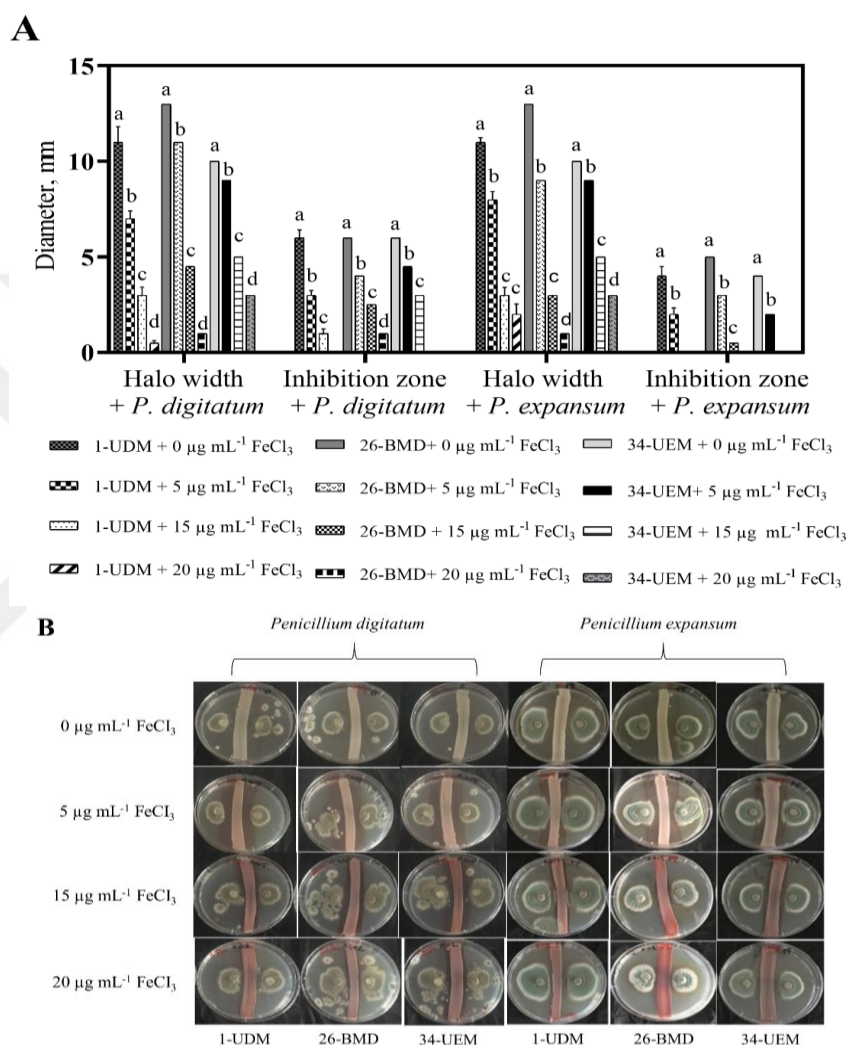


Figure 3.4 : Iron supplementation (A) and its effect on the pigmented halo and inhibition zones (B) of *Metschnikowia* sp. (1-UDM = *M. aff. fructicola* 1-UDM, 26-BMD = *M. pulcherrima* 26-BMD, 34-UEM = *M. pulcherrima*) against *P. digitatum* and *P. expansum*.

3.4.2.5 Biofilm formation

M. aff. fructicola 1-UDM had the highest biofilm formation capability with a significant difference ($p < 0.05$), followed by *M. pulcherrima* 26-BMD and *M. pulcherrima* 34-UEM ($\text{OD } 0.84 \pm 0.08$, 0.69 ± 0.14 and 0.43 ± 0.03 , respectively) after 72 h incubation.

3.4.2.6 Volatile organic compounds

The secretion of VOCs by three *Metschnikowia* sp. varied significantly at pH 4.5 for *P. digitatum* and *P. expansum* and pH 6.0 for only *P. digitatum* (Table 3.4). At both pH values, *M. pulcherrima* 26-BMD and *M. pulcherrima* 34-UEM showed a significant difference in *P. expansum* and *P. digitatum*, respectively. Additionally, *M. aff. fructicola* 1-UDM exhibited the highest inhibition activity for mycelial growth of *P. digitatum* and *P. expansum* at both pH values. In contrast, *M. pulcherrima* 34-UEM slightly inhibited the mycelial growth of *P. digitatum* at both pH values. *M. aff. fructicola* inhibited the mycelial growth inhibition at pH 4.5, whereas the other two *M. pulcherrima* isolates were more effective at pH 6.0 in controlling *Penicillium* fungi, similar to the findings of Liu et al (2019).

Table 3.4 : *In vitro* antagonism of volatile antifungal metabolites of pectinase-free *Metschnikowia* sp.

Isolates ¹	Mycelial growth inhibition ² (%)			
	<i>P. digitatum</i>		<i>P. expansum</i>	
	pH 4.5	pH 6.0	pH 4.5	pH 6.0
1-UDM	30.22 ± 1.09 ^{a3}	28.44 ± 1.49 ^a	20.79 ± 1.34 ^a	19.88 ± 0.95 ^a
26-BMD	18.33 ± 4.08 ^b	21.77 ± 1.49 ^b	13.83 ± 0.78 ^{c*}	18.66 ± 1.51 ^{a*}
34-UEM	15.77 ± 0.68 ^{b*}	21.66 ± 1.36 ^{b*}	16.61 ± 0.98 ^b	18.55 ± 2.18 ^a

(¹1-UDM = *M. aff. fructicola* 1-UDM, 26-BMD = *M. pulcherrima* 26-BMD, 34-UEM = *M. pulcherrima* 34-UEM) against *P. digitatum* and *P. expansum*. ²The results are presented as the mean ± standard deviation of three replicates. Mean radial growth (mm) of *P. digitatum* and *P. expansum* without VOCs were 30.3 ± 0.3 mm, 44.3 ± 1.2 mm at pH 4.5 and 47.7 ± 0.1, 46.9 ± 1.2 at pH 6.0, respectively.

³Different superscript letters within each column are statistically different by Duncan's multiple range test ($p < 0.05$). Values marked with * within each row show a significant difference according to ANOVA ($p < 0.05$).

3.4.3 *In vivo* assays

3.4.3.1 Antifungal activity of pectinase-free yeast cells against *P.expansum* and *P. digitatum* on lemons

All tested yeasts significantly decreased the DI and LD values caused by *P. digitatum* (PD) and *P. expansum* (PE) on 5th, 6th, and 7th incubation at 25 °C, when compared to the control (Figure 3.5). The incidence of *P. digitatum* was observed to be higher than that of *P. expansum* in lemons. Following 7 d of incubation, *P. digitatum* growth was entirely inhibited by *M. aff. fructicola* 1-UDM. The reduction in DI values by *M. pulcherrima* 26-BMD and *M. pulcherrima* 34-UEM was determined to be 93.34 % and 82.23 %, respectively. The highest LD was observed in *M. pulcherrima* 34-UEM

as 10.5 mm for *P. digitatum* (Figure 3.5A). The disease incidence (DI) and lesion diameter (LD) Columns (treatments) represent the mean of three replicates and vertical bars show the standard error of the mean. Different letters at the same incubation time are statistically significant by Duncan's multiple range test ($p < 0.05$).

A similar trend was observed for *P. expansum* in lemons, and the DI values increased over incubation time for all yeasts. For a 7 d incubation period, *M. pulcherrima* 34-UEM showed the highest DI and LD levels, 33.3 % and 17.1 mm, respectively. Additionally, *M. aff. fructicola* 1-UDM and *M. pulcherrima* 26-BMD had similar efficacy in controlling *P. expansum* on lemons on 7th incubation at 25 °C (Figure 3.5B).

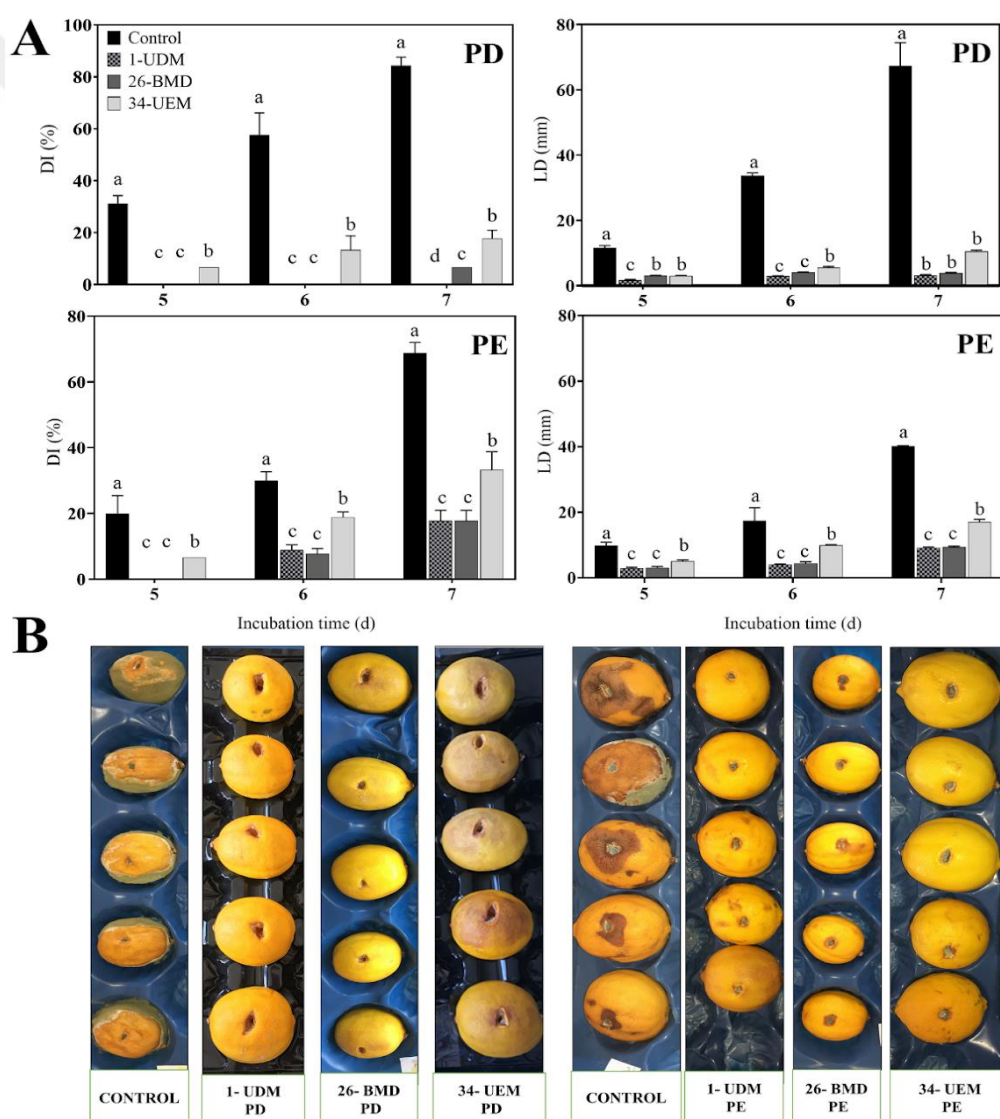


Figure 3.5 : *In vivo* antagonism (A,B) of pectinase-free *Metschnikowia* sp. (1-UDM = *M. aff. fructicola* 1-UDM, 26-BMD = *M. pulcherrima* 26-BMD, 34-UEM = *M. pulcherrima*) in the control of *Penicillium* decay.

3.4.3.2 Iron depletion activity of pectinase-free yeast cells against *P. expansum* and *P. digitatum* on lemons

The fungal growth of *P. digitatum* and *P. expansum* was increased by an excessive amount of iron (20 µg/mL FeCl₃) on artificially-wounded lemons (Figure 3.6). *M. aff. fructicola* 1-UDM showed the highest biocontrol efficacy compared to the other two *M. pulcherrima* yeasts. Moreover, the iron dependence of *P. digitatum* was found higher than that of *P. expansum* for spore germination and fungal growth. The lack of iron in the wounds led to a better biocontrol efficacy, but the iron supplementation increased the DI and LD values for both *Penicillium*.

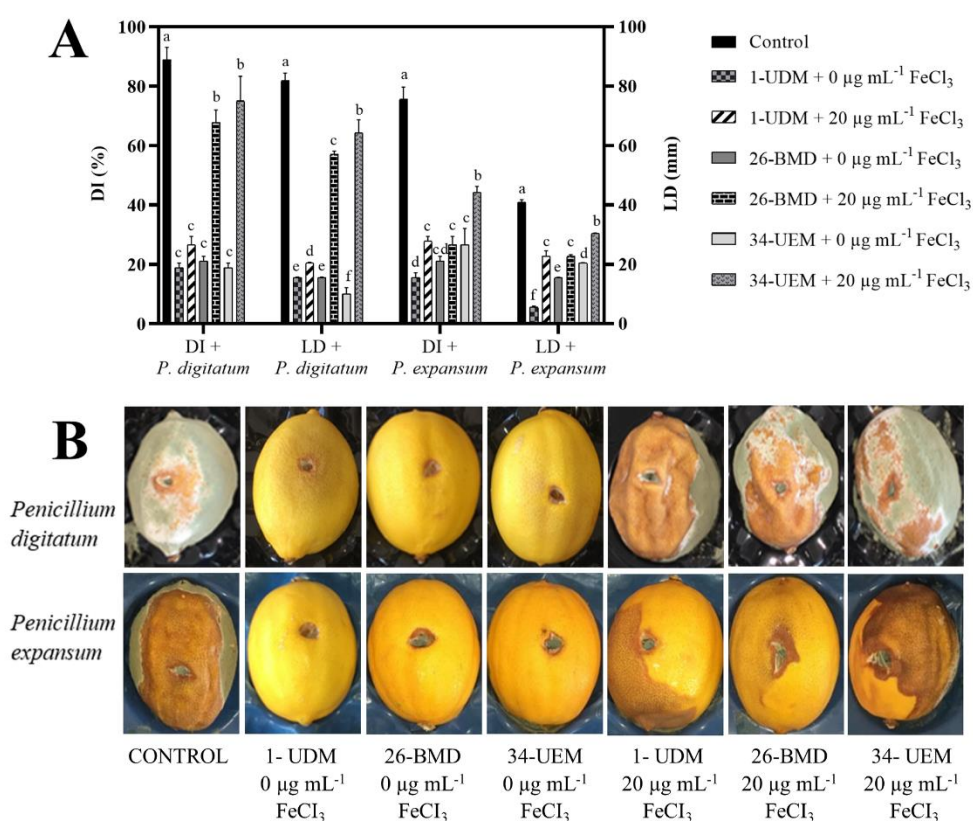


Figure 3.6 : In vivo biocontrolling efficacy (A,B) of pectinase-free *Metschnikowia* sp. (1-UDM = *M. aff. fructicola* 1-UDM, 26-BMD = *M. pulcherrima* 26-BMD, and 34-UEM = *M. pulcherrima* 34-UEM) in the control of *Penicillium* fungi during the lack (0 µg/mL FeCl₃) and presence (20 µg/mL FeCl₃) of iron.

3.5 Discussion

In this study, fruit-isolated *Metschnikowia* yeasts were tested against fungi including *Fusarium*, *Botrytis*, *Alternaria*, and *Penicillium*. Following lytic enzyme activity and

enzymatic profile results, antagonistic pectinase-free yeasts with high biocontrol efficacy against *P. digitatum* and *P. expansum* were selected and examined on lemons.

Although the mechanism of action remains unclear, the antimicrobial activity of *Metschnikowia* sp. lies in its ability to produce pulcherrimin pigment and extracellular hydrolytic enzymes (Saravanakumar et al., 2009). The combined enzymes, such as β -1,3- and β -1,6-glucanases, proteases, and chitinases, act synergistically to lyse pathogenic fungal cell walls (Pawlikowska et al., 2019); therefore the API ZYM test was conducted to determine the enzymatic fingerprinting of yeast isolates. Chitinase, protease, and β -1,3 glucanase were the most widespread activity among *Metschnikowia* yeasts (Table 3.3), which is in line with enzymatic profiles (Figure 3.3). The enzyme activities in *Metschnikowia* were found to be strain-specific, which causes variations in the inhibitory mechanism (Pawlikowska et al., 2019; Sipiczki, 2006). All enzymes can work synergistically to lyse fungal pathogen's cell wall (Pretscher et al., 2018). Since chitin, glucan, protein, and cellulose were the structural components of the fungal cell wall, chitinase, β -1,3 glucanase, protease, and cellulase enzymes were proposed as potential antifungals (Adams, 2004; P.-H. Chen et al., 2018). Chitinase from *M. fructicola* decreased the spore germination, germ tube development, and mycelium diameter of *Monilinia* sp. fungi on peaches (Banani et al., 2015). Protease and gelatinase indicated the proteolytic activity in *Metschnikowia* (Belda et al., 2016; Ianieva & Podgorsky, 2020). Furthermore, the presence of cell-wall degrading α - and β -glucosidase enzymes can contribute to α - and β -glucanase activities (Pretscher et al., 2018). Our findings were in consistent with previous studies that found *Metschnikowia* yeasts to have chitinase (Banani et al., 2015; Saravanakumar et al., 2009), protease (Belda et al., 2016; Reid et al., 2012), cellulase (Escribano et al., 2017), β -1,3 glucanase (Pretscher et al., 2018), pectinase (Belda et al., 2016) and gelatinase (Ianieva & Podgorsky, 2020) activities. Unlike our results, there were no cellulase (Belda et al., 2016), chitinase (Parafati, Vitale, Restuccia, et al., 2015), protease and β -1,3 glucanase (Y. Liu, Yao, et al., 2019) activities in *Metschnikowia* sp.

The pectinase activity of biocontrol yeasts should be considered when applied to fruit since pectinase induces softening in lemons by destabilizing the fruit cell wall (Jayani et al., 2005) and forming pores by supporting fungal colonization (Miedes & Lorences, 2006). Droby (1997) stated that *P. digitatum*'s pectinolytic enzymes damaged

grapefruit. Besides, *P. expansum* infection in apples and tomatoes degraded the pectin in the cell wall leading to a porous structure that promoted fungal growth and colonization (Miedes & Lorences, 2006). Although we found higher *in vitro* antagonistic activity in other *Metschnikowia* isolates, it did not seem feasible to use them on lemons due to their intense pectinase activity. The application of three pulcherrimin-producing yeasts (*M. aff. fructicola* 1-UDM, *M. pulcherrima* 26-BMD, and *M. pulcherrima* 34-UEM) lacked pectinase activity may take advantage in the postharvest treatments of lemons or other pectin-rich fruit. While some papers (Kinay & Yildiz, 2008; Y. Liu, Yao, et al., 2019) investigated the biocontrol efficacy of *Metschnikowia* yeasts on citrus, pectinase-negative *Metschnikowia* yeasts were considered for the first time on lemons.

For the production of pulcherriminic acid, cyclodileucine (cyclo(Leu-Leu)) is needed, and leucine arylamidase helped to produce leucine (Sipiczki, 2006), and all of our yeasts showed high leucine arylamidase activity. The pigmentless mutants of *Metschnikowia* yeasts did not exert antagonistic behavior compared to the wild form (Sipiczki, 2006). In contrast, Gore-Lloyd et al. (2019) suggested that mutant pigmentless *Metschnikowia* yeasts exhibited antagonism to some extent, indicating that other mechanisms may also play a role. Pulcherrimin was secreted around the yeast cells by creating a competition between yeast and fungi since iron is an essential micronutrient, also acts as a cofactor for various enzymes, particularly for conidial germination and fungal growth (Gore-Lloyd et al., 2019; Saravanakumar et al., 2008). The media was supplemented with an increasing amount of ferric ions (0, 5, 15, and 20 $\mu\text{g/mL}$ FeCl_3) to observe the effect of pulcherrimin, resulting in a decrease in the halo and inhibition zones of pigmented yeasts. The elevated amount of ferric ions prevents pulcherriminic acid from being diffused so far so that iron binds to nearby yeasts by forming pulcherrimin, and the remaining free iron can trigger the fungal proliferation.

In contrast, lower iron concentrations in the media increased the biocontrol efficacy of *Metschnikowia* due to the iron starvation of fungi. Therefore, iron was considered a key factor in the fruit before applying biocontrol yeast (Saravanakumar et al., 2008). *In vitro* iron depletion experiments complied with *in vivo* experiments. Consequently, *Metschnikowia* antagonism was found to be iron-dependent and could be reversed by adding ferric ions.

To elucidate the yeast antagonism, selected yeasts were investigated for their biofilm and antifungal VOCs formation capabilities. According to Liu et al. (Y. Liu, Yao, et al., 2019; Y. Liu, Yi, et al., 2019) and Parafati et al. (2015), the antagonistic activity of VOCs could be influenced by pH, hence the selected yeasts were tested at pH 4.5 and pH 6.0. Among the tested isolates, *M. aff. fructicola* 1-UDM displayed the highest antagonism on *P. digitatum* and *P. expansum* with its elevated β -1,3 glucanase activity, biofilm, and antifungal VOCs production capacity. However, *M. pulcherrima* 34-UEM showed the lowest biofilm and VOCs formation, but it had better protease, chitinase, and β -1,3 glucanase activities than *M. pulcherrima* 26-BMD on *P. digitatum*. In contrast, *M. pulcherrima* 26-BMD showed better iron depletion activity on both *Penicillium* fungi. These findings suggest that biofilm formation and VOCs secretion play a major role in the biocontrol mechanism of *Metschnikowia* sp. as previously stated by Liu et al. (2019). The hurdle effect of these minor activities contributed to the biocontrol efficacy of yeasts.

Biofilm is a network of carbohydrates, proteins, lipids, and nucleic acids bound to biotic and abiotic surfaces (Zara et al., 2020). Recently, Liu et al. (2019) revealed that the biofilm activity of antagonist *M. citrinensis* was similar to our observations. Parafati et al. (2015) documented that *M. pulcherrima* showed a positive correlation between biofilm formation and wound colonization on grapes. It could be concluded that the adhesion and biofilm formation capabilities had a great impact on the biocontrol efficacy of *Metschnikowia* sp.

VOCs production in *M. pulcherrima* was studied by Parafati et al. (Parafati et al., 2017), and mycelial growth inhibition was found to be 65.54 % for *P. digitatum*, being higher than our findings. Oro et al. (2018) stated that ethyl acetate was the main VOC responsible for *M. pulcherrima*. Likewise, following a 5 d incubation period, *M. pulcherrima* released ethanol, ethyl acetate, and carbon dioxide, suggesting a synergistic effect on pathogenic fungi (Contarino et al., 2019). Recently, Yang et al. (2021) indicated that 3-methyl-1-butanol, phenyl ethyl alcohol, and 1-hexanol were the major VOCs of *M. pulcherrima*.

In agreement with this work, Spadaro et al. (2013) found higher efficacy in *M. fructicola* than *M. pulcherrima* in controlling *P. expansum* and reducing patulin in apples. However, Parafati et al. (Parafati et al., 2017) reported that *M. pulcherrima*

weakly managed *P. italicum* in on mandarins. Likewise, Saravanakumar et al. (2008) documented that *M. pulcherrima* poorly controlled *P. expansum* on apples.

3.6 Conclusions

The iron depletion, biofilm formation, and secretion of cell-wall degrading enzymes with volatiles were recognized as the major biocontrol mechanisms of selected *Metschnikowia* yeasts. The present study demonstrated that the biocontrol efficacy could vary in yeasts even for the same species. Since each fungus has different spore germination characteristics and mycelial growth, yeast antagonism may vary depending on the fungi. Among all tested yeasts, *M. aff. fructicola* exhibited the highest antagonistic behavior on lemons for both *P. digitatum* and *P. expansum*. Likewise, the other two *M. pulcherrima* also reduced disease incidence and lesion diameter relative to control. The results indicated that natural yeast-based biocontrol agents could be used to replace pesticides. Yeast-based bioproducts and their modes of action need comprehensive research to develop novel formulations against postharvest decay.

4. BIOLOGICAL CONTROL OF GREEN MOULD ON MANDARIN FRUIT THROUGH THE COMBINED USE OF ANTAGONISTIC YEASTS³

4.1 Abstract

Penicillium digitatum is the primary cause of green mould in mandarins during the postharvest period, which is mainly controlled through the application of synthetic fungicides. This study evaluated the biocontrol of green mould on mandarin fruit with three antagonistic yeasts (*Hanseniaspora uvarum*, *Meyerozyma guilliermondii*, and *Metschnikowia* aff. *pulcherrima* P01A016), alone or in combination, by elucidating their possible mechanisms of action. All yeast cultures alone (73.85% - 80.64%) and their combinations (78.40% - 83.18%) reduced the mycelial growth of green mould *in vitro*. *M. aff. pulcherrima* reduced the disease incidence and lesion diameters by 75.5% and 91.3%, respectively, demonstrating the highest biocontrolling activity alone. *M. guilliermondii* showed the highest biofilm formation ($OD\ 0.93 \pm 0.01$) and antifungal activity (71.13%) through volatile organic compounds (VOCs), while *H. uvarum* displayed cell-wall degrading β -1,3 glucanase activity together with mycocinogenic and VOCs activity. All tested yeasts showed chitinase, protease, and leucine arylamidase activity. Among dual combinations, *M. guilliermondii* and *M. aff. pulcherrima* (M-1) were the most compatible, while *M. aff. pulcherrima* and *H. uvarum* (M-3) had the least compatibility. The combined application of these three yeasts resulted in synergistic co-operation, demonstrating the highest biocontrolling efficacy *in* and *in vivo*). Integrating multiple mechanisms of action in yeasts could create a hurdle approach for inhibiting green mould in citrus as a green alternative to synthetic fungicides. Thus, our study demonstrated that combining biocontrol yeasts can result in a beneficial consortium for the eco-friendly and sustainable management

³ This chapter is based on the paper “Oztekin, S., & Karbancioglu-Guler, F. (2023). Biological control of green mould on mandarin fruit through the combined use of antagonistic yeasts. *Biological Control*, 105186.

of *P. digitatum*-caused postharvest diseases of mandarins, in both the wounded and the intact mandarin fruits.

Keywords: *Penicillium digitatum*; citrus; biological control; postharvest; mycotoxinogenic activity; biofungicide.

4.2 Introduction

Citrus fruits, including orange, tangerine, mandarin, lemon, lime, and grapefruit, are cultivated and exported worldwide for their nutritional and therapeutic benefits (FAO, 2022). Notably, the global production of mandarins (including tangerines, mandarins, clementines, and satsumas) in 2020 was 38.6 million tonnes, and Turkey (1.6 million tonnes) ranked third after China (23 million tonnes) and Spain (2.1 million tonnes) (FAO, 2020). The citrus fruits, particularly wounded ones, are vulnerable to fungal attacks by *Penicillium digitatum* (green mould), *Penicillium italicum* (blue mould), *Geotrichum citri-aurantii*, *Aspergillus flavus*, and *Aspergillus niger* due to their acidic nature and high water content (Costa et al., 2019; Z. Wang et al., 2020). *P. digitatum*, the causative agent of green mould, has been linked to more than 90% of citrus fruit postharvest losses (Costa et al., 2021). Even though it was previously thought green mould did not produce mycotoxins (Frisvad et al., 2004), recent investigations have demonstrated that it produces tremorgenic mycotoxins (tryptoquialanines A and C) (Costa et al., 2021; de Vilhena Araújo et al., 2019). *P. digitatum* can produce a considerable amount of spores that can be released into the air, causing fatal pneumonia (Oshikata et al., 2013) and allergies (Moss, 2008).

An estimated 931 million tons of edible food are wasted annually, posing a threat to the economy, environment, health, food security, and sustainability (UNEP, 2021). Postharvest fungal diseases in fruits and vegetables account for nearly half of global food losses (Hernandez-Montiel et al., 2021), which can occur at any point in the food supply chain, from harvest to transportation (Y. Liu et al., 2017). Although synthetic fungicides (e.g., imazalil, propiconazole, thiabendazole, and prochloraz) are widely used to manage *Penicillium* decay in citrus fruit, their toxic residues can cause a variety of environmental and health issues, as well as the development of resistance in pathogenic fungi (Bhatta, 2022; J. Zhang et al., 2021; H. Zhu et al., 2019). At this point, using microbial antagonists instead of synthetic fungicides offers a more secure way of ensuring food safety and quality, thereby reducing poverty (FAO, 2017).

Reducing the use of pesticides and supporting pesticide-free agriculture were among the Sustainable Development Goals (SDG 2) of the United Nations since pesticide contamination is considered as environmental pollution (UN, 2022). Unfortunately, the use of pesticides climbed by 7% across Europe between 2010 and 2019 (FAOSTAT, 2022). In this regard, the European Food Safety Authority (EFSA) continuously monitored pesticide residues in foods through an EU-coordinated multiannual control programme. According to the 2020 report, 5.1% of the 88,141 food samples exceeded the allowable pesticide residue limits (EFSA, 2022). Additionally, RASSF (Rapid Alert System for Food and Feed) has received 95 notifications about pesticide residue in mandarin fruit since 2021. Nearly half of these notifications (53.7%) came from Turkey-based mandarins, and 25.5% of them posed serious health risks and were classified in the border rejection category. Although the policymakers banned the use of chlorpyrifos pesticide, it was frequently found in citrus fruit. Imazalil, propiconazole, and prochloraz were among the most frequently reported synthetic fungicides that exceeded the legal limits in citrus fruits, while prochloraz and flusilazole were found at excessive levels in 10.5% and 4% of mandarins, respectively, (RASFF, 2022).

As stated in the SDG 13 climate action part (UN, 2022), combining antagonistic microorganisms to manage postharvest diseases can be proposed as a part of integrated pest management and sustainable agriculture as an eco-friendly alternative to synthetic fungicides (A. S. Dukare et al., 2019). Thereby, the trend is shifting toward employing biocontrol microorganisms (yeast, bacteria, and yeast-like fungi) to combat fungi-induced postharvest diseases in a more sustainable, effective, and greener manner (Bhatta, 2022; Wisniewski & Droby, 2019). The biocontrol yeasts raise fewer biosafety concerns and offer viable replacements to synthetic fungicides with their genetically stable (J. Liu et al., 2013), biodegradable (Ocampo-Suarez et al., 2017), GRAS (generally regarded as safe)(FDA, 2021), and safety characteristics (non-pathogenic for humans, animals, and host without producing antibiotics, mycotoxins, and allergenic spores) (Konsue et al., 2020; J. Liu et al., 2013). Thanks to their strong attachability and enzymatic activities, the biocontrol yeasts can exploit a variety of compounds as carbon and energy sources, colonize quickly on wounded fruit, and destroy the hyphal wall structure of pathogenic fungi (Droby et al., 2002; Gamagae et al., 2004; Oztekin & Karbancioglu-Guler, 2021). Furthermore, the biocontrol yeasts

can successfully control the mycotoxins synthesis (Medina et al., 2017) by inhibiting mycotoxigenic fungi, limiting nutrients, and producing inhibitors (Sarrocco & Vannacci, 2018). Although the mechanisms of action of biocontrol yeasts are still unknown and require further study, some mechanisms, including competition for space and nutrients (Y. Liu, Yao, et al., 2019), depletion of iron (Sipiczki, 2020), secretion of lytic enzymes (Agirman & Erten, 2020), formation of biofilm, production of mycocin (killer toxin) (da Cunha et al., 2018), emission of volatile organic compounds (VOCs) (Farbo et al., 2018), antibiosis, mycoparasitism and induction of host resistance (Di Francesco et al., 2016) have been documented.

It was proposed that fungi-infected fruit wounds could be effectively treated with antagonistic yeasts to prevent fungal growth (Y. Liu, Yao, et al., 2019). In this case, some yeast-based biofungicides, including Excellence Bio-Nature (from *Metschnikowia pulcherrima*), Shemer (from *Metschnikowia fructicola*), Nexy (from *Candida oleophila*), and Boni-Protect (from *Aureobasidium pullulans*), were registered and commercialized throughout Europe (Droby et al., 2016; Sipiczki, 2020). Most significantly, Shemer is now commercially available under the name of Noli from Koppert Biological Systems (URL-3), which was approved by EFSA (EFSA, 2017) and found to be as effective as synthetic fungicide imazalil (Piombo et al., 2018). In terms of combined yeast formulations, the biocontrol product Zymaflore Egide (Laffort, France), containing *Torulaspora delbrueckii* and *M.pulcherrima*, was commercialized to bio-protect grapes and juices (Vejarano & Gil-Calderón, 2021).

Integrated disease management strategies were used in conjunction with a combination of physical treatments (heat treatments; controlled atmosphere; illumination and irradiation treatments with ultraviolet light), low-toxicity chemicals (natural compounds like plant extracts and essential oils; food additives and GRAS compounds; chemical resistance inducers like acetylsalicylic acid and β -aminobutyric acid; edible coatings like chitosan and wax) and biological control treatments (microbial antagonists like fungi, bacteria, and yeasts) to increase the effectiveness of biocontrol yeasts. (Palou, 2014; Papoutsis et al., 2019). Among them, consumers do not desire physical and chemical methods because of their adverse effects on health and the environment. Biopreservation through antagonistic microorganisms was proposed as one of the most promising eco-friendly and sustainable antifungal

treatments. In particular, it is simple to apply beneficial microbial communities to different crops and foods (Hernández et al., 2022).

In the current study, three different non-*Saccharomyces* yeast species were recruited alone or in combination to investigate the inhibition of citrus green mould. Firstly, *Meyerozyma guilliermondii* (formerly known as *Pichia guilliermondii* and anamorph *Candida guilliermondii*) was found to be effective in postharvest diseases on apples (He et al., 2020; Y. Huang et al., 2021; C. Sun et al., 2021), broccoli (X. Zhang, Yao, et al., 2022), grapefruit (Droby et al., 1992, 1997), grape (X. Wang et al., 2018), mango (López-Cruz et al., 2020), mandarin (Abraham et al., 2010; Z. Wang, Li, Liu, Tian, et al., 2021), oranges (Lahlali et al., 2014), peach (Y. Zhao et al., 2019), and pear (Fu et al., 2021; Han et al., 2021; Y. Yan, Zhang, Zheng, Tibiru, et al., 2018; Q. Yang et al., 2022). Secondly, *Metschnikowia pulcherrima* was able to control phytopathogenic moulds on fruits, including apples (Bühlmann et al., 2021; Fernandez-San Millan, Gamir, Farran, Larraya, & Veramendi, 2022; Saravanakumar et al., 2008; Settler-Ramírez et al., 2022), cherry (de Paiva et al., 2017; Ruiz-Moyano et al., 2020), chili (Basha & Ramanujam, 2015), grape (Parafati, Vitale, Restuccia, et al., 2015), lemon (Oztekin & Karbancioglu-Guler, 2021), loquat (H. Yang et al., 2021), mandarin (Kinay & Yildiz, 2008; Parafati et al., 2016, 2017), and mango (Tian, Li, Jiang, Jing, Shao, et al., 2018). Thirdly, *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*) inhibited phytopathogenic moulds on citrus fruit (P. Liu et al., 2013; Taqarort et al., 2008), grape (Apaliya et al., 2019; Cabañas et al., 2020; Qin et al., 2015), kiwifruit (L. Cheng et al., 2019), orange (W. Li et al., 2016; Y. Liu et al., 2017), strawberry (Qin et al., 2017; Ruiz-Moyano et al., 2020), and tomato (H. Guo et al., 2019; Z. Liu et al., 2022).

There has been research into using biocontrol microorganisms individually to prevent green mould decay of citrus fruit (Ahima et al., 2019; Arras et al., 1998; Delali et al., 2021; Y. Liu et al., 2017; Y. Liu, Yao, et al., 2019; Parafati et al., 2016), but few on using them in combination (Agirman & Erten, 2020; Panebianco et al., 2015). For example, Panebianco et al. (2015) combined *Pseudomonas* and *Trichoderma* fungal strains to control *P. digitatum* on citrus fruit, while Agirman and Erten (Agirman & Erten, 2020) solely investigated the *in vitro* effect of *Aureobasidium pullulans* and *M. guilliermondii* mixtures on *P. digitatum*. According to our knowledge, this is the first study on using compatible antagonistic yeast mixtures for the bio-management of

green mould decay on mandarin fruits. In addition, elucidating the possible modes of action of antagonistic yeasts and optimizing their compatible combinations remains a challenge (X. Zhang et al., 2020). Such a combination could benefit the reformulation of existing treatments and the development of novel yeast-based formulations for postharvest diseases (X. Zhang et al., 2020).

SDG 2.4.1 of the United Nations seeks to end hunger by 2030 by enhancing food security, sustainable agriculture, and food production systems since 840 million people are anticipated to experience hunger by that year. In addition, SDG 12.3 aims to halve food waste and loss. (UN, 2022). Aligning with these SDGs, biocontrol yeasts and their combinations can provide sustainable solutions for phytopathogen inhibition (Droby et al., 2022; Hernández et al., 2022). Therefore, the focus of this study was to evaluate the biocontrol capability of *M. guilliermondii* 7-BYM, *M. aff. pulcherrima* P01A016 32-AMM and *H. uvarum* 38-KMD, alone or in combination, against *P. digitatum* through *in vitro* and *in vivo* treatments on mandarins, with the involvement of antifungal action mechanisms.

4.3 Material and Methods

4.3.1 Fungal culture

Penicillium digitatum DSM2750, a mouldy orange isolate, was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH). The fungal culture was grown on Potato Dextrose Agar, PDA (Merck, Darmstadt, Germany) at 25 °C and frozen at -80 °C in glycerol stock solutions 20 % (v/v).

4.3.2 Fruit samples

Mandarins (*Citrus reticulata*) were purchased from a local grocery store and selected randomly based on their maturity, uniformity, and defect-free condition. The mandarins were sanitized with 2 % (w/v) of sodium hypochlorite (NaOCl) solution for 2 min, then rinsed twice with pure water and air-dried at 25 °C.

4.3.3 Isolation and molecular identification of yeast cultures

M. aff. pulcherrima P01A016 was previously isolated from hawthorn fruits, and its enzymatic profile and lytic enzyme activity were characterized by Oztekin and Karbancioglu-Guler (Oztekin & Karbancioglu-Guler, 2021). *H. uvarum* 38-KMD and

M. guilliermondii 7-BYM were isolated from fresh cornelian cherry fruits (Muğla, Turkey) and blackberry leaves (İstanbul, Turkey) from local orchards, respectively. Each sample was collected in 500 g portions, labeled, and stored in sterile cups. For yeast isolation, decimal dilutions were prepared, and aliquots were seeded onto yeast extract peptone dextrose agar (YEPD agar) plates containing chloramphenicol (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 20 g/L agar, and 0.1 g/L chloramphenicol). All of the media were supplied by Merck (Darmstadt, Germany). The plates were incubated at 25 °C for 48-72 h, and distinct colonies were randomly selected and re-streaked on YEPD agar media to obtain pure colonies. Then, yeast cultures were stored in slants (at 4 °C) or 20% glycerol stocks (at -80 °C) for further analysis. The Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) was used for genotypic characterization by employing M13 primer, as described previously by Dertli et al. (2016). Following the extraction of genomic DNA with the phenol-chloroform method, a 25 µL reaction mix was prepared to screen different patterns (Oztekin & Karbancioglu-Guler, 2021). Further, selected yeasts were identified by sequencing the D1/D2 domain of the large subunit 26S rRNA using primers NL1 (5'– GCATATCAATAAGCGGAGGAAAAG – 3') and NL4 (5'– GGTCCGTGTTTCAAGACGG – 3') (O'Donnell K, 1993) with PCR program including; 95 °C, 10 min; 40 cycles: 94 °C, 1 min; 54 °C, 30 s; 72 °C, 1 min, and 72 °C, 7 min. Amplicons were electrophoresed using 1% agarose gel and sequenced by MedSanTek (İstanbul, Turkey). Finally, DNA sequences were examined with BioEdit software, and then BLASTed (Basic Local Alignment Research Tool) against the NCBI database (URL-1).

4.3.4 Preparation of yeast cell and fungal spore suspensions

A total of 44 yeast isolates were initially screened for their *in vitro* antifungal activities on *Botrytis cinerea* and *Penicillium digitatum*. Since the highest antagonism was determined on *P. digitatum*, the three yeasts (*H. uvarum*, *M. guilliermondii*, and *M. aff. pulcherrima*) belonging to different species were selected for further experiments. For this purpose, yeast cultures were inoculated (1 % v/v) into YEPD Broth (YEPD- 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) and incubated at 28 °C for 24 h at 200 rpm. Yeast cells were collected by centrifugation, resuspended in sterile distilled water (SDW), and counted with a hemocytometer (Marienfeld, Germany). The required concentrations (1×10^8 , 1×10^7 cells/mL) were prepared for subsequent

analysis (Ruiz-Moyano et al., 2016). Yeast cultures (*M. guilliermondii* 7-BYM; *M. aff. pulcherrima* P01A016 32-AMM; *H. uvarum* 38-KMD) and their mixtures (M1 = 7-BYM + 32-AMM; M2 = 7-BYM + 38-KMD; M3 = 32-AMM + 38-KMD (1:1, v/v; 1×10^8 cells/mL), and M4 = 7-BYM + 32-AMM + 38-KMD (1:1:1, v/v/v, 1×10^8 cells/mL)) were prepared by combining equal volumes of suspensions of each antagonist (Agirman & Erten, 2020). Then, they were tested to see if they were compatible (Oro et al., 2014; Panebianco et al., 2015). *P. digitatum* was cultured on PDA plates at 25 °C for 7 d until sporulation, and the fungal spore suspensions were prepared in SDW containing 0.1 % (v/v) Tween 80 (Merck, Germany). The conidia were counted using a hemocytometer, and proper concentrations (1×10^4 conidia/mL, 1×10^6 conidia/mL) were prepared (Y. Liu, Yao, et al., 2019).

4.3.5 Dual culture assay of yeast cultures, alone or in combination

Yeast cultures and their combinations were evaluated for their *in vitro* antagonism against *P. digitatum*, as described by Pantelides et al. (2015) and Restuccia et al. (2020). For this purpose, yeast suspensions (1×10^8 cells/mL) were prepared from freshly grown (48–72 h on PDA medium) yeast cultures. Then, the yeast suspensions were streaked orthogonally on a PDA medium, 2 cm away from the rim of the Petri dish (90 mm diameter). Following yeast growth for 48 h, a 5-mm² mycelium disk of green mould was put 2 cm from the opposite side, and the plates were incubated at 25°C for 7 d. The plates only inoculated with the green mould were used as controls. When the pathogen reached the dish border, the mycelial growth towards the yeast strip was measured using an electronic digital caliper (Neiko 01407A, China). The mycelial growth reduction was calculated with the following equation (4.1).

$$I (\%) = \frac{(C - T)}{C} \times 100 \quad (4.1)$$

where I (%) represented the inhibition of the mycelial growth, C was the mycelial growth in the control (mm), and T was the mycelial growth of pathogenic fungi in the presence of yeast cultures (mm). The experiments were carried out three times for each yeast culture and repeated twice.

4.3.6 Effect of yeast cultures and their combinations on fungal conidia germination

In order to test conidia germination of *P. digitatum*, yeast cultures and their combinations (M-1, M-2, M-3 (1:1, v/v), and M-4 (1:1:1, v/v/v)) were evaluated using the methods of Ruiz-Moyano et al. (2016) and Nally et al. (2015). First, YEPD Broth media was prepared in 5 mL glass tubes, and spore suspensions of *P. digitatum* (100 μ L, 1×10^8 cells/mL) were inoculated into the media. Then, yeast cultures and their mixtures were added at the same level (100 μ L, 1×10^8 cells/mL). After 12 h at 100 rpm at 25 °C, conidia germination was determined when the germ tube length equaled or exceeded the diameter of the spore. SDW served as a control instead of yeast suspensions. The number of germinated conidia was determined by observing at least 100 spores under a light microscope (Olympia) and calculating the percentage of spore germination with equation (4.2).

$$I (\%) = [(1 - S_t \%) / S_c \%) \times 100 \quad (4.2)$$

where I (%) represented the percent inhibition, S_t % represented the percentage of germinated spores under yeast treatment, and S_c % referred to control without yeast treatment. Each treatment had three replicates, and the experiments were performed twice.

4.3.7 Investigations into the mechanism of action of yeast cultures

4.3.7.1 Extracellular lytic enzymes activity

The yeast cultures were investigated for their extracellular cell wall lytic enzymes, including chitinase, protease, gelatinase, pectinase, β -1,3 glucanase, and cellulase. Fresh yeast suspensions (1×10^7 cells/mL) were spotted (10 μ L) on a suitable medium containing the corresponding substrate for the following experiments (Parafati, Vitale, Restuccia, et al., 2015). a) chitinase detection media was prepared using colloidal chitin (pH 7.0) from shrimp shells (Sigma C7170, USA), as described in Souza et al. (2009). After 7 d at 25 °C, extracellular chitinase activity was determined with the clear lysis zones around yeast colonies after flooding with Lugol's solution (Pretscher et al., 2018). b) For the detection of protease activity, 10% skim milk powder (Sigma-Aldrich 70166, Switzerland) was employed as a substrate. Following one week of incubation at 25 °C, forming a clear zone around the colonies indicated a positive result

(Gardini et al., 2006; Vidyasagar et al., 2006). c) Gelatinase enzyme was observed based on gelatin liquefaction in test tubes containing 5 g/L of glucose and 100 g/L of gelatin (Sigma-Aldrich, Steinheim, Germany). The yeast suspensions ($100\ \mu\text{L}$, 1×10^8 cells/mL) were added into tubes containing 9 mL of the medium and incubated for about 3 weeks with a regular examination. After incubation, a positive reaction was interpreted with gelatin liquefaction for gelatinase activity, while the tubes without yeast served as negative controls (Gardini et al., 2006). d) A substrate of citrus pectin (Sigma P9135, Denmark) was used to detect pectinase activity as previously described by Merín et al. (2011). Positive pectinase activity was detected after 48 hours of incubation at $28\ ^\circ\text{C}$, forming a clear halo around colonies when flooded with iodine solution. e) β -1,3 glucanase activity was tested with 5 g/L laminarin (Sigma L9634, USA) in 6.7 g/L Yeast Nitrogen Base (BD, Franklin Lakes, NJ, USA) and 15 g/L agar (Lutz et al., 2013). Following the incubation at $25\ ^\circ\text{C}$ for 72 h, the colonies were stained with 0.6 g/L Congo Red (Sigma C6277, USA) solution for 60 min and washed with 1M NaCl for 30 min. The formation of yellow-orange zones was determined as a positive activity. f) Cellulase activity was determined in YEPD agar containing 0.4 % carboxymethylcellulose sodium salt (CMC) (Sigma C4888, Denmark), and the inoculated yeasts were incubated at $30\ ^\circ\text{C}$ for 5 d. Then, the plates were washed with SDW for 30 min, followed by 0.1 % of Congo Red and 1M of NaCl for 15 min (Escribano et al., 2017).

4.3.7.2 Enzymatic fingerprinting

Screening of enzymatic profiles was conducted using the API ZYM test (BioMérieux, Lyon, France), which allows for the characterization of 19 distinct enzymes, including alkaline phosphatase, esterase (C 4), esterase lipase (C 8), lipase (C 14), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase. The color of the hydrolyzed substrate was measured in duplicate and scored on a five-point scale, i.e., 1 = 5 nmol, 2 = 10 nmol, 3 = 20 nmol, 4 = 30 nmol, and $5 \geq 40$ nmol of API ZYM substrate metabolized (Pini et al., 2017). Heat maps of enzymatic profiles were created using the ClustVis web program (URL-2).

4.3.7.3 Iron depletion activity

As described by Saravanakumar et al. (2008), *in vitro* iron depletion activity was investigated in PDA medium at three FeCl₃ levels; 5, 15, and 20 µg/mL. First, the center of the plate was seeded with yeast suspension (1×10^8 cells/mL) and incubated at 30 °C for 48 h. Actively growing fungal mycelial discs (5-mm² plugs) were symmetrically placed 25 mm apart on both sides of the plate and cultured for 7 days at 25 °C. Yeast-free plates served as control, and mycelial growth was calculated with the following equation (4.3).

$$I (\%) = \frac{(C - T)}{C} \times 100 \quad (4.3)$$

where I (%) represented the growth inhibition, C was the mycelial growth of control (mm), and T was the mycelial growth of fungi with yeast treatment (mm). An iron-chelator substance, tropolone, served as a positive control (Sipiczki, 2006), while the iron-free plates were considered the negative control. All treatments comprised three replicates, and the experiments were repeated twice. In addition, pulcherrimin production of *M. aff. pulcherrima* P01A016 32-AMM was evaluated on yeast extract agar (YEA; 1% yeast extract, 2% glucose, 2% agar) supplemented with pectin (0.5% and 1%) at 20 °C for 5 d (Horváth et al., 2021). The pectin-free medium was employed as a control, and *Metschnikowia chrysoperlae*, a non-pigment-producing yeast, was utilized as a negative control. The color intensity and pigmented zones of pulcherrimin were compared to the control plates.

4.3.7.4 Mycocinogenic activity

The ability of yeast cultures to produce a killer toxin (mycocin) was studied using the method of Ceccato-Antonini et al. (2004) and Izgü et al. (Izgü et al., 2006) with slight modifications. *Saccharomyces cerevisiae* NCYC 1006 served as a killer-sensitive reference culture to detect mycocinogenic yeasts. Freshly grown *S. cerevisiae* (100 µL, 1×10^5 cells/mL) cells were pour-plated in YEPD-methylene blue agar (YEPD-MBA; 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 20 g/L agar, and 0.03 g/L methylene blue), which was buffered to pH 4.5 with 100 mM citrate–phosphate buffer. Then, aliquots of 10 µL yeast culture (1×10^8 cells/mL) were deposited onto sensitive culture-seeded YEPD-MBA plates and left for incubation at 28 °C for 72 h. Finally, the presence of a clear zone (referring to inhibitory activity) or blue zone (indication

of cell death) around the colonies was evaluated as a sign of mycocinogenic (killer) activity. Using same methodology above, the yeasts were applied against *P. digitatum* (1×10^5 conidia/mL) on YEPD-MBA at pH 4.5.

4.3.7.5 Biofilm formation

The yeast cultures were investigated for their ability to form biofilm on polystyrene surfaces through the method of Jin et al. (2003). Yeast nitrogen base (YNB) containing glucose (100 mM) was used to cultivate yeast cultures at 28 °C. Following washing twice with phosphate-buffered saline (PBS, pH 7.2), the yeast cells were resuspended (1×10^7 cells/mL) in YNB media. Then, 100 μ L of yeast suspensions were pipetted into 96-well polystyrene plates and incubated at 28 °C, 75 rpm for 3 h. The wells were washed twice with 150 μ L PBS, and 100 μ L of YNB was pipetted into each well, and the plates were incubated at 28 °C, 75 rpm for 72 h. Throughout the incubation, fresh media was used to replenish the cells daily, and the yeast-free wells served as controls. After incubation, the wells were re-washed with PBS and treated with 100 μ L of crystal violet (0.4 %) for 45 min. The stained cells were washed with SDW four times to remove excess dye, and 200 μ L of 95 % ethanol was added to wells for 45 min for decolorization. Finally, 100 μ L of the purple solution was transferred to a new polystyrene 96-well plate to read the optical density (OD) at 590 nm. The net absorbance values were calculated based on the equation (4.4), and the analysis was carried out twice.

$$A_{\text{net}} = OD_{\text{control}} - OD_{\text{sample}} \quad (4.4)$$

where A_{net} is the net absorbance value, OD_{control} is the absorbance value of control and OD_{sample} is the absorbance value of sample.

4.3.7.6 Volatile organic compounds (VOCs)

The capacity of the yeast cultures and their combinations to release VOCs against *P. digitatum* was tested (Farbo et al., 2018; Parafati, Vitale, Restuccia, et al., 2015). For this purpose, YEPD agar plates were prepared at two different pH values (4.5 and 6.0). Aliquots of 100 μ L of each yeast suspension (1×10^8 cells/mL) were seeded on the center of plates and incubated overnight at 25 °C, with SDW serving as a control. *P. digitatum*'s fungal spore suspension (20 μ L, 1×10^6 conidia/mL) was seeded on the center of the plate and allowed for drying 30 min at room temperature. Then, two

separate plates containing yeast and fungus were covered face-to-face with a double layer of Parafilm® and a single layer of tape and left for incubation at 25 °C for 9 d. The yeast-free plates served as control. The radial growth reduction of fungal mycelia (%) was calculated as in the equation (4.5).

$$I (\%) = \frac{(C - T)}{C} \times 100 \quad (4.5)$$

where I (%) represented the inhibition of the radial mycelial growth, C was the radial mycelial growth in control (mm), and T was the radial mycelial growth of pathogenic fungi in the presence of yeast cultures (mm). The experiment was conducted three times and repeated twice.

4.3.8 *In vivo* biocontrol activities of yeast cultures against *P. digitatum*

The method reported by Liu et al. (2017) and Parafati et al. (2016) was employed to test *in vivo* antagonism of yeast cultures and their combinations against green mould on mandarins. A sterile needle was used to make one 2-mm deep and 3-mm wide wound around the blossom end along the mandarin's basal area. Each wound was inoculated with 20 µL of yeast suspension (1×10^8 cells/mL) and dried at room temperature for 4 hours, while SDW served as a control. Then, artificial wounds of mandarins were inoculated with conidia suspensions of *P. digitatum* (10 µL, 1×10^4 conidia/mL). The mandarins were kept in sealed plastic bags with wet paper towels at 20 °C for 7 d to ensure high relative humidity. The mandarins were checked daily, and measurements were taken when the diameters of the control lesions reached quantifiable sizes. After the fifth day of incubation, the disease incidence (DI) and the lesion diameter (LD) values were calculated using the equation (4.6).

$$DI (\%) = \frac{DF}{TW} \times 100 \quad (4.6)$$

where DF is the number of decayed fruits and TW is for numbers of total wounds. The average measurement of two perpendicular diameters surrounding the decay was also used to calculate LD. Yeast cultures and their combinations were tested in triplicate, using five mandarins per replication with double repetition, and a total of 70 mandarins were used in a randomized complete block design.

In vivo biocontrolling assay was also conducted in the intact mandarin fruits (Perez et al., 2019). In the same manner as described before, whole mandarins were dipped into yeast suspensions (1 × 10⁸ cells/mL), dried for 4 hours at room temperature. Then, they were dipped into *P. digitatum* (1 × 10⁴ conidia/mL) solution. Using five mandarins per replication, yeast cultures and their combinations were tested in triplicate and the experiment was repeated two times. The mandarin fruits were given 15 seconds of exposure to a synthetic fungicide named Fungaflor® 75 WSG (0.13 g/L), which was equivalent to 75% imazalil base. Furthermore, SDW-treated mandarins were used as a control. They were then immersed in a green mould solution (*P. digitatum*, 1 × 10⁴ conidia/mL). Under the identical incubation conditions as above, the protection efficacy of treatments was determined using the equation (4.7).

$$PE (\%) = \frac{HF}{TF} \times 100 \quad (4.7)$$

where PE is the protection efficacy (%), HF is number of healthy fruits, and TF is the number of total fruits.

4.3.9 Statistical analysis

The phylogenetic analysis was carried out with the “Neighbor-Joining” method from the Molecular Evolutionary Genetics Analysis (MEGA Version 7.0.21) using the “Bootstrap” method with 1000 bootstrap replicates. The data were analyzed using statistical software SPSS 22.0 (SPSS Inc., Chicago, IL, USA), and the results were expressed as mean ± standard deviation. Different treatments were compared with a one-way analysis of variance (ANOVA) test. Duncan’s multiple range test was performed to separate means, and the differences were considered statistically significant at $p < 0.05$.

Limpel’s and Colby’s formulas were used to determine if there was synergism in the double and triple yeast mixtures, respectively. The expected effects of two different yeasts, X and Y, were computed for double combinations (M-1, M-2, and M-3), whereas Z was included as a third yeast treatment for triple combinations (M-4). Limpel’s equation (4.8) (Richer, 1987) and Colby’s equation (4.9) (Colby, 1967).

$$Ee = X + Y - \frac{XY}{100} \quad (4.8)$$

$$E_e = X + Y + Z - \frac{XY + XZ + YZ}{100} + \frac{XYZ}{10,000} \quad (4.9)$$

in which formulas the expected effect (E_e) was compared with the observed value (E_o). Therefore, if the combination of the yeasts produces a decay reduction value superior to E_e , then synergy exists.

4.4 Results

4.4.1 Molecular identification of yeast cultures

Phylogenetic analysis of nucleotide sequences from domain D1/D2 of large subunit 26S rDNA resulted in three clades with $\geq 98\%$ identity (Figure 4.1). Table 4.1 displays isolation code, source, accession numbers, and percentage identity information of the yeast isolates. Figure B.1 depicts the RAPD-PCR and conventional PCR images. The biocompatibility and interactions of the selected three antagonistic yeasts need to be examined before using them on fruits. Because non-competitive yeast combinations may have a broader range of activity and be more effective at treating postharvest diseases (Oro et al., 2014; Panebianco et al., 2015). The findings showed that the combined use of yeasts resulted in good biocompatibility, especially for M-1, M-2, and M-4 (Figure 4.2). However, M-3 showed different behavior when grown in mixed culture, which was found to be lower its component curves.

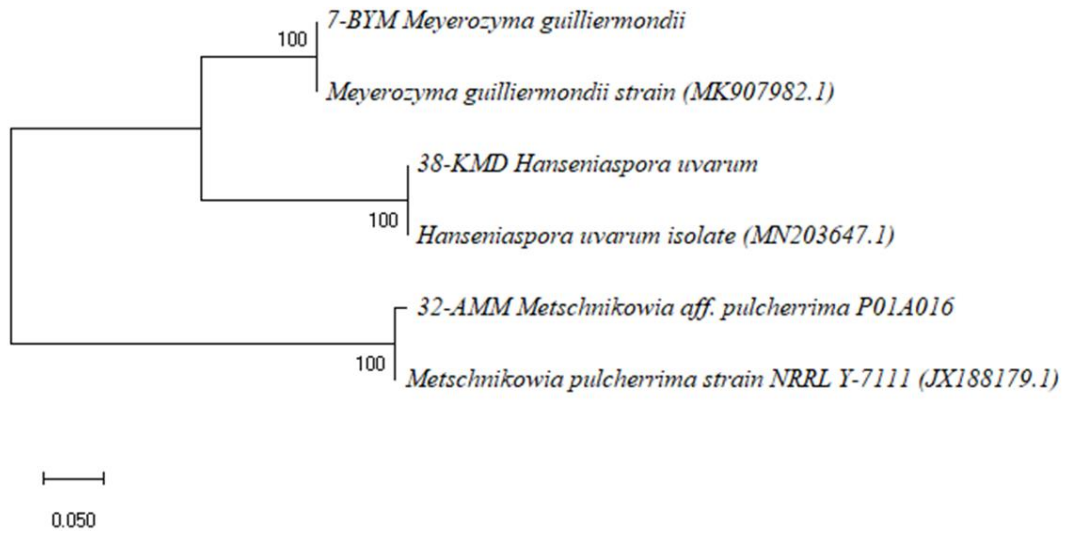


Figure 4.1 : Phylogenetic tree based on sequences of the D1/D2 domains of the large subunit 26S rDNA region.

Table 4.1 : Molecular identification of yeast cultures by comparing the genomic sequences with the GenBank database using the BLAST algorithm.

Isolate Code	Source	Culture	Accession Number	Identity (%)
7-BYM	Blackberry leaves (<i>Rubus fruticosus</i> L.)	<i>Meyerozyma guilliermondii</i>	MT645353.1	98.48
38-KMD	Cornelian cherry (<i>Cornus mas</i> L.)	<i>Hanseniaspora uvarum</i>	MT537584.1	100
32-AMM ¹	Hawthorn (<i>Crataegus pontica</i>)	<i>Metschnikowia</i> aff. <i>pulcherrima</i> P01A016	JX188181.1	100

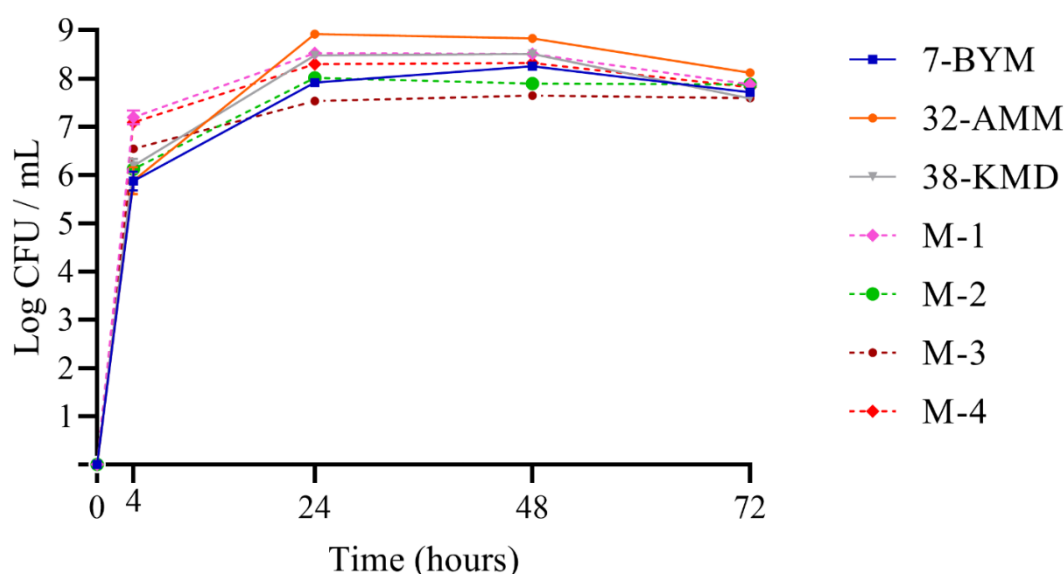


Figure 4.2 : Compatibility of yeast cultures (*M. guilliermondii* 7-BYM; *M. aff. pulcherrima* P01A016 32-AMM; *H. uvarum* 38-KMD) and their combinations (M-1 = 7-BYM + 32-AMM; M-2 = 7-BYM + 38-KMD; M-3 = 32-AMM + 38-KMD; M-4 = 7-BYM + 32-AMM + 38-KMD) in YEPD broth at 28 °C.

4.4.2 *In vitro* inhibitory activities of yeast cultures, alone or in combination

Table 4.2 shows *in vitro* antagonism data of *M. guilliermondii* 7-BYM, *M. aff. pulcherrima* P01A016 32-AMM, *H. uvarum* 38-KMD, and their combined treatments on *P. digitatum* (Figure B.2). All yeast cultures (73.85% - 80.64%) and their combinations (78.40% - 83.18%) reduced the mycelial growth of green mould. The highest biocontrolling activity was found in *Metschnikowia* aff. *pulcherrima* (80.64%). The combination of three yeasts (M-4) demonstrated the highest *in vitro* efficacy, inhibiting mycelial growth by 83.18%.

Table 4.2 : *In vitro* mycelial growth inhibition of *P. digitatum* DSM2750 by tested yeast cultures and their mixtures.

Isolate	Mycelial growth inhibition of <i>P. digitatum</i> DSM2750 (%)
<i>M. guilliermondii</i> 7-BYM	77.70 ± 0.11 ^c
<i>M. aff. pulcherrima</i> P01A016 32-AMM	80.64 ± 0.65 ^b
<i>H. uvarum</i> 38-KMD	73.85 ± 0.31 ^d
M-1 ¹	81.06 ± 0.42 ^b
M-2	78.40 ± 0.09 ^c
M-3	81.22 ± 0.70 ^b
M-4	83.18 ± 0.82 ^a

¹ M-1 = 7-BYM + 32-AMM; M-2 = 7-BYM + 38-KMD; M-3 = 32-AMM + 38-KMD; M-4 = 7-BYM + 32-AMM + 38-KMD.

4.4.3 Inhibition of fungal spore germination

Yeast cultures and their combinations were investigated for their capability to inhibit the conidial germination of *P. digitatum* DSM2750 after 12 h at 100 rpm at 25 °C (Figure 4.3). When compared to the control, the mixture of three yeast cultures (M-4) reduced *P. digitatum*'s conidial germination by 92.03%. Among dual combinations of yeast cultures, M-3, M-1, and M-2 reduced the spore germination by 88.56%, 88.16%, and 86.06%, respectively. Regarding individual treatments, 32-AMM retarded the spore germination of *P. digitatum* by 87.43%. Following this, 7-BYM and 38-KMD inhibited spore germination by 85.23% and 80.46%, respectively ($p < 0.05$).

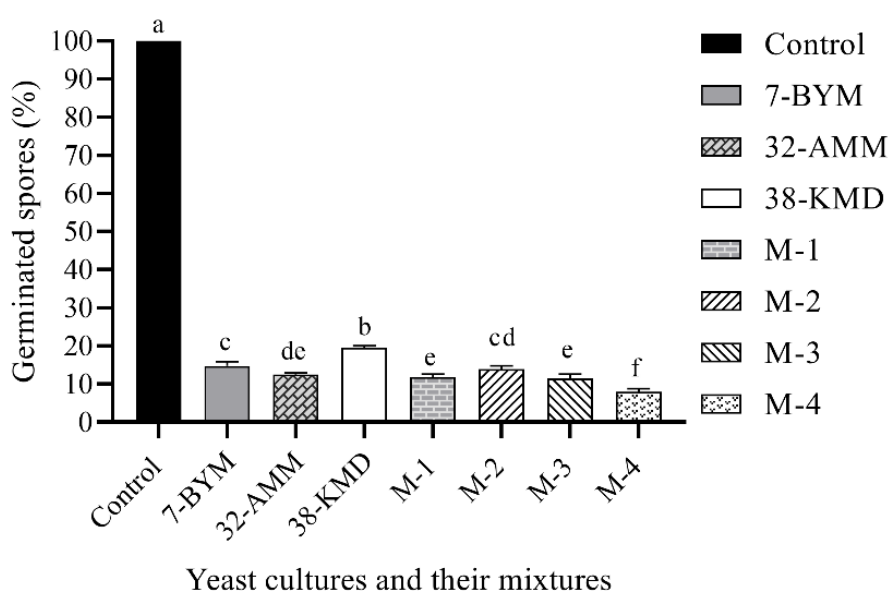


Figure 4.3 : The percent of germinated spore of *P. digitatum* DSM2750.

4.4.4 Possible action mechanisms of yeasts

4.4.4.1 Extracellular lytic enzymes activity

The screening of lytic enzymes is crucial for elucidating the mechanisms of action of yeasts (Y. Liu, Yao, et al., 2019). Table 4.3 lists the cell-wall degrading enzymes, including chitinase, β -1,3 glucanase, cellulase, pectinase, protease, and gelatinase. It was previously found that *M. aff. pulcherrima* P01A016 32-AMM produced all enzymes of interest (Oztekin & Karbancioglu-Guler, 2021).

Table 4.3 : *In vitro* lytic enzyme activities of yeast cultures in the presence (+) and absence (-) of the corresponding enzymes.

Yeast culture	Chitinase	β -1,3 glucanase	Cellulase	Pectinase	Protease	Gelatinase
<i>M. guilliermondii</i> 7-BYM	+	-	-	-	+	+
<i>H. uvarum</i> 38-KMD	+	+	-	-	+	-

4.4.4.2 Enzymatic fingerprinting

The API ZYM showed that all yeast cultures displayed strong (≥ 40 nmol) leucine arylamidase activity. *M. aff. pulcherrima* P01A016 32-AMM was had intense (≥ 40 nmol) α -glucosidase and moderate (20 nmol) β -glucosidase, esterase, and valine arylamidase activities (Oztekin & Karbancioglu-Guler, 2021). *H. uvarum* 38-KMD had strong alkaline phosphatase and β -glucosidase activity. Esterase and valine arylamidase levels were high (30 nmol) in both *M. guilliermondii* 7-BYM and *H. uvarum* 38-KMD, while *M. guilliermondii* 7-BYM exhibited intense alkaline phosphatase. Both *M. guilliermondii* 7-BYM and *M. aff. pulcherrima* P01A016 32-AMM had moderate acid phosphatase activity, and esterase lipase activity was also moderate in all yeasts. However, none of the yeast cultures had lipase, α -mannosidase, α -galactosidase, α -glucosidase, N-acetyl- β -glucosaminidase, β -galactosidase, β -glucuronidase, α -chymotrypsin, trypsin, and α -fucosidase activities (Figure 4.4). The results showed the hierarchical clustering of positive (1–5) and negative (0) results. Distance measure: Euclidean, clustering algorithm: Ward. The yeast cultures and enzymes were shown in rows and columns, respectively (Figure 4.4).

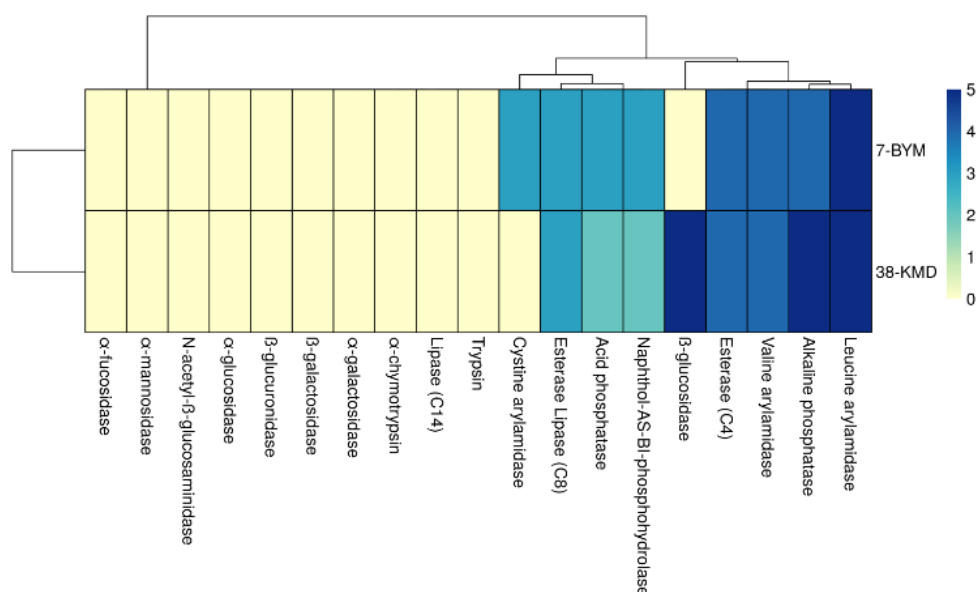


Figure 4.4 : Enzymatic profiles of *M. guilliermondii* 7-BYM and *H. uvarum* 38-KMD.

4.4.4.3 Iron depletion activity

Metschnikowia sp. is known for its strong iron-binding abilities, and its maroon-red pulcherrimin pigment exerts high antimicrobial activity (Sipiczki, 2020). *M. aff. pulcherrima* P01A016 32-AMM was studied for its iron depletion activity at increasing ferric ions (0, 5, 15, and 20 $\mu\text{g/mL}$ FeCl_3) to determine the inhibition zones along with pigmented halos (Figure 4.5). Tropolone was employed as a positive control, and the formation of ferric tropolone precipitate verified the iron depletion effect on *P. digitatum* (Sipiczki, 2006) (Figure B.3). In addition, the pectin-containing medium (0.5% or 1% pectin) promoted the production of antifungal pulcherrimin pigment (Figure B.4).

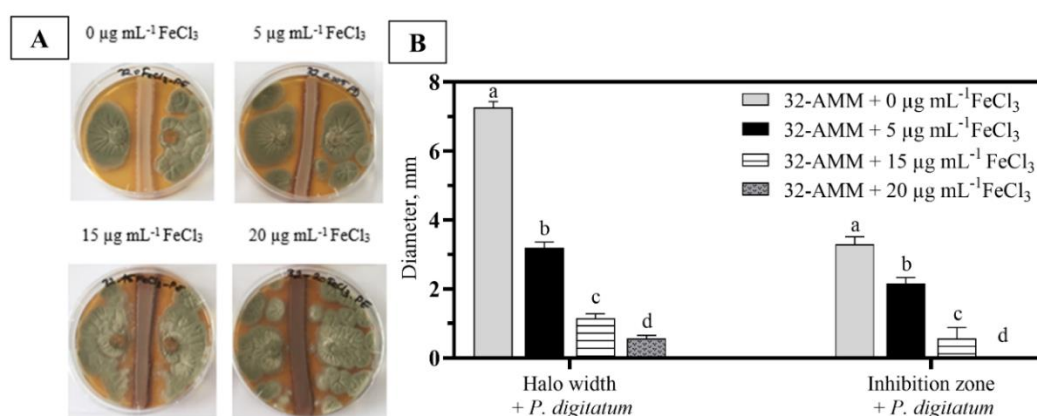


Figure 4.5 : The pigmented halo and inhibition zones (A,B) of *M. aff. pulcherrima* P01A016 32-AMM in the absence (0 $\mu\text{g/mL}$) and the presence (5, 15, and 20 $\mu\text{g/mL}$) of iron (FeCl_3) against *P. digitatum* DSM2750.

4.4.4.4 Mycocinogenic activity

All yeast cultures formed a blue zone (indicating cell death) around the colonies, whereas *M. aff. pulcherrima* P01A016 32-AMM and *H. uvarum* 38-KMD formed an adjacent clear zone (indicating growth inhibition) (Figure 4.6). The results showed considerable mycocinogenic activity against *S. cerevisiae* NCYC 1006, indicating the possibility of these yeast isolates as biocontrol agents. In addition, the mycocinogenic activity of yeasts was shown against *P. digitatum* on YEPD-MBA at pH 4.5 (Figure B.5), and the results indicated that all yeasts antagonized with the green mould, but *M. aff. pulcherrima* had clear inhibition zones (about 3 mm).

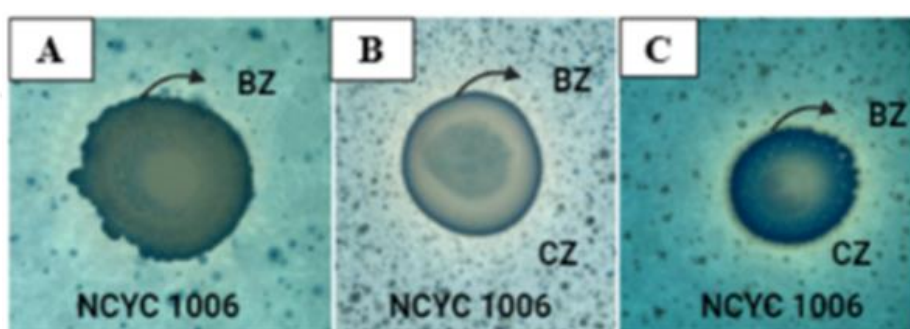


Figure 4.6 : Mycocinogenic activity of (A) *M. guilliermondii* 7-BYM, (B) *M.aff. pulcherrima* P01A016 32-AMM, (C) *H.uvarum* 38-KMD on *Saccharomyces cerevisiae* NCYC 1006) and a blue zone (BZ) (representing the cell death of *S. cerevisiae* NCYC 1006) on YEPD-methylene blue medium (pH 4.5) at 28 °C.

4.4.4.5 Biofilm formation

The biofilm activity was indicated by the absorbance value of the adhering cells at the bottom of the microtiter plate, which turned blue colour when dyed with crystal violet (Jin et al., 2003). After 72 h incubation, *M. guilliermondii* 7-BYM successfully formed biofilm ($OD\ 0.93 \pm 0.01$), followed by *M. aff. pulcherrima* P01A016 32-AMM ($OD\ 0.66 \pm 0.08$), and *H. uvarum* 38-KMD ($OD\ 0.45 \pm 0.02$).

4.4.4.6 Volatile organic compounds (VOCs)

At pH levels of 4.5 and 6, the *in vitro* VOC activities of yeast cultures and their mixtures on *P. digitatum* DSM2750 were found to be significantly different (Figure B.6). As compared to pH 6.0, VOCs significantly reduced the mycelial growth of *P. digitatum* under acidic conditions (at pH 4.5). The combined application of three yeast cultures (M-4) showed better inhibition of *P. digitatum*. *M. guilliermondii* 7-BYM had the highest inhibition rate, followed by *H. uvarum* 38-KMD and *M. aff. pulcherrima*

P01A016 32-AMM. M-2 had the highest volatile activity among dual yeast combinations at pH 4.5 and 6.0 (Table 4.4).

Table 4.4 : *In vitro* antagonism of yeast cultures and their mixtures by means of VOCs against *P. digitatum* DSM2750.

Yeast culture	Mycelial growth inhibition (%) of <i>P. digitatum</i> through yeast VOCs	
	pH 4.5	pH 6.0
<i>M. guilliermondii</i> 7-BYM	71.13 ± 1.38 ^{a*}	36.68 ± 1.48 ^{a*}
<i>M. aff. pulcherrima</i> P01A016 32-AMM	47.19 ± 0.31 ^{c*}	28.73 ± 0.15 ^{c*}
<i>H. uvarum</i> 38-KMD	63.91 ± 0.73 ^{b*}	30.85 ± 0.26 ^{b*}
M-1 ¹	64.70 ± 1.17 ^{b*}	32.04 ± 0.57 ^{b*}
M-2	70.06 ± 0.13 ^{a*}	35.82 ± 0.39 ^{a*}
M-3	62.90 ± 1.21 ^{b*}	30.83 ± 0.86 ^{b*}
M-4	71.80 ± 0.95 ^{a*}	37.01 ± 1.5 ^{a*}

¹M-1 = 7-BYM + 32-AMM; M-2 = 7-BYM + 38-KMD; M-3 = 32-AMM + 38-KMD; M-4 = 7-BYM + 32-AMM + 38-KMD. Data are means of three replicates ± standard error of the mean. The control groups' mean radial growth (mm) measurements were 41.26 ± 0.29 and 55.27 ± 0.19 at pH 4.5 and 6.0, respectively. Duncan's multiple range test indicates different superscript letters show statistically significant differences ($p < 0.05$). According to ANOVA, values marked with * within each row are significantly different ($p < 0.05$).

4.4.4.7 Biocontrolling of *P. digitatum* on mandarins

The disease incidence and lesion diameter values were measured between the 5th - 7th days of storage at 20 °C. On the 7th day, *M. guilliermondii* 7-BYM, *M. aff. pulcherrima* P01A016 32-AMM and *H. uvarum* 38-KMD reduced the disease incidence of the mandarin fruits by 73.3%, 75.5%, and 60%, respectively. In comparison to the control, the combined yeast treatments reduced the disease incidence by 73.3%, 66.7%, 62.2%, and 77.7%, for M-1, M-2, M-3, and M-4, respectively (Figure 4.7). DI and LD values caused by *P. digitatum* after 5th, 6th, and 7th day of incubation at 20 °C (Figure 4.7A). The corresponding reduction values for lesion diameters (%) were calculated to be 88.5%, 91.3%, and 69.1% for individual yeast treatments; 90.6%, 77.8%, and 78.4% for dual yeast combinations; and 92.9% for the mixture of three yeasts (Figure 4.7B). The fungicidal activity of yeasts and their mixtures upon experimental infection of mandarins with *P. digitatum* DSM2750 is displayed on the 7th day. Asterisks indicate synergistic activity was present according to Limpel's and Colby's formula (Figure 4.7C). These results provided evidence that the combined use of three yeasts

successfully reduced the disease incidence and lesion diameters of green mould decay compared to stand-alone treatments. These three yeasts collaborated effectively, and their distinct or similar modes of action (lytic enzymes, enzymatic profiles, iron depletion activity, VOCs, biofilm, and mycocin production) enhanced their bioprotective abilities. Furthermore, the mandarin fruits were treated only with pectinase-positive *M. aff. pulcherrima* P01A016 32-AMM to determine whether these yeasts had any phytopathogenicity (Figure B.7). The application of *M. aff. pulcherrima* P01A016 32-AMM did not damage the mandarins.

Figure B.8 depicts the biological control effect of synthetic fungicide, single yeast cultures, or yeast combinations on intact mandarin fruits (dip application). In general, the findings were found to be consistent with the wounded-mandarins. The biocontrolling effects of the yeast *Metschnikowia aff. pulcherrima* P01A016 32-AMM (97.6%) and M-4 (98.2%) combination were comparable to that of mandarins treated with Fungaflor (98.6%). However, control samples were found to be infected with green mould, and the least protection efficacy was observed in mandarins treated with *Hanseniaspora uvarum* 38-KMD alone.

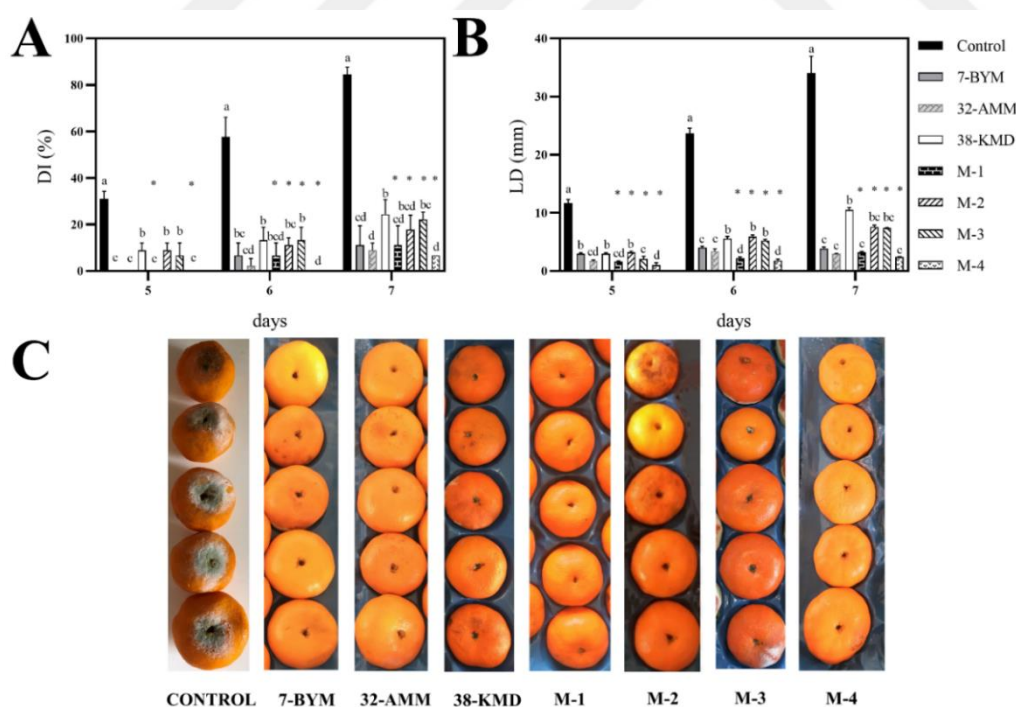


Figure 4.7 : *In vivo* antagonism (A,B,C) of three different yeast cultures (*M. guilliermondii* 7-BYM; *M. aff. pulcherrima* P01A016 32-AMM; *H. uvarum* 38-KMD) and their combinations (M-1 = 7-BYM + 32-AMM; M-2 = 7-BYM + 38-KMD; M-3 = 32-AMM + 38-KMD; M-4 = 7-BYM + 32-AMM + 38-KMD) in controlling of *P. digitatum* DSM2750 decay.

4.5 Discussion

In this study, *M. guilliermondii* 7-BYM, *M. aff. pulcherrima* P01A016 32-AMM and *H. uvarum* 38-KMD, alone or in combination, were tested on *P. digitatum* *in vitro* and *in vivo* on mandarins, as well as investigating the action mechanisms involved in the biocontrolling of green mould decay.

Dual and triple combinations of *M. guilliermondii* 7-BYM, *M. aff. pulcherrima* P01A016 32-AMM and *H. uvarum* 38-KMD were grown for 72 h to determine their compatibility (Figure 4.2) (Oro et al., 2014). The results showed that M-1 was the most compatible, followed by M-4; this could be attributed to the use of *M. aff. pulcherrima*'s enzymatic hydrolyzates promoted the growth of accompanying yeast culture. However, M-3 demonstrated the least compatibility, suggesting that yeast cultures were able to compete by preferring similar nutrients as carbon and energy source. The biocompatibility of yeasts can be explained by a niche differentiation, which indicates how *in vitro* carbon and nitrogen utilization profiles facilitate their cohabitation or *vice versa*. Since these biocontrol yeasts are living organisms, their efficacy may change over time (Janisiewicz, 1996; P. Liu et al., 2013). Furthermore, combining biocontrol yeasts with diverse modes of action can boost the biocontrol efficacy in an additive or synergistic manner (Agirman & Erten, 2020; Delali et al., 2021; Feng et al., 2021). A compatible combination of them can lower fungicide doses and prevent the development of fungal resistance (Perez et al., 2017; Vero et al., 2013).

Most fungi have cell walls that contain chitin, mannoproteins, cellulose, β -1,3- and β -1,6-glucans, and the cell-wall degrading enzymes can contribute to the antifungal mechanism of action (D. Wang et al., 2018). All the yeasts tested in this study displayed chitinase activity as a part of their biocontrol activity, which inhibited the germination of fungi spores and the development of germ tubes (Banani et al., 2015). Remarkably, *M. aff. pulcherrima* P01A016 32-AMM produced a variety of enzymes, including chitinase, protease, gelatinase, cellulase, pectinase, and β -1,3 glucanase (Belda et al., 2016; Escribano et al., 2017; Ianieva & Podgorsky, 2020; Oztekin & Karbancioglu-Guler, 2021; Pretscher et al., 2018).

Together with hydrolytic enzymes, the results of enzymatic fingerprinting can help to elucidate how yeasts act as antifungals. The leucine arylamidase, which belongs to the group of aminopeptidases and is mainly associated with the virulence of yeasts, had

the highest activity in all yeast samples (Figure 4). However, little is known about this enzyme, which could lead to new research avenues in the future (Pini et al., 2017). In addition, Sipiczki (2006) stated that leucine was produced by leucine arylamidase, which was used in the production of pulcherriminic acid, a precursor of pulcherrimin. Pretschner et al. (2018) documented that there was a link between α - and β -glucosidase enzymes and α - and β -glucanase activities. Accordingly, β -glucosidase activity was observed in both *M. aff. pulcherrima* P01A016 32-AMM and *H. uvarum* 38-KMD (Figure 4.4), which were also positive for β -1,3 glucanase (Table 4.3). Conversely, *M. guilliermondii* 7-BYM lacked both α - and β -glucosidase and β -1,3 glucanase.

Meyerozyma guilliermondii suppressed the *Colletotrichum capsici*-caused chili anthracnose using its hydrolytic enzymes. To elucidate the mechanism, the media was supplemented with hyphal cell wall fragments of *C. capsici* instead of laminarin and glucose as a carbon source, which resulted in more chitinase and β -1,3-glucanase production (Chanchaichaovivat et al., 2008). Similarly, Zhang et al. (2011) reported that β -1,3-glucanase and chitinase enzymes were involved in *M. guilliermondii*'s inhibitory mechanism against *Botrytis cinerea*. The application of *M. guilliermondii* to pear fruit induced the production of defense-related enzymes such as phenylalanine ammonia-lyase, peroxidase, catalase, and β -1,3 glucanase against *Penicillium expansum*. *Meyerozyma guilliermondii* suppressed blue mould in pears at 4 °C and 20 °C thanks to its rapid colonization and nutrient competition (Y. Yan, Zhang, Zheng, Tibiru, et al., 2018). Furthermore, protease and gelatinase activities indicated proteolytic activity, and protease activity was mostly observed in *Metschnikowia* and *Hanseniaspora* yeasts (Belda et al., 2016). Additionally, Horváth et al. (2021) detected a positive correlation between protease activity and biocontrol capacity of *Metschnikowia* yeast. In agreement with the current literature, gelatinase activity was observed in *M. guilliermondii* 7-BYM (Agirman & Erten, 2020) and *M. aff. pulcherrima* P01A016 32-AMM (Oztekin & Karbancioglu-Guler, 2021). Zajc et al. (2019) found comparable results for *M. guilliermondii*'s hydrolytic enzymes, except for gelatinase activity. Our findings suggested that different enzymes from different biocontrol yeasts could work synergistically to lyse and alter the permeability of *P. digitatum*'s cell membrane (Di Francesco et al., 2016).

In addition to yeasts, *P. digitatum* can secrete pH-lowering organic acids and pectinolytic enzymes (Droby et al., 1997; Moraes Bazioli et al., 2019), thereby

facilitating its colonization in the fruit by forming a porous structure (Miedes & Lorences, 2006). Furthermore, *P. digitatum* has been shown to produce catalase and decompose hydrogen peroxide, which is the main defense mechanism in citrus (Moraes Bazioli et al., 2019). In our investigation, pectinase-positive *M. aff. pulcherrima* P01A016 32-AMM was used, which also had other enzymes of interest. However, the enzyme activities were found to be strain-specific, resulting in differences in the inhibitory mechanism (Oztekin & Karbancioglu-Guler, 2021; Pawlikowska et al., 2019). Pulcherrimin, a maroon-red pigment produced by the ferric ions and pulcherriminic acid complex, was associated with high antimicrobial activity. Pulcherrimin exhibits antifungal activity by depleting iron from the surrounding media and causing hyphal cracks. As a result, iron deficiency prevented pathogenic fungi from growing normally and germinating conidia (Saravanakumar et al., 2008). Only *M. aff. pulcherrima* P01A016 32-AMM was capable of producing pulcherrimin in this study. Recently, pulcherrimin- and extracellular enzymes-producing *Metschnikowia andauensis* was tested under different culture conditions to understand its antagonistic behavior (Horváth et al., 2021). We hypothesized that these features were similar to the *M. aff. pulcherrima*'s properties. For this reason, *M. aff. pulcherrima* was cultured on YEA supplemented with pectin (0.5% or 1% pectin), and the results indicated that the pectin-containing medium supported the pigmentation of pulcherrimin, thereby the antifungal activity, when compared to the control (0% pectin) (Figure B.4). Furthermore, the reddish zones around the fruit wounds indicated the pulcherrimin formation after 5 d of incubation at 20 °C, indicating that *M. aff. pulcherrima* could be an effective biocontrol agent for green mould on mandarins (Figure B.7). This could be explained by the presence of pectin and nutrients in mandarins induced the production of pulcherrimin (Horváth et al., 2021). It was also hypothesized that *M. aff. pulcherrima*'s pectinolytic activity would facilitate its colonization in the mandarin cell wall, acting as a protective barrier. In some cases, the biocontrol yeasts can acquire extra carbon sources via producing pectinase, thereby ensuring their capacity to successfully adopt and colonize the plants' inner tissue (Pinto et al., 2018). For example, the pectinolytic activity of *Aureobasidium pullulans* yeast was correlated with the ability to penetrate and colonize plant cell walls, thus enhancing its ability to compete with pathogens for nutrients (Parafati, Vitale, Polizzi, et al., 2015). In another study, pectin-containing yeasts, including *Schwanniomyces vanrijiae*, *Galactomyces geotrichum*, *Pichia kudriavzevii*, *Debaryomyces hansenii*, and *Rhodotorula glutinis*

were successfully applied to apple fruit to inhibit *Monilinia fructigena*, the causal agent of brown rot (Madbouly et al., 2020).

In a recent study, apples were bio-controlled against *P. expansum* by *M. pulcherrima*-containing edible coatings (Settier-Ramírez et al., 2022). The viability of *M. pulcherrima* was found to be the highest in apple residues-containing films, which were rich in pectin, cellulose, starch and lignin, acid, and sugars. Surprisingly, the population of *M. pulcherrima* was higher than the initial number of inoculated cells, indicating that cell growth occurred in the apple residue-based films. The natural-based films made from apple residues pretend to be a fruit, thereby supporting the viability of the antifungal yeast *M. pulcherrima*. In line with our work, these edible films were successfully used on pectin-containing apple surfaces as a biocontrol agent.

Iron is a key component for fungal development and conidial germination, which also acts as a co-factor in fungal enzymes. *Metschnikowia* spp. can chelate iron from the medium, making it unavailable to other microorganisms (Y. Liu et al., 2017). To test the effect of iron depletion *in vitro*, PDA plates were supplemented with ferric ions at three different levels (5, 15, and 20 µg/mL FeCl₃) (Figure 4.5). As ferric ion concentrations increased, the halo and inhibition zones of yeasts decreased proportionally (Saravanakumar et al., 2008). *M. aff. pulcherrima*'s antifungal activity was lost when there was an excess of iron in the environment (in our case, 20 µg/mL FeCl₃) because the leftover iron after yeast binding supported fungal proliferation. Contrarily, when ferric ions were scarce, limited ferric ions were shared by yeast and fungus, resulting in iron deficiency in fungi and thereby increased antifungal activity. Based on this phenomenon, some fruits were reported to lower their iron concentrations to activate their defense mechanisms (Saravanakumar et al., 2008). Furthermore, researchers investigated whether the antimicrobial effect of *Metschnikowia* yeasts was solely due to the pigmented pulcherrimin compound. To achieve this, pigmentless *Metschnikowia* mutants were investigated for their antagonistic activity, and they were found to be antagonistic, indicating that other inhibition mechanisms may be involved (Gore-Lloyd et al., 2019). In agreement with our work, Liu et al. (2019) and Horváth et al. (2021) noted that *M. aff. pulcherrima*'s antifungal activity was attributed to the formation of pulcherrimin and hydrolytic enzymes, and their synthesis is strain-specific and culture factor-dependent.

Regarding the spore germination test, yeast cultures, alone or in combination, significantly reduced it by at least 80%, and the co-inoculation of three yeast cultures had the highest inhibition rate of 92.03% on *P. digitatum*. Most notably, *M. aff. pulcherrima*-containing combinations (M-1, M-3, and M-4) lacked sporulation, as shown in Figure B.2, indicating that it had a preventive effect on the spore germination of *P. digitatum*. When the pathogen and antagonist were in cell-to-cell contact in a culture, conidia germination was significantly reduced (Long & Yuan, 2009). Nutrients are essential for conidial spore germination, and the combination of yeast cultures may result in nutrient competition, directly affecting spore germination. The presence of nutrients improved conidial germination (Delali et al., 2021); for example, the addition of fresh orange juice (by 0.5 and 5%) significantly reversed the suppressed conidial germination of *P. italicum* (P. Liu et al., 2013). In addition, Coelho et al. (2009) reported that *M. guilliermondii* inhibited the conidial germination of *P. expansum* by 58.15%, which was lower than our findings on *P. digitatum*.

The ability of yeasts to form antifungal VOCs was investigated for a better understanding of yeast antagonism. These VOCs can be classified into alcohols, esters, organic acids, alkenes, ketones, and aldehydes (Qin et al., 2017). All yeast combinations generally showed higher antifungal activity via VOCs at pH 4.5. Among individual treatments, *M. guilliermondii* 7-BYM had the highest VOC antifungal activity with 71.13 (± 1.38) % and 36.68 (± 1.48) % inhibition rates on *P. digitatum* at pH 4.5 and 6.0, respectively. This could be due to the release of ethyl-acetate and ethanol (Coda et al., 2013). The antifungal activity of VOCs produced by *H. uvarum* 38-KMD and *M. aff. pulcherrima* P01A016 32-AMM were determined to be 63.91 (± 0.73)% and 47.19 (± 0.31)%, respectively (Table 4.4). M-2 had the highest antagonism through VOCs at 70.06 (± 0.13)%. However, M-3 showed relatively lower antifungal activity on green mould via VOCs, with 62.9 (± 1.21) % and 30.83 (± 0.86) %, at pH 4.5 and 6.0, respectively. Most notably, co-inoculation of three yeasts (M-4) had the highest mycelial growth inhibition rate through VOCs, 71.8 (± 0.95)% and 37.01 (± 1.5)% at pH 4.5 and 6.0, respectively. The main antifungal VOCs of *H. uvarum* were reported to be isoamyl alcohol, 2-methyl butanol (Tejero et al., 2021), ethyl acetate, and 1,3,5,7-cyclooctatetraene (Qin et al., 2017), which were released as a part of the defense mechanism in the presence of pathogenic fungi. When *M. pulcherrima*'s antifungal VOCs were tested on *P. digitatum*, the inhibition rate (65.54%) was higher

than ours, possibly because of strain-specific characteristics (Parafati et al., 2017). Hence, the major VOCs of *M. pulcherrima* were identified as ethyl acetate, ethanol, 1-hexanol, and 3-methyl-1-butanol (Contarino et al., 2019; H. Yang et al., 2021).

Biofilm has been proposed as a key feature of the biocontrol mechanism of yeasts. Biofilm was defined as a bunch of adherable microbial cells enclosed in a hydrated matrix of extracellular polymeric substances, including nucleic acids, lipids, proteins, and polysaccharides (Zara et al., 2020). Yeasts with the biofilm-forming ability provide clues about how well they can cling to fruit tissues or pathogen cell walls. In this way, biocontrol yeasts can also produce fungal cell-wall degrading enzymes, deplete iron, and compete for nutrients in the area they clung (Y. Liu, Yao, et al., 2019; Lutz et al., 2013). According to our research, *M. guilliermondii* 7-BYM was able to form a biofilm ($OD\ 0.93 \pm 0.01$) that lasted for 72 hours. Few studies have provided comparable data about *M. guilliermondii*'s biofilm activity. For example, Zajc et al. (2019) found that *M. guilliermondii* produced biofilm at low levels ($\sim OD = 0.4$). Al-Maawali et al. (2021) have reported that *M. guilliermondii* could form biofilm in tomatoes against *Alternaria alternata*. In another research, Wisniewski et al. (1991) used a light microscope to find evidence of *M. guilliermondii*'s strong adhesion to hyphal cell walls of *B. cinerea* on apples, which could indicate its biofilm activity.

Conversely, *M. guilliermondii* from wild grapes (Cordero-Bueso et al., 2017) and rice phylloplane (Into et al., 2020) failed to form biofilms. Recently, Bertini et al. (2021) performed proteomic analyses, showing the quorum sensing (QS) regulation between *Agrobacterium tumefaciens* and *M. guilliermondii* dual culture. The results showed that yeast proteome was altered by the bacterial QS activity leading to QS-influenced proteins, or *vice versa*. Supporting this, Larralde-Corona et al. (2011) proposed that when *M. guilliermondii* was in direct contact with *P. digitatum*, liberated fungal metabolites altered the defensive responses of the yeast, causing yeast to optimize limited nutrients and produce antifungal metabolites. In addition, *M. guilliermondii* was reported to exhibit antagonistic behavior by competing for nutrients. For example, when it was co-cultivated with *P. digitatum*, the addition of external nutrients was shown to reverse its antagonism (Spadaro & Droby, 2016).

The biofilm formation of *Metschnikowia* spp. varies, and the biofilm activity of *M. aff. pulcherrima* P01A016 32-AMM ($OD\ 0.66 \pm 0.08$) was comparable to our previous results ($OD\ 0.69 \pm 0.14$) (Oztekin & Karbancioglu-Guler, 2021). Likewise, upon 72 h

incubation, *M. pulcherrima* was reported to form biofilms at $OD\ 0.73 \pm 0.03$ (H. Yang et al., 2021) and $OD\ 1.12 \pm 0.16$ (Parafati, Vitale, Restuccia, et al., 2015); which were higher than the current findings. The biofilm activity has also contributed to *H. uvarum*'s biocontrolling activity, in line with the findings of Cabañas et al. (2020) and Cordero-Bueso et al. (2017). According to Pu et al. (2014), the biofilm formation of *H. uvarum* was controlled by the presence of phenylethanol, a volatile QS molecule, in the controlling of *P. italicum*. Similarly, 2-phenylethanol generated by *H. uvarum* can stimulate the adhesion-related gene, promoting the formation of a biofilm barrier between the wound surface and the *Penicillium* fungi. With the help of this barrier, fungi may not receive nutrients, resulting in cell death (P. Liu et al., 2014). Besides, Zhu et al. (2022) reported that *H. uvarum* controlled *P. expansum* in apples through nutrient competition, induced resistance, space competition, and parasitism. In another study, *H. uvarum* challenged for nutrients and vitamins in preventing *P. italicum*-induced citrus deterioration (P. Liu et al., 2013).

Several yeasts (including *Saccharomyces*, *Metschnikowia*, *Candida*, *Hansenula*, *Kluyveromyces*, and *Pichia*) can produce antifungal killer proteins (mycocin), which are predominantly active at acidic pH values and have lethal activity against sensitive microorganisms (Izgü & Altinbay, 2004). The inner pH of the citrus fruit is lower than its surface and drops dramatically when the fruit is injured or wounded (Citrus fruit has a pH of around 5 when measured from about 2mm below the surface). Under these circumstances, pH fluctuations may reduce the efficacy of conventional fungicides; alternatively, integrating mycocin-producing biocontrol yeasts could be a promising and effective approach for the citrus fruit (pH 3.0-5.5) (López-García et al., 2003). While some studies found that *M. guilliermondii* could produce killer toxins (Coelho et al., 2009; Lima et al., 2013), others showed that it could not (Chanchaichaovivat et al., 2008), indicating that it was a strain-dependent activity. *H. uvarum* (Schmitt & Neuhausen, 1994) and *M. pulcherrima* (Bedir & Kuleaşan, 2021) were discovered to produce killer toxins, which are consistent with the present observations. Conversely, Oro et al. (2014) concluded that *M. pulcherrima*'s antimicrobial action was due to its pulcherrimin production rather than its killer effect. Additionally, Figure B.5 demonstrates that all yeasts antagonized with the citrus green mould and *M. aff. pulcherrima* P01A016 32-AMM had clear inhibition zones, showing their biocontrol potential at acidic environments.

Among these three yeast isolates tested on mandarin, *M. aff. pulcherrima* P01A016 32-AMM was the most promising one, which exhibited iron depletion, mycocinogenic, and biofilm formation activities with a broad spectrum of enzymatic action as biocontrol mechanisms. Furthermore, *M. guilliermondii* 7-BYM capable of producing VOCs, biofilm, and cell-wall degrading enzymes, including chitinase, protease, and gelatinase. In addition, *H. uvarum* 38-KMD had chitinase, protease, and β -1,3 glucanase enzymes together with mycocinogenic activity and antifungal VOCs emission against *P. digitatum*. Yeast-treated mandarins had a longer shelf life with less decay and quality change than the control (Figure 4.7). By lowering disease incidence and lesion diameters (75.5% and 91.3%, respectively) throughout 7 d at 20 °C, *M. aff. pulcherrima* P01A016 showed the best efficacy on green mould decay of mandarins when compared to other treatments. The main acting mechanisms of biocontrol yeasts to inhibit green mould on mandarins were thought to be wound colonization and nutrition competition. Wound colonization and nutrition competition were considered to be the main acting mechanisms of biocontrol yeasts to inhibit green mould on mandarins. Among dual treatments, M-1 showed the best biocontrolling efficacy on green mould, having 73.3% and 90.6% values for incidence and lesion diameters. The results of this study appear to be very promising since combining three different yeasts demonstrated enhanced biocontrolling activity over individual isolates, as well as broadening its spectrum of activity (Janisiewicz, 1996). Our findings revealed that the combined application of three yeasts (M-4) was far superior to individual antagonists in controlling green mould on mandarins, reducing disease incidence and lesion diameters by 77.7% and 92.9%, respectively. It has been shown that the synergism between three yeasts against green mould could reduce the use of chemical fungicides following the SDG 13 climate action plan (UN, 2022). Employing antagonistic yeast combinations provided a green and sustainable solution for the environment, health, and economy and decreased food loss and waste (Bhatta, 2022; A. S. Dukare et al., 2019).

4.6 Conclusions

Understanding the individual antifungal mechanisms of antagonistic yeasts is critical for developing commercial formulations and effective application strategies by taking advantage of diverse action mechanisms. Employing different yeasts with distinct

modes of action could create a hurdle effect in reducing the decay of citrus green mould, which could boost the capacity for biological control. When *Hanseniaspora uvarum*, *Meyerozyma guilliermondii*, and *Metschnikowia* aff. *pulcherrima* P01A016 yeasts were used together (M-4), and their inhibitory effects increased in both *in vitro* and *in vivo* treatments of the mandarin fruit wounds and the whole fruits. *P. digitatum*, demonstrating synergism between these compatible yeasts. Overall, integrating multiple antagonistic yeasts can open new avenues to design effective microbial consortiums for a tailored bio-fungicide, which could help manage postharvest diseases in a sustainable and environmentally friendly way. Further investigation is required on taxonomically distinct but functionally compatible strains with their large-scale application.





5. RECRUITING GRAPE-ISOLATED ANTAGONISTIC YEASTS FOR THE SUSTAINABLE BIO-MANAGEMENT OF *BOTRYTIS CINEREA* ON GRAPES

5.1 Abstract

Botrytis cinerea is the causative agent of grey mould disease in grapes, which was linked to significant postharvest losses. This study examined three grape-isolated yeasts (*Metschnikowia* aff. *fructicola*, *Metschnikowia pulcherrima*, and *Hansenispora uvarum*) through *in vitro* and *in vivo* tests on detached grape berries against grey mould, as well as the elucidation of their possible mechanisms of action. The antifungal mechanism of action of yeasts was determined by the lytic enzyme activity, inhibition of spore germination, biofilm activity, iron depletion, diffusible metabolites, wound-site colonisation, mycocin, and volatile organic compounds (VOCs) production. The highest *in vitro* efficacy (83.13%) was observed on *M. aff. fructicola*, followed by *M. pulcherrima* (82.10%) and *H. uvarum* (71.66%). *Metschnikowia* yeasts exhibited comparable enzyme activities, including protease, β -1,3 glucanase, gelatinase, chitinase, and cellulase, while *H. uvarum* had a poor enzymatic activity with chitinase and gelatinase. *M. aff. fructicola* showed relatively higher iron depletion activity than *M. pulcherrima*, while *M. pulcherrima* outperformed via diffusible metabolites. All yeast cultures significantly reduced spore germination by at least 86%. Overall, *M. aff. fructicola* exhibited the highest biocontrolling activity with its iron depletion, inhibition of conidial germination, biofilm formation, VOCs, and well colonisation on grape berries. *M. aff. fructicola* 1-UDM outperformed all other yeasts by significantly reducing disease incidence and lesion diameter values (93.4% and 94.3%, respectively). Interestingly, *H. uvarum* VOCs demonstrated potential as a biofumigant for suppressing grey mould. All yeasts are well adapted to their ecological niche to bio-protect grapes from grey mould disease.

Keywords: *Botrytis cinerea*; grey mould; biological control; postharvest; grapes, antagonistic yeasts

5.2 Introduction

Grapes (*Vitis vinifera* L.) are one of the most economically valuable fruits in the world, with their distinctive taste, antioxidants, and vital nutrients (Majeed et al., 2023). Globally, grape production averaged 73 million tonnes in 2021. Türkiye ranked sixth in producing 5% of the world's grapes, after China, Italy, Spain, the United States, and France (FAO, 2021). However, grapes are particularly susceptible to fungal infestation during harvesting, storage, and transportation. In this respect, the most common postharvest diseases in grapes can be attributed to grey (Botrytis bunch rot) and blue mould diseases caused by *Botrytis cinerea* and *Penicillium expansum*, respectively (H. Zhang et al., 2020).

B. cinerea, a necrotrophic fungus, is a major viticulture pathogen that can cause massive (up to \$100 billion worldwide) economic losses (Hua et al., 2018; Otto et al., 2022). *B. cinerea* can host various fruits and vegetables, including grapes, strawberries, blueberries, and tomatoes. It can attack with both asexual and sexual spores and survive in harsh environments, even at temperatures as low as -0.5 °C (Choquer et al., 2007; Hua et al., 2018; Karabulut et al., 2004). Although using synthetic fungicides to treat grey mould disease in postharvest fruits was used as a standard method, the acquired drug resistance and residue from these chemicals may harm human health (Droby et al., 2009). Furthermore, the rising popularity of organic products, stricter regulations, and safety concerns about the adverse effects of these toxic chemicals on human health and the environment have pushed scientists to develop efficient, eco-friendly, and sustainable alternatives (H. Zhang et al., 2020). Apart from synthetic fungicides, sulfur dioxide (SO₂) gas is used in the fumigation of grapes to prevent grey mould disease (Karabulut et al., 2004). Sulfites, on the other hand, may harm health, particularly for those who are sulfite intolerant (Timbo et al., 2004), and excessive sulfite residues may jeopardize worker safety and air quality (Karabulut et al., 2004). Moreover, SO₂ can bleach colored grapes when applied at high concentrations, causing early browning and an unpleasant aftertaste, particularly during subsequent winemaking processes (S. Ahmed et al., 2018). To address these concerns, non-*Saccharomyces* yeasts were proposed as a viable alternative to sulfites in grape bioprotection (Windholtz et al., 2021). Puyo et al. (2023), for example, combined *M. pulcherrima* with tannins as an alternative to sulfites to protect grapes and must from microbial spoilage and oxidation.

Antagonistic yeasts are one of the most promising eco-friendly and sustainable alternatives to synthetic fungicides and sulfites in the biopreservation of fruits and vegetables from phytopathogenic fungi (A. S. Dukare et al., 2019; Hernández et al., 2022; Windholtz et al., 2021). These yeasts have numerous advantages in the biocontrolling of fruits with their GRAS (generally recognized as safe) (FDA, 2021) and safe characteristics (without producing mycotoxin, antibiotics, and allergenic spores) (Nunes, 2012; Parafati, Vitale, Restuccia, et al., 2015). Furthermore, antagonistic yeasts can exploit simple nutrients through their diverse lytic enzyme activities. They are also successful at attaching to the surface of fruits, competing for space and nutrients, and limiting the growth of pathogenic fungi. These yeasts can combat fungi through antifungal component synthesis, including the ability to secrete volatile organic compounds (VOCs), iron-chelating compounds, and mycocin (killer toxin) (Agirman & Erten, 2020; Izgü & Altinbay, 2004; Oztekin & Karbancioglu-Guler, 2021; Parafati, Vitale, Restuccia, et al., 2015).

The surface of the fruit is a natural reservoir for epiphytic yeasts (Oztekin & Karbancioglu-Guler, 2021). A biocontrol system is composed of four main elements: host (fruit), pathogen (fungi), yeast (antagonistic yeast), and resident epiphytic flora (environment) (Spadaro & Droby, 2016). The use of antagonistic yeasts as bio-fungicides has been the subject of extensive research (Hernández et al., 2022; J. Liu et al., 2013; Sare et al., 2021). In this sense, the most frequently reported antagonistic yeasts belonged to *Metschnikowia*, *Candida*, *Pichia*, *Hanseniaspora*, *Debaryomyces*, *Meyerozyma*, *Aureobasidium*, and *Saccharomyces* (Hernández et al., 2022; Izgü et al., 2006; Oztekin & Karbancioglu-Guler, 2021; Parafati, Vitale, Restuccia, et al., 2015). Within the focus of the current study, *Metschnikowia pulcherrima* (Fernandez-San Millan, Gamir, Farran, Larraya, Veramendi, et al., 2022; Parafati, Vitale, Restuccia, et al., 2015; Saravanakumar et al., 2008; Sipiczki, 2020; Türkel et al., 2014), *Metschnikowia fructicola* (Guzzon et al., 2014; Karabulut et al., 2003; Kurtzman & Droby, 2001; Oztekin & Karbancioglu-Guler, 2021), and *Hanseniaspora uvarum* (Apaliya et al., 2019; Cordero-Bueso et al., 2017; Guzzon et al., 2014; H. M. Liu et al., 2010; Qin et al., 2015) were used on grapes to biocontrol grey mould disease.

Some of the yeast-based biofungicide products have been approved and marketed by various companies. For example, Excellence B-Nature[®] from *M. pulcherrima* biocontrolled *B. cinerea* while reducing SO₂ dose on grapes (URL-4). Likewise, a

mixture of *Torulaspora delbrueckii* and *M. pulcherrima* was marketed under the brand name Zymaflore® Égide to protect grapes and reduce SO₂ (URL-5). Additionally, the European Union approved *M. fructicola*-based biofungicide to biocontrol *Botrytis* and *Monilinia* in grapes, strawberries, and stone fruits (EFSA, 2017), which was first marketed under the name Shemer®. Then, Koppert Company licensed it under the brand name Noli® (URL-3).

Reducing pesticide use was one of the Sustainable Development Goals (SDG 2) of the United Nations since pesticide contamination is considered to be environmental destruction. Furthermore, biocontrol yeasts can aid in developing sustainable agricultural applications to end hunger and halve postharvest losses (SDG 12) by 2030 (UN, 2022). Fruits are a rich source of antagonistic yeasts for *in situ* management of fungi-induced post-harvest diseases (Oztekin & Karbancioglu-Guler, 2021). Therefore, this study aimed to test grape-isolated antagonistic yeasts (*M. aff. fructicola*, *M. pulcherrima*, and *H.uvarum*) in inhibiting *B. cinerea* through *in vitro* and *in vivo* experiments on grapes while also elucidating their potential mechanisms of action.

5.3 Materials and Methods

5.3.1 Yeast cultures

Metschnikowia aff. fructicola 1-UDM and *M. pulcherrima* 34-UEM were previously isolated and identified from grapes by PCR amplification and sequencing of the D1/D2 domain of the 26S rDNA (Oztekin & Karbancioglu-Guler, 2021). A grape-isolate *Hanseniaspora uvarum* 3-UDM was identified by employing the same methodology above. The sensitive yeast strain *Saccharomyces cerevisiae* NCYC 1006 was obtained from the National Collection of Yeast Cultures (Norwich, UK). Further, the yeast isolates were preserved at 4 °C in yeast extract peptone dextrose agar (YEPD agar- 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar) slants and 20 % glycerol stock at -80 °C for further use.

5.3.2 Fungal culture

Botrytis cinerea was isolated from botrytized grapes and obtained from the Department of Plant Protection, Selçuk University, Konya, Turkey. Mould culture was

grown on Potato Dextrose Agar (PDA) at 25 °C, and stock solutions were prepared in 20 % (v/v) glycerol and stored at -80 °C.

5.3.3 Fruits

Green grapes (*Vitis vinifera* L.) were purchased from an organic market, and bunches were selected randomly based on uniformity, maturity, and absence of injury. The fruits were superficially disinfected using 2 % (w/v) of sodium hypochlorite (NaOCl) solution for 2 min and rinsed twice with pure water and air-dried at 25 °C.

5.3.4 Preparation of yeast cells and fungal spore suspensions

Each yeast culture was inoculated (1 % v/v) into YEPD Broth (YEPD-10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) and incubated at 28 °C for 24 h at 200 rpm. Yeast cells were obtained by centrifugation and subsequently adjusted to desired concentrations (1×10^8 , 1×10^7 , 1×10^6 , 1×10^5 cells/mL) in sterile distilled water (SDW) (Ruiz-Moyano et al., 2016). Additionally, the conidial suspensions of *B. cinerea* were prepared (1×10^6 , 1×10^8 conidia/mL) in SDW containing 0.1 % (v/v) Tween 80 (Merck, Germany). Both yeast cells and conidia were counted using a Neubauer chamber (Marienfeld, Germany).

5.3.5 In vitro assays

5.3.5.1 Dual culture plate assay

Yeast cultures were screened for their *in vitro* antagonistic activity against *B. cinerea* employing the method of Pantelides et al. (2015). First, yeast suspensions (1×10^8 cells/mL) were streaked orthogonally 2 cm away from the Petri dish containing PDA and left for incubation for 48 h. Then, a mycelial disc (5-mm square plugs) of grey mould was placed 2 cm away from the yeast and incubated at 25 °C for 5 d. The control plates contained grey mould only. The mycelial growth reduction was calculated as in the equation (5.1):

$$I (\%) = \frac{(C - T)}{C} \times 100 \quad (5.1)$$

where I (%) represented the inhibition of the mycelial growth, C was the mycelial growth in the control (mm), and T was the mycelial growth of pathogenic fungi in the

presence of yeast cultures (mm). The experiments were performed thrice and repeated twice.

5.3.5.2 Diffusible metabolite assay

Following the *in vitro* antagonism test, antagonistic yeasts were examined for their diffusible compounds to understand the inhibition mechanism better (P.-H. Chen et al., 2018). To achieve this, yeast suspensions (20 μ L, 1×10^8 cells/mL) were pipetted onto YEPD agar, 2 cm away from the plate's edge (90 mm diameter). Fungal mycelium (5×5 mm²) was placed on the opposite side and incubated at 22 °C for 5 d. The plates without yeasts were used as a control, and mycelial growth was measured with a calliper. According to the equation (5.2):

$$I (\%) = \frac{(R_c - R_{exp})}{R_c} \times 100 \quad (5.2)$$

where I % represented the inhibition rate, R_c was the longest diameter of the fungal mycelium (mm), and R_{exp} was the horizontal diameter of the fungal mycelium (mm). The experiment was repeated twice, with three replicates for each treatment.

5.3.5.3 Extracellular lytic enzyme activity

The yeast cultures were investigated for their chitinase (Pretscher et al., 2018; Souza et al., 2009), pectinase (Merín et al., 2011), β -1,3 glucanase (Lutz et al., 2013), cellulase (Escribano et al., 2017), protease (Gardini et al., 2006; Vidyasagar et al., 2006), and gelatinase (Gardini et al., 2006) activities. The presence of these enzymes was determined on specified agar mediums containing colloidal chitin (Sigma C7170, USA), citrus pectin (Sigma P9135, Denmark), laminarin (Sigma L9634, USA), carboxymethylcellulose sodium salt (CMC) (Sigma C4888, Denmark), skim milk powder (Sigma-Aldrich 70166, Switzerland) and gelatin (Sigma-Aldrich, Steinheim, Germany).

For this purpose, 10 μ L of fresh yeast suspensions (1×10^7 cells/mL) were pipetted into the abovementioned agar plates. After incubating at 25 °C for 7 d, extracellular chitinase activity was determined by the clear lysis zones around the yeast colonies upon flooding with Lugol's solution. After 48 h of incubation at 28 °C, pectinase activity was detected by the formation of a distinct halo around the colonies when flooding them with iodine solution. Following the incubation at 25 °C for 72 h, grown

colonies on laminarin-containing agar plates were stained with Congo Red (Sigma C6277, USA) solution for 60 min and washed with 1M NaCl for 30 min. The resulting yellow-orange zones around yeast colonies indicated the presence of β -1,3 glucanase activity. Following the incubation at 30 °C for 5 d, the colonies in CMC-containing plates were rinsed with SDW (1 min), Congo Red (30 min), and 1M of NaCl (15 min). Then, the clear halo formed around yeast colonies indicated cellulase activity. Likewise, the protease activity was determined by clear halos around the yeast colonies after 7 d at 25 °C. Finally, gelatinase activity was observed based on the liquefaction after 3 weeks of incubation at 30 °C.

5.3.5.4 API-ZYM enzymatic profiles

The hydrolytic enzyme profile of yeast isolates was screened using the API ZYM test (BioMérieux, Lyon, France) following the manufacturer's instructions containing 20 cupules with a specific substrate and buffer. Nineteen different enzymes were identified, including alkaline phosphatase, esterase (C 4), esterase lipase (C 8), lipase (C 14), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase. Results were assessed in nanomoles (nmol) of the hydrolysed substrate on a 5-step scale, ranging from 0 (no activity) to 5 (highest activity), with 1 equalling 5 nmol, 2 equalling 10 nmol, 3 equalling 20 nmol, 4 equalling 30 nmol, and 5 equalling 40 nmol of API ZYM substrate (Pini et al., 2017). Heat maps of the enzymatic profiles were produced using the ClustVis web program (URL-2).

5.3.5.5 Spore germination test

The yeast cultures were evaluated for their ability to inhibit the conidial germination of *B. cinerea* based on the method of Ruiz-Moyano et al. (2016). First, yeast cell suspensions (100 μ L, 1×10^8 cells/mL) were mixed with the spore suspensions of *B. cinerea* (100 μ L, 1×10^8 conidia/mL) in a 5 mL YEPD Broth. The conidia germination was measured after 12 h of incubation at 100 rpm and 25 °C. The control was prepared using SDW instead of yeast suspensions. The germinated conidia were observed under a light microscope (Olympia) and calculated based on 100 spores observing at least 5 microscope fields. When the length of the germ tube was greater than the diameter of

the spore, it was regarded to be germinated. The percentage of spore germination was calculated as in the equation (5.3):

$$I (\%) = \frac{(C - T)}{C} \times 100 \quad (5.3)$$

where I (%) represented the percent inhibition, T % represented the percentage of germinated spores under yeast treatment, and C % referred to control without yeast treatment. Each treatment was repeated thrice, and the experiments were carried out twice.

5.3.5.6 Iron depletion assay

To determine their antagonism, yeast cultures were tested on FeCl₃-containing (5, 15, and 20 µg/mL) PDA plates (Saravanakumar et al., 2008). First, the yeast cultures (1 × 10⁸ cells/mL) were streaked orthogonally from the center of the plate and incubated for 48 h at 30 °C. A fungal plug (5 × 5 mm²) from a 10-d-old *B. cinerea* culture cultivated on PDA was placed 25 mm away on both sides of the yeast. Inhibition and halo zones were recorded following 7 days of incubation at 25 °C. Iron-free PDA plates were used as a negative control (Sipiczki, 2006). The experiment was carried out three times with double repetition for each iron level.

5.3.5.7 Volatile organic compounds (VOCs)

The antifungal activity of yeast VOCs was investigated against *B. cinerea* by employing the method of Parafati et al. (2015). The yeast suspensions (100 µL, 1 × 10⁸ cells/mL) were spread into YEPD agar plates prepared at two different pH values (4.5 and 6.0) and incubated overnight at 25 °C. The control plates contained SDW only. The plate was seeded with a *B. cinerea* fungal spore suspension (20 µL, 1 × 10⁶ conidia/mL) and left to dry for 30 min at 25 °C. Then, Parafilm® and tape were used to cover two confronting plates containing fungal and yeast inoculum. The mycelium growth inhibition was observed throughout 9 d at 25 °C. The radial growth reduction of fungal mycelia (%) was calculated as in the equation (5.4):

$$I (\%) = \frac{(C - T)}{C} \times 100 \quad (5.4)$$

where I (%) represented the inhibition of the radial mycelial growth, C was the radial mycelial growth in control (mm), and T was the radial mycelial growth of pathogenic fungi in the presence of yeast cultures (mm). The experiment was carried out thrice and repeated twice.

5.3.5.8 Biofilm formation

Using the Jin et al.'s approach (2003), yeast colonies were examined for their capacity to create biofilms. To achieve this, yeast nitrogen base (YNB) medium supplemented with glucose (100 mM) was used to cultivate yeasts at 28 °C for 24 h in 96-well plates. Then, the wells were washed twice with phosphate-buffered saline (PBS, pH 7.2), and an aliquot of (100 µL) yeast suspensions (1×10^7 cells/mL) were pipetted into wells, then left for incubation at 28 °C, 75 rpm for 3 h. The cells without yeast were used as control. After that, the wells were washed twice with 150 µL of PBS, and 100 µL of YNB was pipetted into the wells and incubated at 28 °C, 75 rpm for 72 h. Again, the wells were washed twice with PBS and stained with 100 µL of crystal violet (0.4 %) for 45 min. The stained cells were washed with SDW four times to drain excess dye, and then 200 µL of 95% ethanol was added and incubated for 45 min. The resulting solution (100 µL) was pipetted into a new polystyrene 96-well plate, and absorbances were recorded at OD₅₉₀ nm. Biofilm formation was indicated to be positive when the mean absorbance of the treatment was higher than that of the negative control. The net absorbance values were determined by subtracting the OD_{control} from OD_{sample} values. The net absorbance values were calculated based on the equation (5.5), and the analysis was carried out twice.

$$A_{\text{net}} = OD_{\text{control}} - OD_{\text{sample}} \quad (5.5)$$

where A_{net} is the net absorbance value, OD_{control} is the absorbance value of control and OD_{sample} is the absorbance value of sample.

5.3.5.9 Mycocinogenic activity

Yeast cultures were examined for their mycocin (killer toxin) activity (Izgü et al., 2006; Öztekin & Karbancioglu-Guler, 2023). *Saccharomyces cerevisiae* NCYC 1006 was used as a killer-sensitive reference culture and grown at 25 °C for 24h h on YEPD agar. *S. cerevisiae* (100 µL, 1×10^5 cells/mL) cells were pour-plated in YEPD-methylene blue agar (YPD-MBA; 10 g/L yeast extract, 20 g/L peptone, 20 g/L

glucose, 20 g/L agar, and 0.03 g/L methylene blue), which was buffered to pH 4.5 with 100 mM citrate–phosphate buffer. Subsequently, yeast cultures (10 μ L, 1×10^8 cells/mL) were pipetted onto *S. cerevisiae*-seeded YEPD-MBA plates and incubated at 28 °C for 72 h. The existence of a clear zone (signifying inhibitory action) or blue zone (signifying cell death) around the colonies was viewed as evidence of mycocinogenic (killer) activity.

5.3.6 *In vivo* assays

5.3.6.1 Yeast colonisation in wounded grapes

The colonisation ability of yeast cultures on grape surfaces was determined by using the method of Parafati et al. (2015). To do this, grape berries were wounded (3 mm deep and 3 mm wide, four wounds/grape) along the equatorial area, and yeast cultures (20 μ L, 1×10^6 cells/mL) were inoculated into these wounds. Then they incubated for 96 h at 25 °C and 95% relative humidity (RH). At certain time intervals (0, 24, 48, 72, and 96 h), the wounds were removed using a sterile knife and transferred into tubes containing 1 mL of buffered peptone water (Merck, Germany). The recovered yeasts were cultivated (at 25 °C for 48h) on chloramphenicol added (100 mg/L)-Sabouraud Dextrose Agar (Darmstadt, Germany), and population dynamics ($\log_{10}$ CFU) were calculated based on the fruit weight. Then, 30 grape berries (three replicates of ten grape fruits) were used per treatment in a completely randomised design, and the experiment was conducted twice.

5.3.6.2 Biocontrolling efficacy of yeast cells

The method reported by Parafati et al. (2015) was employed to test *in vivo* biocontrolling activity of yeast cultures against grey mould on grapes. The grapes were artificially wounded as previously described and inoculated with the conidial suspension of *B. cinerea* (20 μ L, 1×10^6 conidia/mL). After 2 h of air drying, yeast cultures were inoculated (20 μ L, 1×10^8 cells/mL) into grape wounds, while the same amount of SDW was used as a control. The grape berries were enclosed in plastic bags with wet paper towels at 25 °C for 5 days to maintain a high RH. The disease incidence (DI) and the lesion diameter (LD) values were calculated using the equation (5.6):

$$DI (\%) = \frac{DF}{TW} \times 100 \quad (5.6)$$

where DF is the number of decayed fruits and TW is for numbers of total wounds. In addition, lesion diameter (LD) values were calculated using the average diameter surrounding the decay. As previously stated, each treatment consisted of ten grape berries with three replicates in a completely randomised design, and the experiment was repeated twice.

5.3.6.3 Biocontrolling efficacy through VOCs

The method of Parafati et al. (2015) was used to test whether VOCs from yeasts could biocontrol the grey mould on grapes. First, each yeast culture was grown on YEPD agar plates at pH 4.5 and incubated for 48 h at 25 °C. The grape berries were seeded with *B. cinerea* as previously mentioned. Subsequently, yeast-seeded plates (six plates per container) and grape berries (10 fruits) were put into polypropylene containers and sealed in plastic bags to prevent air leakages. The yeast-free plates were used as a control. After 5 days of inoculation at 25 °C, DI and LD values were determined as explained in the equation (5.6). Three biological replicates were performed for each treatment, and the experiment was performed twice.

5.3.7 Statistical analysis

The phylogenetic analysis was performed through the neighbour-joining (NJ) method from The Molecular Evolutionary Genetics Analysis (MEGA Version 7.0.21), including 1000 bootstrap replicates. A clustering analysis was performed using the UPGMA (unweighted pair group method with arithmetic mean) for branch robustness. *In vivo* and *in vitro* data were analysed through the statistical software SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and expressed as mean ± standard deviation. Treatments were compared with a one-way analysis of variance (ANOVA) test. Duncan's multiple range test performed post-hoc comparisons, and the differences were considered statistically significant at $p < 0.05$.

5.4 Results

5.4.1 Phylogenetic tree of yeast cultures

Phylogenetic analysis of nucleotide sequences from domain D1/D2 of large subunit 26S rDNA resulted in three clades with $\geq 99\%$ identity (Figure. 5.1). The numbers at nodes indicate the bootstrap values, and the bar shows genetic distance. Table 5.1 depicts the isolation code, source, culture, GenBank accession numbers, and percentage identities of the three yeast isolates.

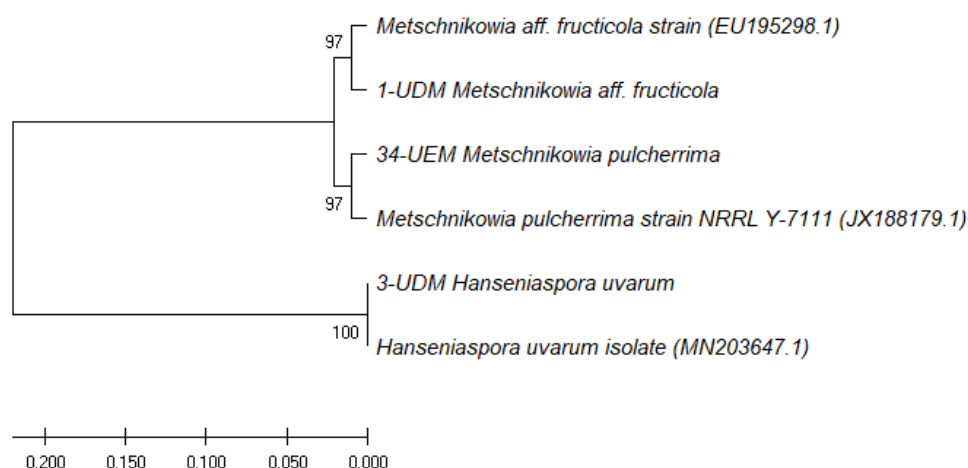


Figure 5.1 : The phylogenetic tree based on sequences of the D1/D2 domains of the large subunit 26S rDNA region.

Table 5.1 : Molecular identification of yeast isolates by comparing the genomic sequences in the GenBank database using the BLAST algorithm.

Isolate code	Source	Culture	Accession Number	Identity (%)
1-UDM*	Green grapes (<i>Vitis vinifera</i> L., Bozcaada Çavuşu)	<i>M. aff. fructicola</i>	KU862650.1	99.54
3-UDM	Green grapes (<i>Vitis vinifera</i> L., Bozcaada Çavuşu)	<i>H. uvarum</i>	OQ305050.1	99.41
34-UEM*	Red grapes (<i>Vitis vinifera</i> L., Erzincan Cimin)	<i>M. pulcherrima</i>	MN371975.1	99.79

* Oztekin & Karbancioglu-Guler (2021).

5.4.2 *In vitro* antagonistic activity of yeast cultures

Table 5.2 depicts three yeast cultures' *in vitro* antagonistic activities on *B. cinerea*. *Metschnikowia aff. fructicola* 1-UDM and *M. pulcherrima* 34-UEM were previously determined by Oztekin and Karbancioglu-Guler (2021), while the mycelial growth

inhibition by *Hanseniaspora uvarum* 3-UDM is presented in Figure 5.2. The highest *in vitro* antagonism was observed in *Metschnikowia* aff. *fructicola* 1-UDM (83.13%), followed by *M. pulcherrima* 34-UEM (82.10%) and *Hanseniaspora uvarum* 3-UDM (71.66%).

Table 5.2 : *In vitro* mycelial growth inhibition of yeast isolates against *B. cinerea*.

Mycelial growth inhibition (%)	
Isolate	<i>B. cinerea</i>
1-UDM*	83.13 ± 3.18 ^a
3-UDM	71.66 ± 0.21 ^b
34-UEM*	82.10 ± 1.45 ^a

* Oztekin & Karbancioglu-Guler (2021). Different letters in each column are statistically different by Duncan's multiple range test ($p < 0.05$). Each value is the mean ± standard deviation of three replicates.

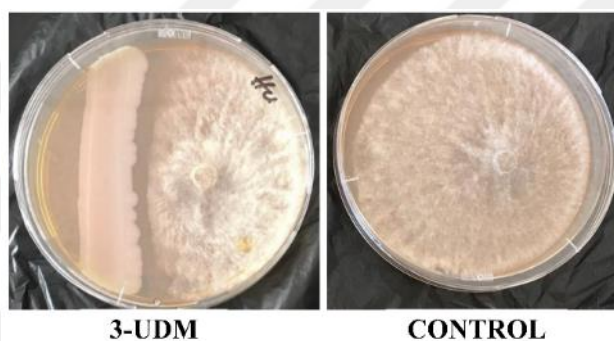


Figure 5.2 : *In vitro* mycelial growth inhibition of *Botrytis cinerea* by *Hanseniaspora uvarum* 3-UDM.

5.4.3 Enzymatic activities of yeast cultures

The yeast cultures were screened for hydrolytic enzymes, including chitinase, pectinase, β -1,3 glucanase, cellulase, protease, and gelatinase (Table 5.3). Pectinase activity was absent in all yeasts tested, whereas chitinase and gelatinase activities were positive. *Metschnikowia* aff. *fructicola* 1-UDM and *M. pulcherrima* 34-UEM had β -1,3 glucanase, cellulase, and protease activity (Oztekin & Karbancioglu-Guler, 2021); however, *Hanseniaspora uvarum* 3-UDM responded negatively to these enzymes.

Table 5.3 : Lytic enzyme activities of yeast isolates.

Isolate	Chitinase	Pectinase	β -1,3 glucanase	Cellulase	Protease	Gelatinase
1UDM*	+	-	+	+	+	+
3UDM	+	-	-	-	-	+
34UEM*	+	-	+	+	+	+

*Oztekin & Karbancioglu-Guler (2021). The presence (+), and absence (-) of enzymes are indicated for the corresponding enzymes.

The enzymatic fingerprinting of yeast cultures, shown in Figure 5.3, revealed that β -glucosidase and leucine arylamidase activities were the highest (≥ 40 nmol) in all yeasts tested. The previous work by Oztekin and Karbancioglu-Guler (2021) on the API ZYM profiles of the yeasts is indicated with an asterisk (*). Hierarchical clustering of positive (1–5) and negative (0) results. Distance measure: Euclidean. Clustering algorithm: Ward (Figure 5.3). The yeast cultures and enzymes are shown in rows and columns, respectively. The API ZYM enzymatic profiles of *Metschnikowia* aff. *fructicola* 1-UDM and *M. pulcherrima* 34-UEM were previously tested by Oztekin and Karbancioglu-Guler (2021), which had intense (≥ 40 nmol) α -glucosidase, moderate (30 nmol) valine arylamidase, esterase (C4), esterase lipase (C8), and weak (10 nmol) cysteine arylamidase and acid phosphatase activity. *Hanseniaspora uvarum* 3-UDM exhibited intense (≥ 40 nmol) alkaline phosphatase, acid phosphatase, esterase (C4), and valine arylamidase with moderate (30 nmol) esterase lipase (C8), and naphthol-AS-BI-phosphohydrolase activity. However, the enzyme activities were lacking for α -fucosidase, α -mannosidase, N-acetyl- β -glucosaminidase, β -glucuronidase, β -galactosidase, α -galactosidase, α -chymotrypsin, trypsin, and lipase (C14) activities.

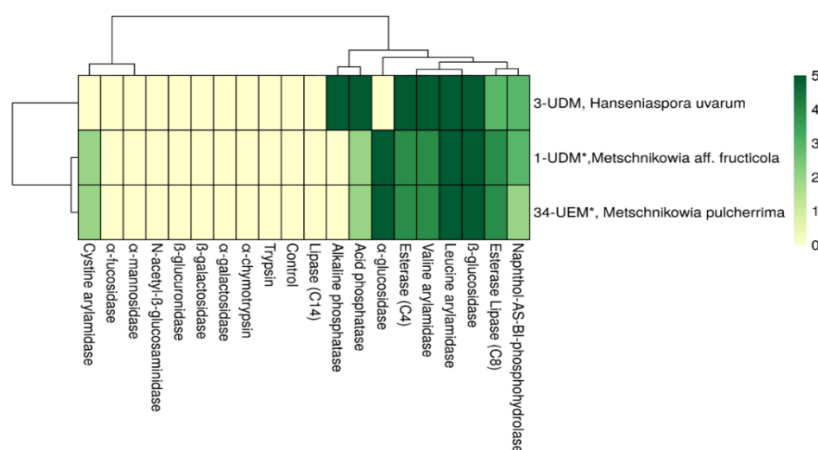


Figure 5.3 : Enzymatic profiles of *Metschnikowia* aff. *fructicola* 1-UDM, *Hanseniaspora uvarum* 3-UDM, and *M. pulcherrima* 34-UEM.

5.4.4 Effect of yeast cultures on spore germination of *B. cinerea*

Yeast cultures were tested for their ability to inhibit the conidial germination of *B. cinerea* after 12 h at 100 rpm at 25 °C (Figure 5.4). Compared to the control, *Metschnikowia* aff. *fructicola* 1-UDM had the highest reduction in the conidial germination of the grey mould by 97.9%, followed by *M. pulcherrima* 34-UEM and *Hanseniaspora uvarum* 3-UDM by 97.2% and 86.8%, respectively ($p < 0.05$).

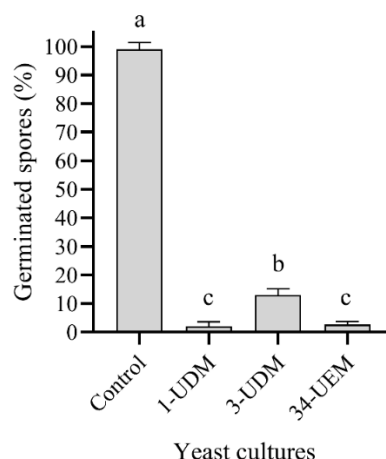


Figure 5.4 : The effect of *Metschnikowia* aff. *fructicola* 1-UDM, *Hanseniaspora uvarum* 3-UDM, and *M. pulcherrima* 34-UEM on the spore germination of *B. cinerea*.

5.4.5 Iron depletion activity of yeast cultures

Metschnikowia spp. can deplete iron from the surrounding media, leaving pathogenic fungi iron-starved (Saravanakumar et al., 2008). For this purpose, *Metschnikowia* aff. *fructicola* 1-UDM and *M. pulcherrima* 34-UEM were tested *in vitro* against *B. cinerea* at increasing concentrations of iron (5, 15, and 20 $\mu\text{g/mL}$ FeCl_3) to determine the inhibition zone and pigmented halo (Figure 5.5). As the concentration of ferric ions in the growing medium increases, the intensity of the maroon-red pigmentation around the yeast increases, indicating the formation of pulcherrimin (Sipiczki, 2020).

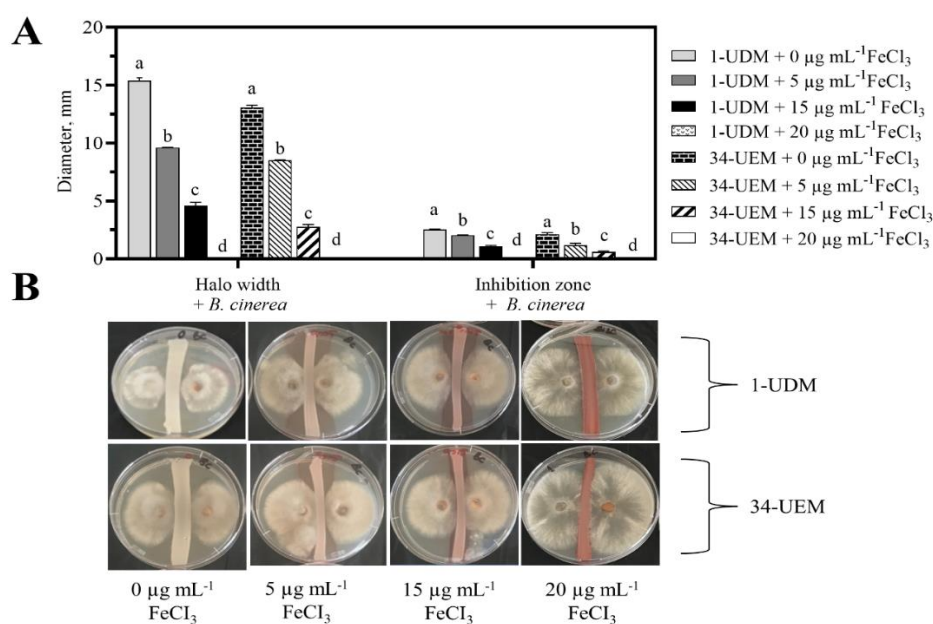


Figure 5.5 : The pigmented halo and inhibition zones (A,B) of *Metschnikowia* aff. *fructicola* 1-UDM and *M. pulcherrima* 34-UEM against *B. cinerea* at different iron concentrations (5, 15, and 20 $\mu\text{g/mL}$).

5.4.6 Diffusible metabolite assay of yeast culture

The yeast cultures produced diffusible antifungal compounds on grey mould at varying levels (Figure 5.6). The inhibition of *B. cinerea* mycelial growth through diffusible metabolites was the highest in *M. pulcherrima* 34-UEM (70.65%), followed by *Metschnikowia* aff. *fructicola* 1-UDM (59.20%) and *Hanseniaspora uvarum* 3-UDM (53.81%) (Table 4).

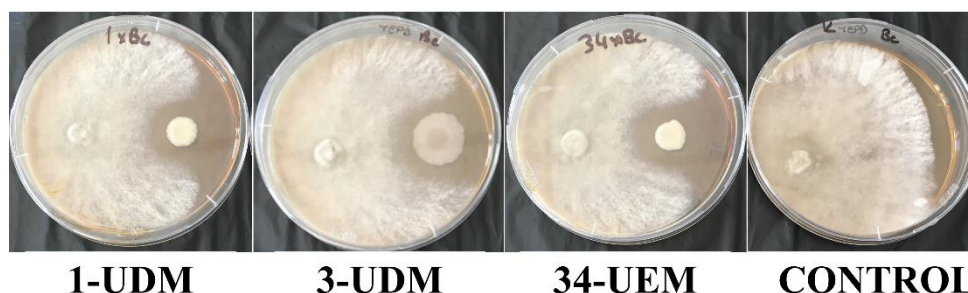


Figure 5.6 : Diffusible metabolite assay of yeast cultures (*Metschnikowia* aff. *fructicola* 1-UDM, *Hanseniaspora uvarum* 3-UDM, and *Metschnikowia pulcherrima* 34-UEM) in dual culture with *Botrytis cinerea*.

5.4.7 Effect of VOCs in the mycelial growth reduction of *B. cinerea*

The mycelial growth inhibition of grey mould using VOCs varied significantly at pH 4.5 and 6.0 (Table 5.4). At pH 4.5, the mycelial growth reduction of *B. cinerea* was higher than that of pH 6.0. *H. uvarum* 3-UDM exhibited the highest (59.96%) inhibition activity for the mycelial growth of *B. cinerea*, while *M. pulcherrima* 34-UEM had the lowest (49.59%). At pH 6.0, *M. aff. fructicola* 1-UDM had the highest inhibition rate (31.29%), while *M. pulcherrima* 34-UEM had the lowest one (18.99%).

Table 5.4 : *In vitro* antagonism of diffusible metabolites and volatile organic compounds (VOCs) of yeasts.

Isolate	Mycelial growth inhibition (%) of <i>B. cinerea</i>		
	Diffusible metabolites	VOCs	
		pH 4.5	pH 6.0
<i>Metschnikowia</i> aff. <i>fructicola</i> 1-UDM	59.20 ± 1.77 ^b	50.20 ± 0.16 ^{b*}	31.29 ± 0.37 ^{c*}
<i>Hanseniaspora uvarum</i> 3-UDM	53.81 ± 0.62 ^c	61.70 ± 0.35 ^{a*}	29.16 ± 0.30 ^{b*}
<i>M. pulcherrima</i> 34-UEM	62.3 ± 0.32 ^a	42.66 ± 0.25 ^{c*}	18.99 ± 0.80 ^{a*}

Asterisks indicate statistical differences compared to control according to Duncan's multiple range test at $p \leq 0.05$.

5.4.8 Biofilm activity

The biofilm formation capability of *M. aff. fructicola* 1-UDM and *M. pulcherrima* 34-UEM were detected previously (Oztekin & Karbancioglu-Guler, 2021). The results showed that *M. aff. fructicola* 1-UDM had the highest biofilm-forming capacity (OD 0.84 ± 0.08), followed by *M. pulcherrima* 34-UEM (OD 0.43 ± 0.03), and *H. uvarum* 3-UDM (OD 0.25 ± 0.02), respectively ($p < 0.05$).

5.4.9 Mycocinogenic activity

All yeast cultures developed blue (showing cell death) and clear (showing growth inhibition) zones to varying degrees (Figure 5.7). The results showed significant mycocinogenic activity against *S. cerevisiae* NCYC 1006, implying that these yeast isolates hold the potential to be used as biocontrol agents.

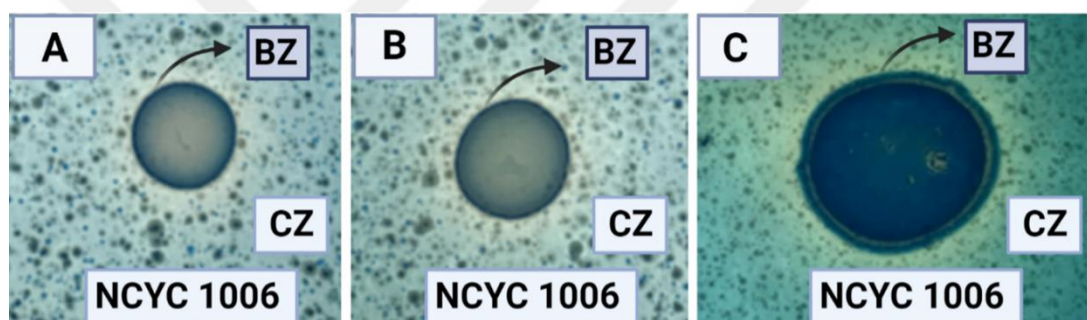


Figure 5.7 : Mycocinogenic activity of (A) *M. aff. fructicola* 1-UDM (B) *M. pulcherrima* 34-UEM M, (C) *H. uvarum* 3-UDM on *Saccharomyces cerevisiae* NCYC 1006) and a blue zone (BZ) (representing the cell death of *S. cerevisiae* NCYC 1006) on YEPD-methylene blue medium (pH 4.5) at 28 °C.

5.4.10 Population dynamics of yeast cultures on grape wounds

Figure 5.8 illustrates the counting of yeast colonies on the wounded grape berries. All yeast cultures began from the same point (4.8 log concentration) and had a logarithmic growth until 1 d. Then, all yeasts followed an increasing path till 4 d. At the end of the incubation period, the population growth rates of *M. aff. fructicola* 1-UDM, *M. pulcherrima* 34-UEM, and *H. uvarum* 3-UDM reached 7.84, 7.53, and 7.90, respectively.

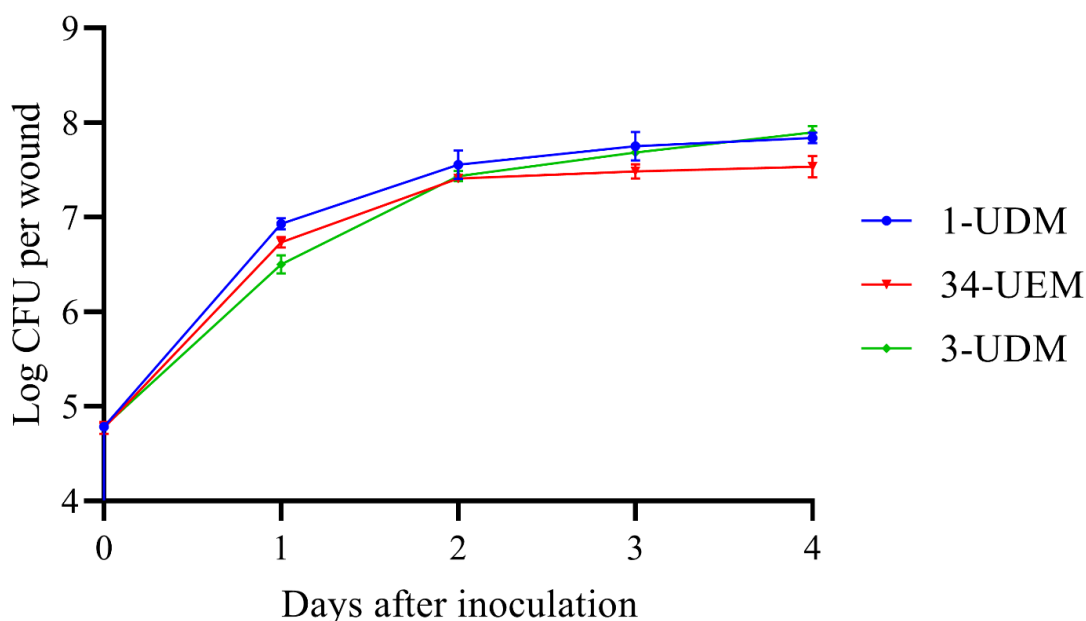


Figure 5.8 : Population dynamics of *Metschnikowia* aff. *fructicola* 1-UDM, *M. pulcherrima* 34-UEM, and *Hanseniaspora uvarum* 3-UDM on grape wounds at 25 °C.

5.4.11 Biocontrolling efficacy of yeast cultures on grapes

The *in vivo* biocontrolling efficacy of yeast cells on wounded grape berries is presented in Figure 5.9. Compared to the control, all yeast cultures significantly reduced DI and LD values at varying levels. *M. aff. fructicola* 1-UDM had the lowest DI and LD values, indicating its high biocontrolling efficacy on *B. cinerea*-inoculated grape berries, followed by *M. pulcherrima* 34-UEM and *H. uvarum* 3-UDM. DI values were reduced by 93.4%, 89.4%, and 68.2%, while LD reduction values were found to be 94.3%, 88.7%, and 73.7% for *M. aff. fructicola* 1-UDM, *M. pulcherrima* 34-UEM, and *H. uvarum* 3-UDM, respectively.

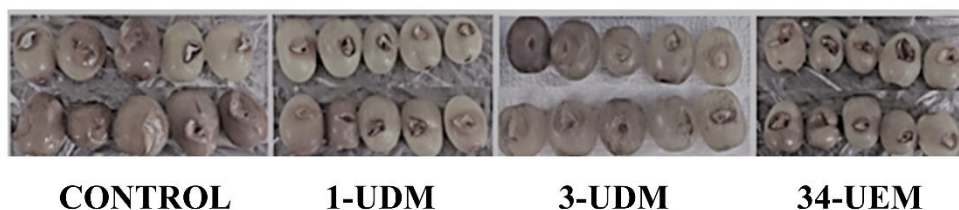


Figure 5.9 : Biocontrol activity of *Metschnikowia* aff. *fructicola* 1-UDM, *Hanseniaspora uvarum* 3-UDM, and *M. pulcherrima* 34-UEM on *Botrytis cinerea*-inoculated grape berries for a 5-d incubation period at 25 °C.

Correspondingly, the grey mould inhibition on grape berries through VOCs is shown in Figure 5.10B. Compared to *in vivo* biocontrolling by yeast cells, *in vivo* VOCs treatments resulted in significantly higher DI and LD values, demonstrating that the

effect of VOCs alone is insufficient. *H. uvarum* 3-UDM VOCs have yielded relatively lower DI and LD values (64.7% and 6.5 mm, respectively), implying that its VOCs played a significant role in the biocontrolling activity on grey mould-inoculated grape berries. The corresponding values were higher for *M. aff. fructicola* 1-UDM (68% and 8.3 mm) and *M. pulcherrima* 34-UEM (69.5% and 9.7 mm), respectively.

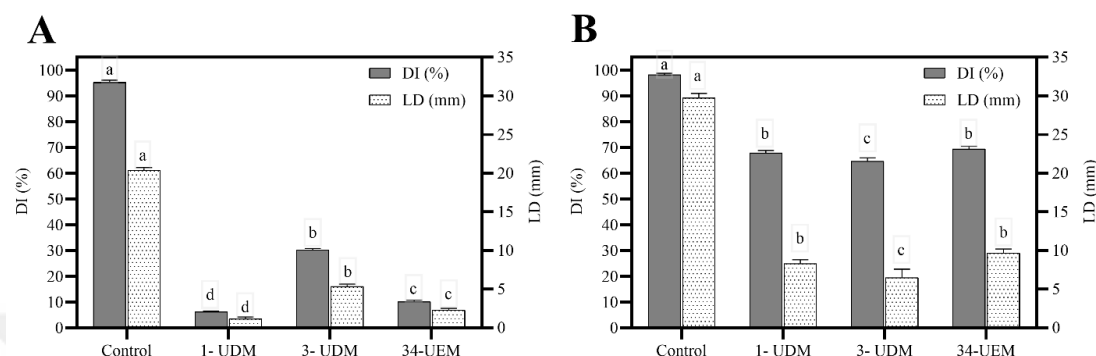


Figure 5.10 : *In vivo* biocontrolling efficacy of yeast cultures A) *Metschnikowia* aff. *fructicola* 1-UDM, *Hanseniaspora uvarum* 3-UDM, and *M. pulcherrima* 34-UEM, and their B) VOCs on *Botrytis cinerea*-inoculated grape berries after 5-d of incubation at 25 °C.

5.5 Discussion

In this study, grape-isolated *M. aff. fructicola* 1-UDM, *H. uvarum* 3-UDM, and *M. pulcherrima* 34-UEM yeasts were tested against *B. cinerea* through *in vitro* and *in vivo* analyses on grapes. Additionally, potential antifungal modes of action in yeast cultures were examined to understand the underlying mechanism better.

All yeast cultures showed varying antagonistic activity on grey mould *in vitro* and *in vivo*. In this regard, several mechanisms have been proposed in the biological control process of antagonistic yeasts, including hydrolytic enzymes secretion, biofilm formation, iron depletion, mycocin production, and VOCs emission (Agirman & Erten, 2020; Farbo et al., 2018; Oztekin & Karbancioglu-Guler, 2021; Öztekin & Karbancioglu-Guler, 2023; Sipiczki, 2020).

The cell walls of most fungi contain chitin, mannoproteins, cellulose, β -1,3- and β -1,6-glucans. Thus the cell-wall degrading enzymes can contribute to the antifungal mechanism of action (D. Wang et al., 2018). Employing antagonistic yeasts with cell-wall degrading enzymes, such as β -1,3 glucanase, protease, gelatinase, chitinase, and cellulase can directly contribute to the antifungal action (Belda et al., 2016; Oztekin & Karbancioglu-Guler, 2021; Pretscher et al., 2018). All relevant enzymes were present

in *M. aff. fructicola* 1-UDM and *M. pulcherrima* 34-UEM, except for pectinase. Other than these, *H. uvarum* 3-UDM exhibited chitinase and gelatinase activity. It was discovered that the enzyme activity in *Metschnikowia* is strain-specific, resulting in changes in the biocontrolling efficacy (Oztekin & Karbancioglu-Guler, 2021; Pawlikowska et al., 2019). For example, Parafati et al. (2015) discovered that *M. pulcherrima* lacked hydrolytic enzymes, whereas some studies found that *Metschnikowia* yeasts were highly active in the production of enzymes (Belda et al., 2016; Horváth et al., 2021; Oztekin & Karbancioglu-Guler, 2021). In this sense, proteolytic activity was detected with the presence of protease and gelatinase enzymes, which indicated the exploitation of *Metschnikowia* and *Hanseniaspora* yeasts was a promising choice in the biological control (Belda et al., 2016; Horváth et al., 2021). Likewise, chitinase activity was related to the inhibition of spore germination and the development of germ tubes (Figure 5.4) (Banani et al., 2015). Most antagonists produced chitinases and glucanases in the presence of *B. cinerea* (Maluleke et al., 2022; D. Zhang et al., 2011).

Apart from hydrolytic enzymes, API-ZYM enzymatic profiles of yeasts were investigated to have detailed information on the inhibition mechanism (Figure 5.3). The findings demonstrated that levels of β -glucosidase and leucine arylamidase activity were observed at the highest level in all yeasts. An aminopeptidase enzyme, leucine arylamidase, produces leucine for use in the production of pulcherrimin (Sipiczki, 2006). Moreover, α - and β -glucosidase enzymes were associated with α - and β -glucanase enzymes (Pretscher et al., 2018). In line with this hypothesis, *M. aff. fructicola* 1-UDM and *M. pulcherrima* 34-UEM exhibited both α - and β -glucosidase activities, as well as β -1,3 glucanase activity. However, *H. uvarum* 3-UDM lacked for α -glucosidase and β -1,3 glucanase despite having β -glucosidase.

Metschnikowia spp. yeasts are known for their iron-chelating activities, which resulted in pulcherrimin synthesis (Saravanakumar et al., 2008; Sipiczki, 2020). Pulcherrimin's maroon-red color intensifies with increasing ferric iron levels in the medium, indicating pulcherrimin's saturation with iron or *vice versa*. Excessive iron levels limit antifungal efficacy because the remaining iron after yeast binding encourages fungal growth. Contrarily, antagonistic yeast showed higher antifungal activity at low iron concentrations due to its capacity to effectively bind ferric ions without becoming saturated. Because iron is a key micronutrient, the fungus may not develop normally

and produce conidia due to iron deficiency. Moreover, it was highlighted that iron deprivation reduced *B. cinerea*'s virulence activity (Saravanakumar et al., 2008).

Accordingly, iron-supplemented (5, 15, and 20 µg/mL FeCl₃) plates showed that increasing ferric ions in the media led to a decrease in the halo and inhibition zones. Considering the above, *Metschnikowia* aff. *fructicola* 1-UDM absorbed iron better than *M. pulcherrima* 34-UEM, which was indicated by the relatively higher inhibition zones and reduction in the mycelial growth of *B. cinerea*. Supporting our findings, *M. fructicola* (Spadaro & Droby, 2016) and *M. pulcherrima* (Agarbatı et al., 2022; Sipiczki, 2020) were reported to produce pulcherrimin in the presence of FeCl₃. Pulcherrimin production, cell-wall degrading enzymes, inhibition of spore germination, biofilm formation, and wound site colonisation mechanisms were proposed as the main modes of action in *Metschnikowia* yeasts on *B. cinerea* (P.-H. Chen et al., 2018; Horváth et al., 2021; Oztekin & Karbancioglu-Guler, 2021). Moreover, *H. uvarum* was employed in biocontrolling due to its ability to compete for living space, suppress spore germination, colonise on fruits, and secrete defense-related enzymes (Qin et al., 2015).

Mycocins were first discovered in *S. cerevisiae* (Makower & Bevan, 1963), then several yeasts (*Saccharomyces*, *Metschnikowia*, *Candida*, *Hanseniaspora*, *Kluyveromyces*, and *Pichia*) were found to produce these proteinaceous killer toxins effective on sensitive strains (Izgü & Altınbay, 2004; Öztekin & Karbancioglu-Guler, 2023). Since mycocins are antifungal proteins and function effectively at acidic pH levels (Izgü et al., 2006), the experiment was carried out at pH 4.5 (Figure 5.7). Furthermore, *B. cinerea* can secrete pH-lowering organic acids (e.g., oxalic acid) and pectinolytic enzymes (e.g., pectin methylesterases) and facilitate its colonisation in the fruit (Manteau et al., 2003). Under these circumstances, mycocin production of yeasts could be effectively employed in biological control. *H. uvarum* (Schmitt & Neuhausen, 1994) and *M. pulcherrima* were discovered to produce killer toxins (Bedir & Kuleaşan, 2021), being consistent with the present observations. Grapes are acidic because they contain significant amounts of malic, tartaric, and, to a lesser extent, citric acids (Robles et al., 2019). Furthermore, the pH of a grape varies (pH 3.18-3.60) depending on its variety (Kamiloğlu & Üstün, 2014). This study suggests that mycocin-producing yeast cultures may be an effective alternative to synthetic fungicides in the biocontrol

of acidic grapes because it has been noted that synthetic fungicides can demonstrate inconsistent efficacy in acidic settings (Cordero-Bueso et al., 2017).

The microbial colonies trapped in a polymeric matrix and adhering to the surface of an experimental plate and fruit are biofilms (P.-H. Chen et al., 2018). In our case, *H. uvarum* 3-UDM ranked first in the wound colonisation on grape wounds (3-UDM>1-UDM>34-UEM) (Figure 5.8) despite having the lowest *in vitro* biofilm activity. A volatile quorum sensing (QS) molecule, 2-phenylethanol, was found to trigger the adhesion-related gene and increase the formation of biofilm in *H. uvarum* (anamorph *Kloeckera apiculata*) (Pu et al., 2014). In another study, *H. uvarum* exhibited comparable colonisation (8 log) in kiwifruit when stored at 25 °C for 4 d, resulting in the induction of defense-related genes and enzymes (e.g., chitinase and β -1,3-glucanase) (L. Cheng et al., 2019). Despite having a high potential for wound colonisation *in vivo*, *H. uvarum* 3-UDM had a poor ability to produce biofilm *in vitro*. The ability of *M. aff. fructicola* 1-UDM to colonise wounds and form biofilms was discovered to be strongly related in this study. Likewise, *M. pulcherrima* produced comparable results when tested on grape berries against grey mould (Parafati, Vitale, Restuccia, et al., 2015). Without considering biofilm activity, Karabulut et al. (2003) reported that the *M. fructicola* on table grapes remained stable at 6 log following 4 weeks of storage at 1 °C. Moreover, it was observed that considerable biofilm production is not necessary for wound protection (Fernandez-San Millan et al., 2021). With moderate biofilm production, *M. pulcherrima* 34-UEM demonstrated comparable colonisation potential on detached grape berries depending on the strain. Some studies have shown that *M. pulcherrima* produced biofilms at OD 0.73 ± 0.03 (H. Yang et al., 2021) and OD 1.85 ± 0.16 (Parafati, Vitale, Restuccia, et al., 2015) lower and higher than that of the current investigation, respectively. Besides, *M. pulcherrima* colonisation on grapes began at 6.2 log and progressed to 7.5 log after 10 days (X. Wang et al., 2018). Since these yeasts are isolated from grapes, they can rapidly colonise and adapt well to their ecological niche (Raspor et al., 2010). Yeast colonisation has been discovered to be advantageous in competition for nutrients and space (niche exclusion), thereby enhancing the efficacy of biological control (Parafati, Vitale, Restuccia, et al., 2015).

All yeast cultures significantly reduced spore germination by at least 86%. Both *Metschnikowia* yeasts significantly reduced the conidial germination of grey mould ($\geq$

97.2%) (Figure 5.4). Liu et al. (2010) reported that *H. uvarum* (1×10^8 cells mL) inhibited spore germination of *B. cinerea* (1×10^6 spores mL) by 87.6%, which was comparable with the current findings. Guo et al. (2019) discovered that trans-cinnamaldehyde, a volatile organic monomer from *H. uvarum*, strongly inhibited mycelium growth and conidial germination of *B. cinerea* in cherry tomatoes. Furthermore, *M. pulcherrima* (5×10^8 cells/mL) inhibited the conidial germination of *B. cinerea* (1×10^8 spores/mL) (Ruiz-Moyano et al., 2016). The spore germination of *B. cinerea* (5×10^6 spores mL) was totally suppressed by live yeast cells of *M. pulcherrima* (2×10^5 and 2×10^6 cells mL), however at lower concentrations (2×10^4 cells mL), the germination rate was reduced by 34% in comparison to control (Piano et al., 1997). Thus, the concentration of antagonistic yeast is a determining factor for the inhibition of spore germination (Ruiz-Moyano et al., 2016). Moreover, conidial germination was significantly reduced when the pathogen and antagonist were in cell-to-cell contact in culture (Long & Yuan, 2009). In this sense, Fernandez-San Millan et al. (Fernandez-San Millan, Gamir, Farran, Larraya, Veramendi, et al., 2022) co-cultured *M. pulcherrima* with *B. cinerea* to induce and investigate the production of novel antifungal metabolites. Also, it was highlighted that although inhibition of spore germination is not necessary for yeast to limit fungal growth, it most likely promotes antagonism (Fernandez-San Millan et al., 2021).

Additionally, all yeasts produced diffusible inhibitory compounds on *B. cinerea* at different levels (34-UEM>1-UDM>3-UDM). The diffusible antifungal compounds involved in the biocontrol of grey mould could be defined as 3-amino-5-methylhexanoic acid, biphenyl-2,3-diol, sinapaldehyde, long chain fatty acids, alkaloids, bile acid, piperidine, antibiotics, phenylpropanoid hydrolytic enzymes, and pulcherriminic acid (P.-H. Chen et al., 2018; Sipiczki, 2006). Cyclic peptides and diketopiperazine were described as non-volatile components of antifungal yeasts that were effective against *B. cinerea* (Maluleke et al., 2022). Fernandez-San Millan et al. (Fernandez-San Millan, Gamir, Farran, Larraya, Veramendi, et al., 2022) reported that these metabolites disrupted the cell membrane, cracked the hyphal form of *B. cinerea*, and increased the intracellular level of reactive oxygen species (ROS).

Antifungal VOCs from yeasts are used as a bio-fumigant to inhibit fungal growth in postharvest fruits (Qin et al., 2017). *In vitro* antifungal activity by VOCs revealed that all yeast cultures exhibited better antifungal activity at pH 4.5 (3-UDM>1-UDM>34-

UEM), which also significantly differed at both pH values (Table 5.4). Among the tested yeasts, the highest VOCs production was detected in *H. uvarum* 3-UDM, which could be explained by the release of isoamyl alcohol, 2-methyl butanol (Tejero et al., 2021), ethyl acetate, and 1,3,5,7-cyclooctatetraene (Qin et al., 2017). In another study, VOCs from *H. uvarum* were linked to different esters (ethyl propionate, n-Propyl acetate, isobutyl acetate, 2-methylbutyl acetate, furfuryl acetate, phenylmethyl acetate, 2-phenylethyl acetate), ketones (Heptan-2-one), and volatile acids (acetic acid and octanoic acid)(Ruiz-Moyano et al., 2020). During 4 days of storage at 25 °C, two *H. uvarum* strains, 531 and 793, reduced the radial growth of grey mould via VOCs by 49.01% and 62.27%, respectively (Ruiz-Moyano et al., 2020), which were lower and higher than that of our findings, indicating that inhibition through VOCs is strain-dependent. VOCs of *H. uvarum* significantly reduced the grey mould incidence on strawberries by 72.1% within 3 d and on cherries by 45.6% after 4 d at 25 °C (Ruiz-Moyano et al., 2020).

Likewise, major VOCs of *Metschnikowia* yeasts can be attributed to the emission of ethyl acetate, ethanol, 1-hexanol, and 3-methyl-1-butanol (Contarino et al., 2019; H. Yang et al., 2021). The observations were compared with those of Parafati et al. (2015), who reported that *M. pulcherrima* resulted in a higher (47.48%) inhibitory effect by VOCs on grey mould at acidic conditions (pH 4.5). *In vitro* antifungal activities through VOCs were compatible with those of *in vivo* (Table 5.4 and Figure 5.10B). Contrary to our findings, Parafati et al. (2015) found no correlation between the activities of VOCs *in vitro* and *in vivo*, which is strain-specific and highly dependent on the environmental and growth conditions (Fernandez-San Millan et al., 2021).

In vivo biocontrolling assay on grape berries showed that DI and LD values were significantly reduced by antagonistic yeast treatments compared to control, with *M. aff. fructicola* 1-UDM having the highest efficacy in reducing grey mould decay (Figure 5.9 and Figure 5.10A). Agarbati et al. (2022) documented that *B. cinerea*-inoculated (2×10^5 spores/mL) and *M. pulcherrima*-treated (1×10^7 cells/mL) grape berries stored for 3 d at 25 °C, which resulted in the lowest decay (12%) among other yeasts. Artificially wounded table grapes inoculated with *B. cinerea* (1.5×10^7 and 1.5×10^4 conidia/mL) were biocontrolled by *M. pulcherrima* (1.5×10^7 cells/mL) following 5 days of incubation at 25 °C (Marsico et al., 2021). Liu et al. (2010)

documented that *H. uvarum* (1×10^8 cells/mL) completely inhibited the grey mould decay after 4 days at 25 °C without impairing the grape quality. According to Ruiz-Moyano et al. (2016), *M. pulcherrima* completely inhibited grey mould on apples, while *Hanseniaspora opuntiae* inhibited it by 76.9%. The iron competition, biofilm formation and VOCs production were discovered to be a key feature of *M. pulcherrima*, which outperformed other yeast species (*Wickerhamomyces anomalus*, *Aureobasidium pullulans*, and *Saccharomyces cerevisiae*) in the biocontrol of grapes against Botrytis rot (Parafati, Vitale, Restuccia, et al., 2015).

The DI and LD values of *B. cinerea*-inoculated (1×10^4 spores/mL) kiwifruits treated with *H. uvarum* (5×10^7 cells/mL) decreased by 47% and 37%, respectively, during 4 days of storage at 25 °C, which is less than recent findings (L. Cheng et al., 2019). The difference in the biological control effectiveness of *H. uvarum* may be attributed to the strain, environmental conditions or the applied fruit referring to the substrate (Ruiz-Moyano et al., 2020).

Our findings support the hypothesis that yeast antifungal activities are influenced by a variety of mechanisms; however not all those mechanisms together were found in a single yeast alone. Thus, iron depletion, secretion of hydrolytic enzymes, diffusible compounds, inhibition of conidial germination, biofilm formation, wound-site colonisation, and VOCs emission all collectively contributed significantly to the reduction of the grey mould on grape berries (Parafati, Vitale, Restuccia, et al., 2015; Ruiz-Moyano et al., 2020). Furthermore, other inhibiting mechanisms that were not investigated in this study, such as induction of host defense, should be considered as well (L. Cheng et al., 2019).

5.6 Conclusion

The fruit microbiome is a precious source of antagonistic yeasts for *in situ* management of fungi-induced postharvest diseases. In this respect, grape-isolated yeasts (*M. aff. fructicola*, *M. pulcherrima*, and *H. uvarum*) inhibited grey mould through *in vitro* and *in vivo* applications on grapes via different mechanisms. Although both *Metschnikowia* yeasts yielded comparable results, *M. aff. fructicola* 1-UDM outperformed all other yeasts. Interestingly, *H. uvarum* 3-UDM VOCs hold considerable potential as a biofumigant for preventing *B. cinerea*-caused postharvest grape rot. Additional investigation is required to identify and improve these VOCs

under various environmental settings and on different fruits in the presence of *B. cinerea*. These biocontrol yeasts show promise as bio-based agrochemicals in organic viticulture, replacing synthetic counterparts. Further investigation is needed to elucidate these biocontrol yeasts' precise mechanisms of action, considering the interactions between the host, pathogen, antagonist, and microbial flora, together with large-scale applications.



6. A NOVEL EXOPOLYSACCHARIDE FROM COLD-ADAPTED YEAST *RHODOTORULA GLUTINIS*, ALONG WITH STRUCTURAL, RHEOLOGICAL, ANTIOXIDANT, AND ANTIBIOFILM PROPERTIES

6.1 Abstract

In the present study, a novel exopolysaccharide EPS-BMD26 produced by cold-adapted yeast *Rhodotorula glutinis* was structurally examined, along with its rheological, antioxidant, and antibiofilm properties. High-performance liquid chromatography (HPLC) analysis revealed glucose and galactose as two monosaccharide constituents. The functional groups (O-H, C-H, C=O, -COO, and C-O-C) within the EPS-BMD26 structure were detected by Fourier transform infrared (FT-IR) spectroscopy. The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra of EPS-BMD26 also confirmed its β -D-glucose and β -D-galactose components with an average molecular weight of 118 kDa. Differential scanning calorimetry (DSC) analysis revealed that EPS-BMD26 had a high level of thermal stability, up to 326.16 °C. Scanning electron microscopy (SEM) analysis demonstrated that EPS-BMD26 had a porous microstructure with fissures. X-ray diffraction (XRD) analysis revealed its semi-crystalline nature. EPS-BMD26 showed moderate and concentration-dependent antioxidant potential recruiting different methods, including •OH (hydroxyl), DPPH• (2,2-diphenyl-1-picrylhydrazyl), CUPRAC (cupric ion reducing antioxidant capacity), and ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activities. Water-soluble EPS-BMD26 exhibited high water holding capacity of $190 \pm 0.22\%$ with a water solubility index of $60.6 \pm 8.3\%$. The EPS-BMD26 was also capable of inhibiting biofilm formation by *Staphylococcus aureus* ATCC 25923 (79.5% of biofilm inhibition at 1250 ppm). Rheological characterization revealed that the aqueous solution of EPS-BMD26 had shear thinning and pseudoplastic behavior. Given these properties, EPS-BMD26 may be a promising bio-hydrocolloid, antibiofilm agent, and food additive for biotechnological and industrial applications.

Keywords

Rhodotorula glutinis, exopolysaccharide, structural analysis, rheology, antioxidant, antibiofilm.

6.2 Introduction

Exopolysaccharides (EPS) are extracellular polymers composed of sugars together with proteins or nucleic acids (Gientka et al., 2015). The bacteria, fungi, and microalgae can secrete EPSs as homopolysaccharides or heteropolysaccharides, depending on whether their EPS repeating units include one or more sugar monomers, respectively (Ma et al., 2018). Among these, bacteria-derived EPSs and their characterization are well-documented. However, data on the fungal and yeast-derived EPSs are scarce (Hamidi et al., 2022; Rahbar Saadat et al., 2021), and they need to be thoroughly searched for novel EPSs. Besides, yeast-derived EPSs have an advantage over bacteria-derived EPSs in that they are less expensive, easier to extract, higher yielding, sustainable, non-toxic, and biodegradable (Pavlova et al., 2009; Seveiri et al., 2020). In this regard, *Rhodotorula* (Cho et al., 2001; Hamidi et al., 2020), *Lipomyces* (W. Li et al., 2021; Ragavan & Das, 2019), *Cryptococcus* (Pavlova et al., 2009), *Saccharomyces* (L. Liu et al., 2022), *Sporobolomyces* (Poli et al., 2010), *Hanseniaspora* and *Metschnikowia* (Yildiran et al., 2019) were among the EPS-producing yeasts. Yeast-derived EPSs were mainly found in the form of mannan, phosphomannan, glucan, galactomannan, and glucuronoxylomannan (Rahbar Saadat et al., 2021), which are reported to have antioxidant, antibiofilm, antiviral, antithrombotic, antitumor, antiproliferative, and anticoagulant activities (Hamidi et al., 2020; Kheyrandish et al., 2022; Ma et al., 2018; Rahbar Saadat et al., 2021; Vazquez-Rodriguez et al., 2018).

Studies on the psychrophile and cold-adapted EPS-producing yeasts have drawn increasing attention since their EPSs can be used for many industrial and medical purposes (Hamidi et al., 2020; Kheyrandish et al., 2022; Rusinova-Videva et al., 2022). Important biological metabolites, including carotenoids, unsaturated fatty acids, enzymes, and EPSs, can be biosynthesized by cold-adapted yeasts (Daskaya-Dikmen et al., 2018; Hamidi et al., 2020; Taskin et al., 2016; Touchette et al., 2022). *Rhodotorula glutinis*-derived EPSs have undergone various investigations (Hamidi et al., 2020; Ma et al., 2018; Seveiri et al., 2020; Silambarasan et al., 2019; Vazquez-

Rodriguez et al., 2018); however, there is still a lack of information on their structural, physical, and bioactive characteristics (Ghada et al., 2012). In addition, there is no report on the antibiofilm activity of *R. glutinis* on biofilm-forming pathogenic bacteria. As a result, the focus of this research was on the structural characterization of novel EPS-BMD26 from cold-adapted yeast *R. glutinis* together with antioxidant, antibiofilm, and rheological analyses. The functional groups and monomeric sugars of EPS-BMD26 were detected using Fourier-transform infrared (FTIR) spectra and high-performance liquid chromatography (HPLC) analyses, respectively. Further structural characterization was performed by scanning electron microscopy (SEM) and nuclear magnetic resonance (NMR) spectroscopy analyses. Moreover, X-ray diffraction (XRD) patterns revealed the physical properties of the EPS-BMD26. Finally, the antioxidant and antibiofilm tests were conducted to understand the bioactive properties of EPS-BMD26, along with a rheological investigation. Research into the novel EPS-BMD26 extracted from cold-adapted yeast *R. glutinis* will help to understand its versatile technological functions to be utilized in various industrial settings.

6.3 Materials and Methods

6.3.1 Culture conditions and molecular identification of *Rhodotorula glutinis*

Rhodotorula glutinis was isolated from fresh blackberry (*Rubus fruticosus*) fruits (Bursa, Turkey) from local orchards. The fruit sample was collected in 500 g portions, labeled, and stored in sterile cups. For isolation of yeasts, decimal dilutions were prepared and seeded on yeast extract peptone dextrose agar (YEPD agar) plates with chloramphenicol (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 20 g/L agar, and 0.1 g/L chloramphenicol). Following a 7-14 day incubation at 4 °C, orange-colored colonies were randomly selected for their ability to synthesize polysaccharides, as determined by their jelly-like slimy appearance (Taskin et al., 2016). Then, re-streaked pure yeast cultures were stored in slants (at 4 °C) or in 20% glycerol stocks (at -80 °C) for further analysis. All media and chemicals were purchased from Merck (Darmstadt, Germany). As previously outlined by Oztekin and Karbancioglu-Guler (2021), the RAPD-PCR employing M13 primer was used to characterize the genotype. MEGA Version 7.0.21 software was used to construct the phylogenetic tree of *R. glutinis* through the neighbor-joining (NJ) method (Tamura et al., 2007). Further, the

nucleotide sequence of *R. glutinis* was compared with *Rhodotorula* species in the GenBank database.

6.3.2 Exopolysaccharide (EPS) production, extraction, and purification

An optimized medium with 25 g/L sucrose and 100 g/L ammonium sulfate buffered at pH 5.5 was used to inoculate *R. glutinis* yeast (10%;v/v) from an overnight culture grown at 25 °C, OD = 0.8), which was left to incubation at 150 rpm at 22°C up to 120 h for EPS production. The EPS-containing cell-free supernatant was then extracted from the resultant medium by centrifuging it at $8500 \times g$ for 30 min at 4 °C. After adding 10% trichloroacetic acid (TCA), proteins were removed after precipitating at 4 °C for 4 h with gentle agitation. Next, centrifugation (Eppendorf Centrifuge 5804R, Hamburg, Germany) at $14,000 \times g$ for 10 min at 4 °C was performed to separate the proteins from the mixture. Then, 96% of cold ethanol (1:3, v/v) was added and kept at 4 °C overnight. Subsequently, the EPS sediment was washed twice with cold ethanol before being centrifuged again under identical conditions. Finally, the resulting EPS was dialyzed for 48 h (8000–14,000 Da dialysis membrane, Solarbio) and freeze-dried overnight in a lyophilizer (Biobase Freeze-Dryer, China) at -56 °C. Following that, the total EPS was weighed to calculate the yield (Cho et al., 2001; Hamidi et al., 2020; Mohammed et al., 2021; Okoro et al., 2021). The total carbohydrate and protein of the purified EPS were determined by the phenol-sulphuric acid (Dubois et al., 1956) and Bradford (Bradford, 1976) methods. The UV-vis spectrophotometer was utilized to check the possible protein and nucleic acid contaminations through the corresponding absorption peaks at 260 and 280 nm (Lakra et al., 2020).

6.3.3 Solubility and technological properties of EPS-BMD26

The EPS-BMD26 solubility was evaluated in different solvents, including distilled water, chloroform, and methanol. For this reason, a small amount of EPS pellet was vortexed with the solvents in a 2 mL microtube for 1 min to see if EPS was soluble (Castellane et al., 2015). Furthermore, the water solubility index (WSI) was calculated through Ahmed et al.'s (2013) method with minor modifications. First, an aqueous solution of lyophilized EPS-BMD26 (50 mg/mL) was incubated overnight at 37 °C with continuous stirring. To collect the supernatant, centrifugation was applied at $5000 \times g$ for 15 min (0.2 mL), followed by ethanol precipitation (0.6 mL). Subsequently, the centrifugation step was repeated at $10,000 \times g$ for 20 min at 4 °C. After vacuum-

drying for 48 h at 50 °C, the EPS was weighed. The WSI% was calculated with the following equation (6.1) below:

$$\text{WSI (\%)} = \frac{\text{Total EPS concentration in supernatant}}{\text{Weight of EPS at dry weight basis}} \times 100 \quad (6.1)$$

Further, Niknezhad et al. (2018) were used to assess the water holding capacity (WHC) of EPS-BMD26. To remove the unbounded water, 0.02 g/mL of EPS aqueous solution was vortexed and centrifuged at $16,000 \times g$ for 25 min. Then, the remaining excess water was removed using filter paper. The differences in EPS weight were calculated using equation (6.2) below:

$$\text{WHC (\%)} = \frac{\text{Total EPS weight after water absorption}}{\text{Total weight of dry EPS}} \times 100 \quad (6.2)$$

6.3.4 Monosaccharide composition of EPS-BMD26

The hydrolyzation of EPS-BMD26 was conducted based on Dertli et al.'s (2018) method. For this purpose, H_2SO_4 (0.5 M) was used to hydrolyze the lyophilized EPS at 95 °C for 12 h, and NaOH (4 M) was used for neutralization, then the hydrolysate was filtered (0.45 μm pore-size). Following the method of Zhu et al. (2013), the monosaccharide composition of EPS was determined by HPLC (Shimadzu, Japan). The sugars were separated on a transgenomic CARBOsep CoreGel 87C column (300 $\times$ 7.8 mm, CHO-99-5860) at 85 °C and a RID-10A refractive index detector to monitor the eluted sugars. The ultra-pure water was used as a mobile phase at a flow rate of 0.6 mL min⁻¹. Glucose, galactose, fructose, and mannose were employed as the monosaccharide standards.

6.3.5 Molecular weight determination of EPS-BMD26

The viscometric method was used to calculate the average molecular weight of EPS-BMD26 using the correlation of the intrinsic viscosity (η) (Beer et al., 1999; Masuelli, 2018). To achieve this, the viscosity of EPS aqueous solutions at different concentrations was measured at 25 °C using a rheometer (Haake RheoStress 1, Germany). The running time of polymer solutions (t_1) and the solvent (t_0) was used to calculate the specific viscosity with the following equation (6.3) (Fan et al., 2020).

$$\eta_{sp} = (t_1 - t_0)/t_0 \quad (6.3)$$

The intrinsic viscosity ($[\eta]$) was obtained by the following equation (6.4).

$$[\eta] = \lim_{c \rightarrow 0} \eta_{sp}/c \quad (6.4)$$

where c is the concentration of the EPS-BMD26 solution, η_{sp} is the specific viscosity of the EPS-BMD26. Then, the molecular weight of the EPS-BMD26 was calculated using the following Mark–Houwink equation (6.5):

$$[\eta] = k \times M_w^\alpha \quad (6.5)$$

where both k and α are constants for EPS-BMD26 solvent system, M_w is the molecular weight, and $[\eta]$ is the intrinsic viscosity.

6.3.6 Fourier transform infrared (FT-IR) spectroscopy analysis of EPS-BMD26

The EPS-BMD26's functional groups were identified by FT-IR analysis, which was performed with Perkin Elmer Spectrum 65 instrument. The FT-IR spectra of the EPS-BMD26 were run in transmission mode and a resolution of 4 cm^{-1} at 25°C , between the wavelengths of $4000\text{--}400 \text{ cm}^{-1}$.

6.3.7 Differential scanning calorimetry (DSC) analysis of EPS-BMD26

The EPS-BMD26's thermal behavior was determined using a differential scanning calorimeter (DSC 8000, Perkin-Elmer instruments, Waltham, MA, USA). Lyophilized EPS-BMD26 (3 mg) was hermetically sealed in an aluminum pan, while an empty pan served as a control. The change in enthalpy and melting point was investigated by scanning the sample from 30 to 500°C with the increment of the temperature for $10^\circ\text{C min}^{-1}$ (Nambiar et al., 2018).

6.3.8 Scanning electron microscopy (SEM) analysis of EPS-BMD26

The microstructural characterization of EPS-BMD26 was conducted through scanning electron microscopy (FEI Nova Nano SEM 450 Instrument, USA). The EPS-BMD26 was coated with a 10 nm thick gold layer and subjected to an accelerated voltage of $10\text{--}20 \text{ kV}$ prior to analysis (Hamidi et al., 2020). The SEM analysis was performed at magnification levels between $10,000$ and $100,000\times$.

6.3.9 X-ray diffraction (XRD) analysis of EPS-BMD26

X-ray diffractometer (XRD, Bruker D8 Discover AXS, Germany) was utilized to characterize the crystallographic structure of EPS-BMD26. The scanning was performed in different ranges of 2θ angles (5-90°) with a scanning step of 0.03° (Singh et al., 2011).

6.3.10 Nuclear magnetic resonance (NMR) analysis of EPS-BMD26

NMR spectrometer (Agilent VNMRs 500 MHz) was used to monitor ^1H , and ^{13}C and gHSQCAD NMR spectra. The spectra is referenced through the solvent lock (2H) signal according to IUPAC recommended secondary referencing method. Briefly, the lyophilized EPS-BMD26 (60 mg) was dissolved in 1 ml of D_2O before being transferred to NMR tubes (5 mm). MNova 14.3.0 (Mestrelab Research, Spain) software was used for data interpretation (Ma et al., 2018).

6.3.11 Antioxidant activity of EPS BMD26

The antioxidant properties of EPS-BMD26 were investigated by hydroxyl ($\bullet\text{OH}$) radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH $\bullet$) radical scavenging, cupric ion reducing antioxidant capacity (CUPRAC), and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assays. The antioxidant level of EPS-BMD26 was expressed as a percentage of $\bullet\text{OH}$, DPPH, CUPRAC, and ABTS radical scavenging activities at different EPS-BMD26 aqueous solutions (0.1, 0.25, 0.5, 1, 2.5, 5, and 7.5 mg/mL). The ascorbic acid was used as a positive control. The EPS BMD26's capacity to scavenge radicals ($\bullet\text{OH}$, DPPH, CUPRAC and ABTS) was calculated using the equation (6.6).

$$\text{RSA (\%)} = A_{\text{blank}} - \frac{(A_{\text{sample}} - A_{\text{control}})}{A_{\text{blank}}} \times 100 \quad (6.6)$$

where RSA is for radical scavenging activity (%), A_{sample} represents the absorbance of the solution with reaction mixture and EPS BMD26, A_{blank} represents the absorbance of the solution with reaction mixture and distilled water (instead of EPS-BMD26), A_{control} represents the absorbance of the solution with EPS-BMD26 and distilled water (instead of the reaction mixture).

6.3.11.1 Hydroxyl ($\bullet$ OH) radical scavenging activity

The FeSO_4 -salicylic acid method was used to determine the EPS-BMD26's hydroxyl ($\bullet$ OH) radical scavenging activity based on (Niknezhad et al., 2018). Briefly, 40 μL of EPS aqueous solutions (0.1-7.5 mg/mL) were mixed with equal volumes (40 μL) of the following solutions: FeSO_4 (9 mM), salicylic acid in ethanol (9 mM), and H_2O_2 (8.8 mM). The solutions were incubated for 30 minutes at 37 $^\circ\text{C}$, and the absorbances were measured at 510 nm in comparison to a control sample. The blank solution was prepared with distilled water instead of salicylic acid.

6.3.11.2 DPPH radical scavenging activity

DPPH $\bullet$ free radical scavenging activity was used to assess the antioxidant activity of EPS-BMD26, as previously outlined by Xu et al. (2019). For this purpose, 1 mL of EPS-BMD26 solutions (0.1-7.5 mg/mL) and 1 mL of DPPH ethanolic solution (0.2 mM) were combined and incubated for 30 minutes at room temperature in the dark. The blank solution was achieved by substituting EPS with distilled water, and the absorbances were recorded at 517 nm.

6.3.11.3 CUPRAC radical scavenging activity

CUPRAC radical scavenging activity of EPS-BMD26 was examined based on Apak et al. (2004). First, 0.1 mL of the EPS-BMD26 (0.1-7.5 mg/mL) were mixed with 1 mL of CuCl_2 solution (0.01 mM), 1 mL of Neocuproine solution (0.0075 M, in ethanol), 1 mL of NH_4Ac buffer solution (1 M, pH 7) and 1 mL of distilled water. Following 30 min of incubation, the absorbances were read against distilled water at 450 nm.

6.3.11.4 ABTS radical scavenging activity

The antioxidant activity of EPS-BMD26 was determined by ABTS $\bullet$ radical scavenging activity assay, according to Cheng et al. (2013). Prior to the analysis, ABTS (7 mM) and potassium persulfate (2.45 mM) solutions were combined and allowed to stand at room temperature in the dark for 16 hours for the formation of ABTS free radical ($\text{ABTS}\bullet^+$). Then, $\text{ABTS}\bullet^+$ solution was diluted with phosphate buffered saline (pH 7.4) to obtain an absorbance of 0.70 ± 0.02 at 734 nm before use. Following that, 3.6 mL of the $\text{ABTS}\bullet^+$ solution was added to 0.4 mL EPS-BMD26 of various concentrations (0.1-7.5 mg/mL). The blank solution was prepared using

ABTS^{•+} and distilled water. After incubation for 20 minutes, the absorbance of the solutions was measured at 734 nm against a blank solution.

6.3.12 Rheological properties of EPS-BMD26

Rheological measurements were taken in duplicate for each EPS solution using a rheometer. (Haake RheoStress 1, Germany) with a cylindrical spindle (222-1266) (Nambiar et al., 2018). An aqueous solution (2 mg/mL) of the lyophilized EPS-BMD26 was examined at two different pH conditions (4.0 and 7.0). The viscosity and temperature relationship were examined under three different temperature values, 30 °C, 40 °C, and 50 °C, and the samples' apparent viscosity(μ) was determined as a function of shear rate between 0.1 s⁻¹ and 300 s⁻¹.

6.3.13 Antibiofilm activity

The crystal violet staining method was used to determine whether EPS-BMD26 had an antibiofilm effect on *Staphylococcus aureus* ATCC 25923 (Venkatesh et al., 2016). Briefly, *S. aureus* was incubated at 37 °C for 24 h in a tryptic soy broth (TSB) medium. The microtiter wells were filled with 180 μ L of sterile TSB, 10 μ L of EPS-BMD26 at different concentrations (0-1250 ppm), and finally with 10 μ L of the bacterial suspension (OD = 1, corresponding to $\sim 1.5 \times 10^8$ CFU/mL). After the incubation at 37 °C for 48 h, the reaction mixture was discarded and washed with 200 μ L of PBS (Phosphate buffer saline, pH 7.2). For biofilm fixation, 50 μ L of methanol was pipetted into wells and waited for 15 min, then followed by a PBS-washing. The crystal violet (0.2%) was used for 15 min to stain the biofilm in the wells, which were then flooded with distilled water for the removal of excess stain. The absorbances were noted at 570 nm after 100 μ L of acetic acid (0.5 M) was introduced to dissolve the adhering blue biofilm. The wells with bacterial suspensions served as positive controls (0 ppm), and the antibiofilm activity (%) was calculated using the following equation (6.7).

$$A = \frac{OD_{\text{control}} - OD_{\text{sample}}}{OD_{\text{control}}} \times 100 \quad (6.7)$$

where A is the antibiofilm activity (%), OD_{control} is the absorbance value of control and OD_{sample} is the absorbance value of sample.

6.3.14 Statistical analysis

The antioxidant and antibiofilm activity, water holding capacity, and water solubility index analyses were conducted in triplicate, and the results were expressed as the mean and the standard deviation. The comparison between the means of the test was calculated by Duncan's test at $p < 0.05$.

6.4 Results and Discussion

6.4.1 Phylogenetic tree of *Rhodotorula glutinis*

The sequence analysis of 5.8S-ITS rRNA for *Rhodotorula* clade showed that *R. glutinis* isolate (under the accession number of MT635318.1) was highly related (99%) to *R. glutinis* strains in the GenBank database. Utilizing the neighbor-joining method, the evolutionary distance was calculated in relation to sequences found in the GenBank database, where accession numbers are presented following reference species names. The numbers at nodes indicate the bootstrap values (1000 replicates), and the bar shows genetic distance. The scale bar indicates 0.21 substitutions per nucleotide position (Figure 6.1).

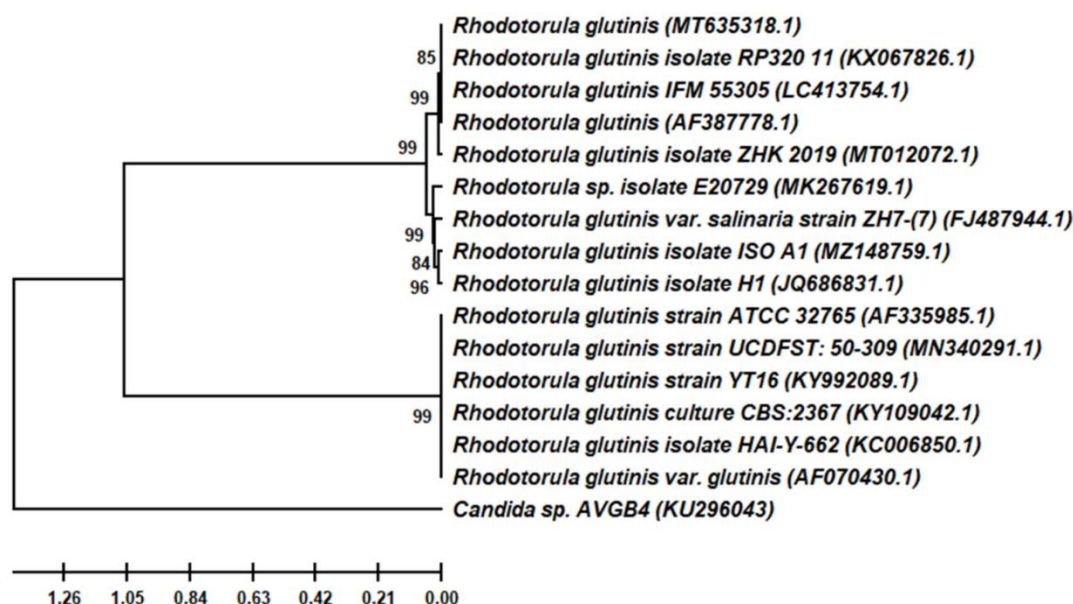


Figure 6.1 : Phylogenetic tree based on sequences of the D1/D2 domains of the large subunit 26S rDNA region of *Rhodotorula glutinis* (MT635318.1). *Candida sp.* AVGB4 was used as an outgroup.

6.4.2 The yield and technological characteristics of EPS-BMD26

The total yield of EPS-BMD26 from cold-adapted *R. glutinis* was found to be 31.8 g/L under specified conditions in section 6.3.2, which was far higher than many other yeast-derived (Hamidi et al., 2020; H. Li et al., 2020; W. Li et al., 2021; L. Liu et al., 2022; Ma et al., 2018; Mirzaei et al., 2019; Mohammed et al., 2021; Pavlova et al., 2009; Poli et al., 2010; Ragavan & Das, 2019; Ramirez & Ramirez, 2015; Seveiri et al., 2020; Silambarasan et al., 2019; Takita et al., 2004; Vazquez-Rodriguez et al., 2018) or bacteria-derived (Jurášková et al., 2022; Lakra et al., 2020). EPSs. Furthermore, there have been few studies on the amount of EPS produced and characterized by the cold-adapted yeast *Rhodotorula* spp. (Hamidi et al., 2020; Okoro et al., 2021). For example, the total EPS yield of *R. mucilaginosa* sp. GUMS16 was found to be lower (28.5 g/L) and higher (134.8 g/L) than our findings (Hamidi et al., 2020; Okoro et al., 2021). The cold-adapted yeasts are other than *Rhodotoula* spp. genus had a relatively lower level of EPS; for example, Antarctic yeasts *Cryptococcus flavus* A51 and *Sporobolomyces salmonicolor* AL1 produced 5.75 g/L (Pavlova et al., 2009) and 5.64 g/L (Poli et al., 2010) of EPS, respectively. More recently, the EPSs yields of *Vishniacozyma victoriae* and *Tremellomycetes* sp. were calculated to be 4 g/L and 4.5 g/L levels, respectively (Rusinova-Videva et al., 2022). These variations in EPS yield could be attributed to the culture itself, as well as the composition of culture media, temperature, agitation speed, pH, and incubation time (Gientka et al., 2015; Okoro et al., 2021). According to earlier investigations, the best sources of carbon and nitrogen for the synthesis of EPSs obtained from *Rhodotorula* spp. were reported to be sucrose and ammonium sulfate, respectively (Hamidi et al., 2020; Okoro et al., 2021; Samadlouie et al., 2020). Yeast-derived EPSs had larger yields and were easier and simpler to extract from growth media than their bacterial counterparts (L. Liu et al., 2022; Rahbar Saadat et al., 2021). Therefore, the utilization of EPS-BMD26 from *R. glutinis* holds great potential for large-scale production.

According to phenol-sulphuric acid and Bradford analysis, EPS-BMD26 contained $77.26 \pm 2.5\%$ carbohydrate and $0.087\% \pm 0.012$ protein, respectively. These trace amounts of the protein content of EPS-BMD26 indicated its high purity (Mirzaei et al., 2019). Furthermore, the EPS from *Rhodotorula mucilaginosa* sp. GUMS16 was found to be less pure than ours, with 61.7% of carbohydrate and 0.8% of protein content (Hamidi et al., 2020). Lyophilized EPS-BMD26 was insoluble in chloroform

and methanol but soluble in distilled water. The results matched those of Hamidi et al. (2020), as polysaccharides are typically water-soluble and insoluble in organic solvents. The hydroxyl, carboxyl, amine, sulfate, and phosphate groups, glycosidic linkages, non-polar regions, and glucose units within the EPS structure could contribute to its hydrophilic/hydrophobic properties and thus solubility (Moran, 2009; J. Wang et al., 2016). Besides, EPSs with low molecular weight showed superior antioxidant and anticancer properties with high solubility in water (L. Liu et al., 2022). The EPS-BMD26 had WHC and WSI values of $190.0 \pm 0.2\%$ and $60.6 \pm 8.3\%$, respectively. Since data on *Rhodotorula* sp. is limited for WHC and WSI, the current findings were compared to EPSs from yeasts and bacteria. According to comparable data from Li et al. (2021), the yeast *Lipomyces starkeyi*'s EPS had a WHC of 2.10 g/g (210%), meaning it absorbed twice as much water as it weighed. Another yeast-derived EPS from *Rhodospiridium babjevae* had a WHC of about $519 \pm 18.5\%$ (Seveiri et al., 2020), suggesting that yeast-EPSs could be suitable candidates as bio-thickeners and stabilizers with their great water-holding capability. When compared to the WHC of bacteria-derived EPSs, the present results were higher than *Leuconostoc lactis* (117%) (Saravanan & Shetty, 2016) but lower than *Lactobacillus kefiranofaciens* (496%) (Z. Ahmed et al., 2013). In addition, a bacteria-derived EPS from *Pantoea* sp. had higher WHC and lower WSI; $662 \pm 12.5\%$ and $15.6 \pm 0.22\%$, respectively (Niknezhad et al., 2018). Again, the present WSI results were superior to those listed in the earlier studies (14.2%) (Z. Ahmed et al., 2013; Saravanan & Shetty, 2016) but lower than galactan EPS produced by *Weissella confusa* (100%) (Kavitake et al., 2016).

According to the current findings, EPS-BMD26 absorbed about twice as much water as its weight due to strong hydrogen bonding and had high solubility in water. Therefore, EPS-BMD26 could be recruited as a bio-hydrocolloid in the food and pharmaceutical industries. Furthermore, lactose-negative *R. glutinis* culture could be used alone or in combination with starter cultures for the enhancement of texture in fermented dairy products (Simova et al., 2004; Tiwari et al., 2021).

6.4.3 Sugar composition and molecular weight of EPS-BMD26

When compared to standard chromatograms (Figure C.1), EPS-BMD26 from *R. glutinis* was composed of glucose and galactose monomeric units (Figure 6.2), indicating its heteropolysaccharide nature. NMR and FT-IR analyses confirmed that

the EPS-BMD26 structure had glucose and galactose without mannose, making it a novel biopolymer. Since EPS from *Rhodotorula* spp. was primarily constituted of glucose, mannose, and galactose (Ma et al., 2018; Silambarasan et al., 2019; Vazquez-Rodriguez et al., 2018) with arabinose (Ma et al., 2018), fucose (Vazquez-Rodriguez et al., 2018), and rhamnose (Mirzaei et al., 2019) at lower levels, the results were comparable with earlier studies, except for mannose. In this regard, it seems feasible to produce bacteria-derived EPSs in yeasts at higher yields; for instance, the composition EPS from *Lactobacillus kefir* was composed of galactose and glucose units (Riaz Rajoka et al., 2019), similar to EPS-BMD26. The monosaccharide composition of EPS was linked with the nutrients in the growth medium and environmental factors (temperature, pH, a_w) as well as species and strains (Zhou et al., 2019). The variations in the monosaccharide composition may influence the EPS's thermal, rheological, and antioxidative functions (Ayyash, Abu-Jdayil, Itsaranuwat, et al., 2020; Hamidi et al., 2020). For example, the presence of galactose within the structure of EPS was associated with anti-inflammatory effects on the macrophages (Y.-C. Chen et al., 2019).

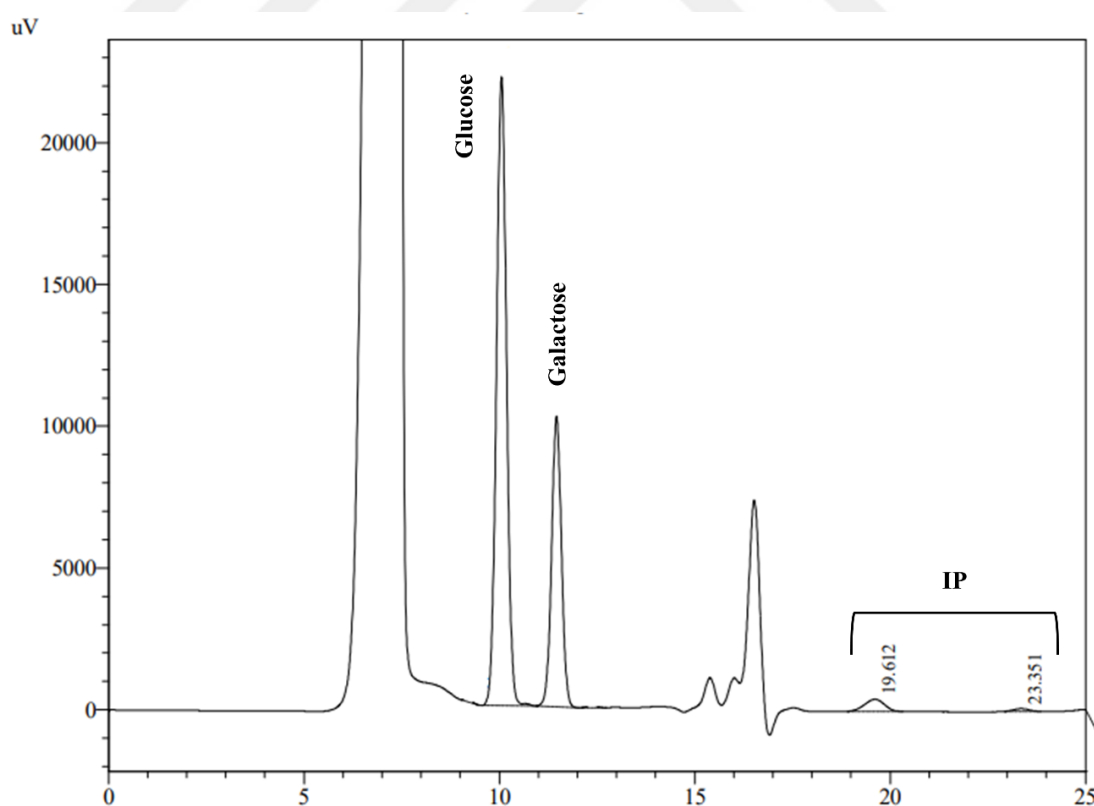


Figure 6.2 : HPLC chromatogram of EPS-BMD26 from *Rhodotorula glutinis* demonstrating the presence of glucose and galactose as sugar monomers, with IP representing impurities.

The EPS-BMD26 had an average molecular weight of 118 kDa. In agreement with this, EPS from *R. glutinis* had a molecular weight between 100 – 380 kDa (Cho et al., 2001). When compared to other *Rhodotorula* spp., *R. mucilaginosa*-derived EPS had a molecular weight of 84 kDa (Hamidi et al., 2020), which was lower than our finding. Furthermore, the molecular weight of *R. mucilaginosa* CICC 33013-derived EPS was 7125 kDa (Ma et al., 2018), and Wang et al. (2021) detected a molecular weight of 59020 kDa for *Rhodotorula* sp. RY1801-derived EPS, being far higher than the present findings. Similarly, Li et al. (2020) noted that *R. mucilaginosa* YL-1 had a mean molecular mass of 1200 kDa, suggesting that varied culture techniques led to variances in molecular weights that were also strain-specific. As discussed in sections 6.4.2, 6.4.9, and 6.4.10, the lower molecular weight of EPS-BMD26 could improve solubility, antioxidant capabilities, and rheological behavior.

6.4.4 Detection of functional groups of EPS-BMD26 by FT-IR analysis

Figure 6.3 shows the FT-IR spectrum of the EPS-BMD26 from *R. glutinis*. The absorption bands at 3301.66, 2926.79, 1644.15, 1429.88, and 1089.11 cm^{-1} followed the characteristic polysaccharide patterns (Hamidi et al., 2020; Ma et al., 2018; Silambarasan et al., 2019). The typical broad peak at 3301.66 cm^{-1} corresponded to O-H (hydroxyl) stretching, a characteristic signal of carbohydrate ring, indicating the hydrophilic nature of EPS (Silambarasan et al., 2019). The existence of organic substances like sugar and proteins was indicated by the following peaks at 2926.79, 2114.92, and 1987.88 cm^{-1} that were linked to the C-H stretching (aliphatic CH_2 group) (Nambiar et al., 2018; Silambarasan et al., 2019). The absorption at 1644.15 cm^{-1} indicated the C=O stretching associated with the carboxyl groups (Ma et al., 2018), which may correspond to galactose (Nambiar et al., 2018). The signal at 1429.88 cm^{-1} was characterized by the symmetric stretching of the $-\text{COO}^-$ and C=O groups (Nambiar et al., 2018). The existence of a significant peak at 1089.11 cm^{-1} within 1200-950 cm^{-1} regions indicated that EPS is a polysaccharide (Mohammed et al., 2021; Ramirez, 2016). Furthermore, the presence of C-O-C and C-O groups in polysaccharides could be associated with 1191.87 cm^{-1} and 1089.11 cm^{-1} signals, implying the EPS's monosaccharides structure was in pyranose form. The absorption peak around 747.42 cm^{-1} might be associated with C-O-C bending vibration, and the following peak around 550-650 cm^{-1} might be attributed to the stretching of alkyl halides (S-F) (Nambiar et al., 2018; Sardari et al., 2017). Additionally, there was no

absorption peak at 1730 cm^{-1} , proving that the EPS-BMD26 was free of uronic acid (Ma et al., 2018).

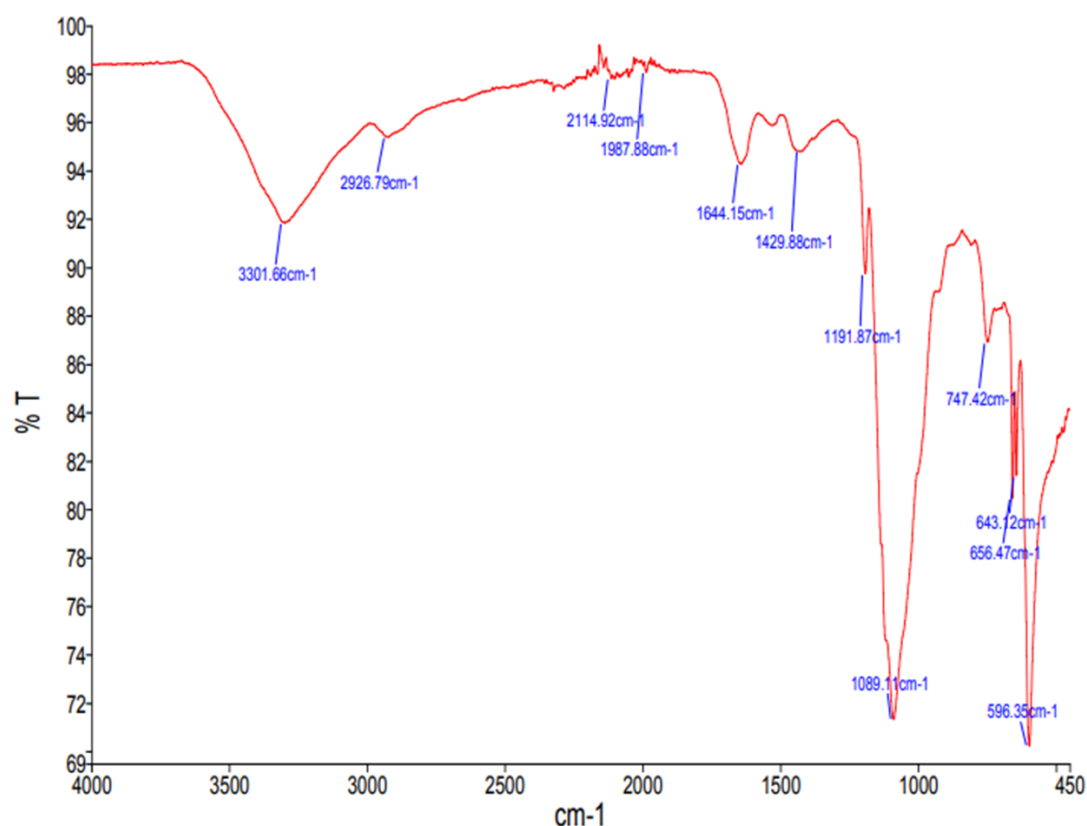


Figure 6.3 : FT-IR spectrum of the EPS-BMD26 from *Rhodotorula glutinis* displays the functional groups at wavelength of $400\text{--}4000\text{ cm}^{-1}$ at a resolution of 4 cm^{-1} .

6.4.5 Thermal characteristics of EPS-BMD26 by DSC analysis

Figure 6.4 displays the DSC curve of EPS-BMD26 scanned between 30 and $500\text{ }^{\circ}\text{C}$. Its high thermal stability was evidenced by the fact that degradation began at around 326.16°C . Once the temperature reached $500\text{ }^{\circ}\text{C}$, EPS-BMD26 began to degrade rapidly. Besides, the commercial applicability of EPS is greatly influenced by its thermal stability (Marinho-Soriano & Bourret, 2005). EPS-BMD26 from *Rhodotorula glutinis* exhibited promising characteristics for use in thermally processed foods. Since no information about the DSC analysis of *Rhodotorula*-derived EPS is available, the current findings were compared to other yeast- and bacteria-derived EPSs. According to a recent study, *Saccharomyces cerevisiae* Y3 EPS degraded at $275\text{ }^{\circ}\text{C}$ (L. Liu et al., 2022), demonstrating that the current EPS-BMD26 had one of the highest melting points. Likewise, fungal EPSs tended to have higher thermal properties; for example, EPS from *Fomitopsis meliae* AGDP-2 started to degrade after reaching $288\text{ }^{\circ}\text{C}$ (Prajapati et al., 2022). In contrast, different EPSs from bacteria had relatively lower

melting points; for example, melting and degradation temperatures for bacterial EPSs from *Pediococcus pentosaceus* M41 were detected to be 158.82 °C and 235.64 °C, respectively (Ayyash, Abu-Jdayil, Olaimat, et al., 2020). Similarly, the melting point of *Lactobacillus plantarum* HM47 was found to be 96.08 °C (Nambiar et al., 2018). The molecular structure and monosaccharide composition of EPS, which are dependent on growth medium, environmental factors, and species- and strain-dependent factors, may influence thermal behavior (Ayyash, Abu-Jdayil, Olaimat, et al., 2020; Nambiar et al., 2018).

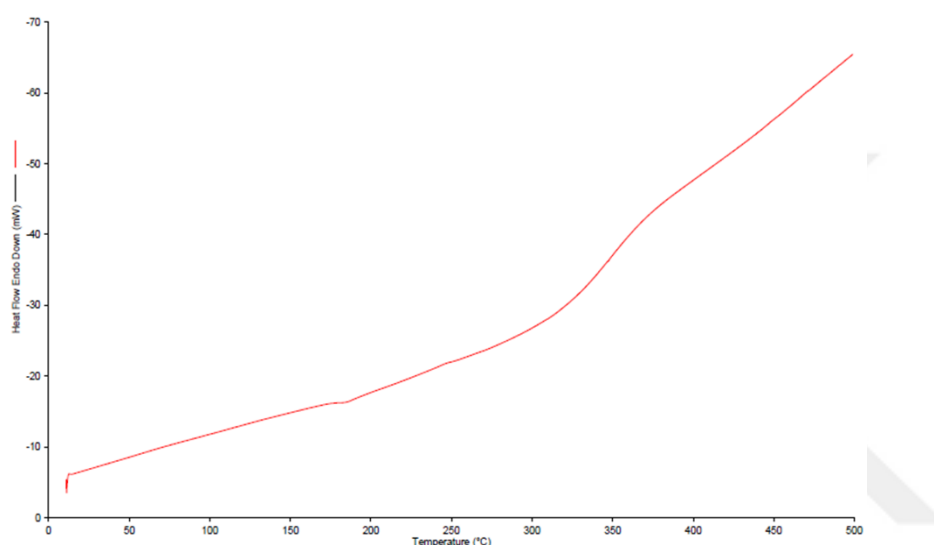


Figure 6.4 : DSC thermogram of EPS-BMD26 from *Rhodotorula glutinis* demonstrating its high thermal stability.

6.4.6 Morphological characterization of EPS-BMD26 by SEM analysis

Multiple pores and fissures were found to be the primary microstructural surface properties of EPS-BMD26 during SEM analysis, which was confirmed at various magnification levels (10,000 to 100,000) (Figure 6.5). Identical findings were also reported by Hamidi et al. (2020) for *Rhodotorula mucilaginosa* sp. GUMS16. The microstructure of EPS can reveal information about its technological and functional roles (Nambiar et al., 2018). With its amorphous globular nature and porous structure, EPS-BMD26 has been proposed as a promising adsorbent, viscosifier, stabilizer, and thickener agent to be used in the pharmaceutical and food industry (Garza et al., 2016; Nambiar et al., 2018; Ramirez, 2016). Besides, EPS with a porous microstructure might be useful for biomaterials and drug delivery (Seveiri et al., 2020). Furthermore, the rough globular surface of EPS-BMD26 could contribute to its good solubility in water (Y. Xu et al., 2019), which was consistent with section 6.4.2.

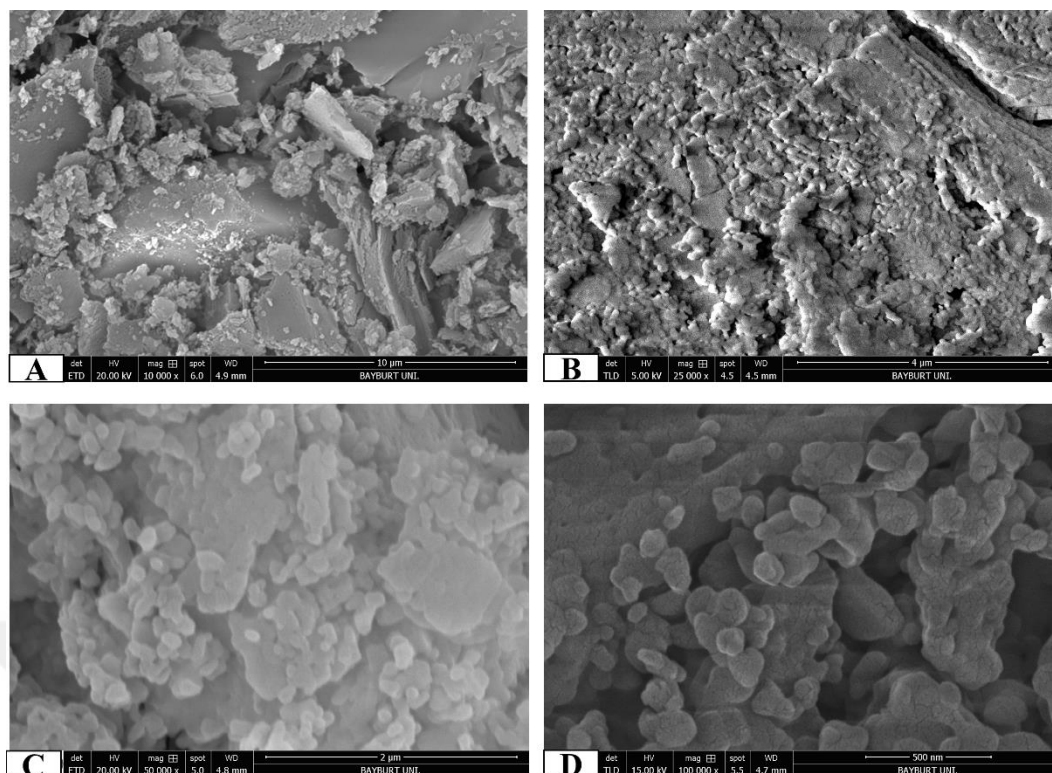


Figure 6.5 : SEM images of EPS-BMD26 from *Rhodotorula glutinis* at different magnification levels $10,000 \times$ (A), $25,000 \times$ (B), $50,000 \times$ (C), and $100,000 \times$ (D), displaying its porous microstructure with fissures.

6.4.7 Physical nature of EPS-BMD26 by XRD analysis

XRD analysis was used to predict whether EPS was amorphous or crystalline (Ragavan & Das, 2019). Figure 6.6 represents the XRD patterns of EPS-BMD26, with the sharp peaks representing the crystallinity, whereas the broad peaks indicate the amorphous nature. The intense distinct peaks were observed at 17.67° , 21.39° , 23.82° , 29.82° , 30.84° , 31.85° , 34° , 35.66° , 37.88° , 40.89° , 43.48° , 48.15° , and 64.12° in the 2θ area, attributed to interplanar spacing (d-spacing) of 5.01, 4.15, 3.73, 2.99, 2.71, 2.81, 2.80, 2.64, 2.52, 2.37, 2.20, 1.89, and 1.45 Å, respectively. The major diffraction peak was observed at 30.84° , indicating that EPS-BMD26 had a partial crystalline nature. The crystallinity index (CI) is a quantitative indicator of crystallinity, which was computed by dividing the area of crystalline peaks by the combined crystalline and amorphous area under the XRD curve with the help of OriginLab Software 2022 (MA, United States) (Rani et al., 2017). According to the CI results (0.72), the crystallinity of EPS-BMD26 was calculated to be 72%, indicating its semi-crystalline nature. This type of semi-crystalline structure can affect its viscosity, solubility, swelling, and flexibility in aqueous solutions (W. Li et al., 2021; Y. X. Wang et al., 2020).

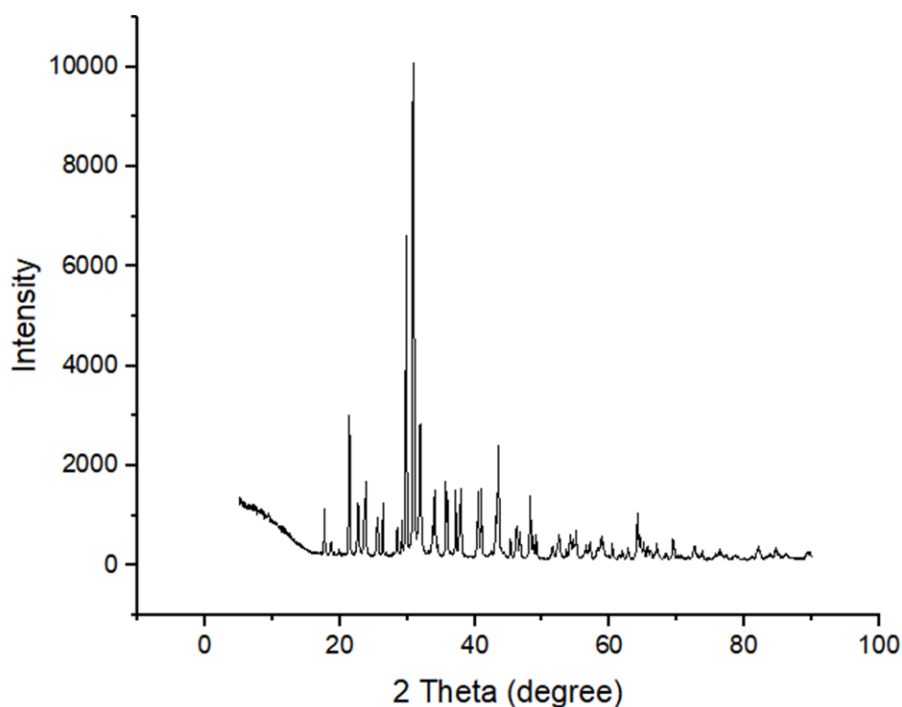


Figure 6.6 : XRD patterns of EPS-BMD26 from *Rhodotorula glutinis* confirming its semi-crystalline nature.

These EPSs could be feasible for use in edible films and food coatings for a range of food preparations (Lakra et al., 2020). A yeast-derived EPS from *Lipomyces starkeyi* exhibited a semi-crystalline structure with consistent CI values of 60.48% (W. Li et al., 2021) and 67.4% (Ragavan & Das, 2019), respectively. Accordingly, Riaz Rajoka et al. (2019) stated that *Lactobacillus kefir*'s EPS also had a semi-crystalline feature. On the contrary, *Saccharomyces cerevisiae* Y3 was reported to have a noncrystalline amorphous nature (L. Liu et al., 2022). The crystallinity of the bacteria-derived EPSs from *Bacillus tequilensis* (Rani et al., 2017) and *Bacillus licheniformis* (Singh et al., 2011) was detected to be 15.6% and 39.75%, respectively, lower than the present findings. It can be concluded that crystallinity could be affected by strain, species, environment, and produced metabolites.

6.4.8 Structural analysis of EPS-BMD26 by NMR analysis

Figures 6.7A and Figures 6.7B show the ^1H and ^{13}C NMR spectra of EPS-BMD26 produced by *R. glutinis*. The ^1H NMR (Figure 6.7A) of EPS comprised of anomeric (δ 4.4–5.2 ppm), ring proton (δ 3.1–3.8 ppm), and alkyl regions (δ 2.1–2.2 ppm) (Nambiar et al., 2018). In the anomeric region of ^1H NMR, the resonances at 4.56 and 4.64 ppm could be attributed to pyranose ring forms in β -D-(1,3)-galactose and β -D-glucose, respectively, in comparison with the available literature (Agrawal, 1992; Ma

et al., 2018). The absence of mannose and rhamnose was confirmed by the absence of peaks at δ 5.28 and δ 5.34, respectively (Ragavan & Das, 2019). As Hamidi et al. (2020) stated, there was no peak at 4.86 ppm, referring to β -D-mannan. In the ^{13}C NMR spectrum (Figure 6.7B), main signals at 95.81, 71.40, 75.85, 69.52, 74.01, and 60.53 ppm corresponded to C-1, C-2, C-3, C-4, C-5 and C-6 carbon residues of β -D-glucose (Agrawal, 1992; Hamidi et al., 2020; Silambarasan et al., 2019). Furthermore, the absence of a peak around 104 ppm indicated the absence of mannose in the EPS (Silambarasan et al., 2019). The ^{13}C NMR spectrum of EPS-BMD26 revealed anomeric C-1 signals at δ 92.04 and 95.81 ppm, indicating the linkages for the galactose and glucose residues (Nambiar et al., 2018). Moreover, the peaks at 92.04, 71.27, 73.97, 72.65, 75.63, and 60.66 ppm matched with β -D-galactose bound to glucose corresponding to C-1, C-2, C-3, C-4, C-5, and C-6 carbon residues (Agrawal, 1992; Silambarasan et al., 2019). The lack of carbon signals above 170 ppm and below 20 ppm in the ^{13}C NMR spectrum confirmed the absence of uronic acid and rhamnose, respectively (Ma et al., 2018). Furthermore, 2D HMQC data from both ^1H , ^{13}C NMR confirmed the ability of *R. glutinis* to produce EPS containing (1 $\rightarrow$ 3)-linked β -D-galactose and β -D-glucose units as branching points.

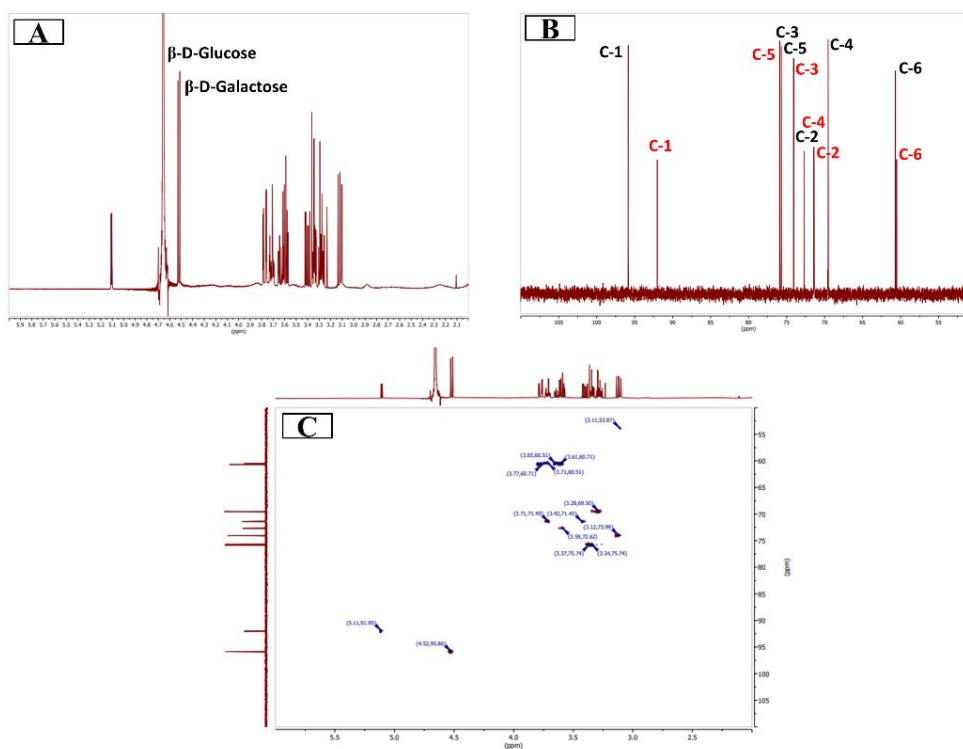


Figure 6.7 : (A) ^1H and (B) ^{13}C NMR and (C) $^{13}\text{C}/^1\text{H}$ 2D HMQC NMR spectra of EPS-BMD26 produced by *Rhodotorula glutinis* in D_2O at 25 $^\circ\text{C}$.

6.4.9 Antioxidant activity of EPS-BMD26

Antioxidant activity is one of the bioactive properties of microbial EPSs that needs to be addressed (Ma et al., 2018). For this reason, the $\bullet\text{OH}$, DPPH $\bullet$, CUPRAC, and ABTS radical scavenging activities were employed to examine the antioxidant properties of EPS-BMD26 compared to ascorbic acid (Figure 6.8A-D). The higher absorbance implies higher reducing capacity, indicating its concentration-dependent antioxidant activity (Figure 6.8). Values are means $\pm$ standard deviations (n=3). At 7.5 mg/mL concentration level, $\bullet\text{OH}$, DPPH $\bullet$, CUPRAC, and ABTS radical scavenging activities reached 72.53 ± 4.70 %, 34.31 ± 3.61 %, 39.05 ± 1.70 %, and 60.15 ± 1.55 %, respectively, relatively lower than ascorbic acid. Furthermore, the CUPRAC radical scavenging test result of EPS-BMD26 (Figure 6.8C) was comparable to that of ascorbic acid. Hydrophilic and hydrophobic interactions between antioxidant assays and EPS-BMD26 could explain the variations in antioxidant activity results. The primary component of EPS is a carbohydrate, which is responsible for the polysaccharide's distinct antioxidant properties (Niknezhad et al., 2018). It is obvious that DPPH yielded the lowest levels of scavenging activity, which could be attributed to its hydrophobic nature when measuring lipophilic antioxidants (Capanoglu et al., 2020; Y. Xu et al., 2019). The CUPRAC and hydroxyl ($\bullet\text{OH}$) radical scavengers can be used to test both hydrophilic and hydrophobic antioxidants; advantageously, the CUPRAC method does not oxidize simple sugars (Apak et al., 2022; Capanoglu et al., 2020). Besides, ABTS was considered to interact more with the hydrophilic antioxidants of EPS-BMD26 (Y. Xu et al., 2019). FT-IR and solubility tests also demonstrated that EPS-BMD26 had a hydrophilic behavior (sections 6.4.2 and 6.4.4). At 7.5 mg/mL concentration level, a glucose-mannose containing EPS from *Rhodotorula mucilaginosa* sp. GUMS16 had lower antioxidant activities through the DPPH $\bullet$ and $\bullet\text{OH}$ scavenging activities, which were detected at around 29% and 48%, respectively (Hamidi et al., 2020). In contrast to the current findings, Mirzaei et al. (2019) reported that *Rhodotorula minuta*'s EPS (containing glucose, mannose, and rhamnose) exhibited 22% and 25% of the DPPH $\bullet$ and $\bullet\text{OH}$ scavenging activities at 10 mg/mL, respectively. Likewise, at 8 mg/mL concentration level, EPS (containing galactose, arabinose, glucose, and mannose) from *Rhodotorula mucilaginosa* CICC 33013 had approximately 60% DPPH $\bullet$ and ABTS scavenging activity (Ma et al., 2018). These results were lower than the current DPPH $\bullet$ results but similar to the

ABTS scavenging results, implying that antioxidant activity could be related to monosaccharide composition and structural differences (hydroxyl groups, glycosidic linkages) of EPSs among *Rhodotorula* species (Hamidi et al., 2020). Therefore, these results demonstrated that EPS-BMD26 from *R. glutinis* exhibited considerable antioxidant activity with important bioactive attributes. Understanding the structure-function-guided exploration of EPS antioxidant activity and their *in vivo* antioxidant activity mechanisms will require more study.

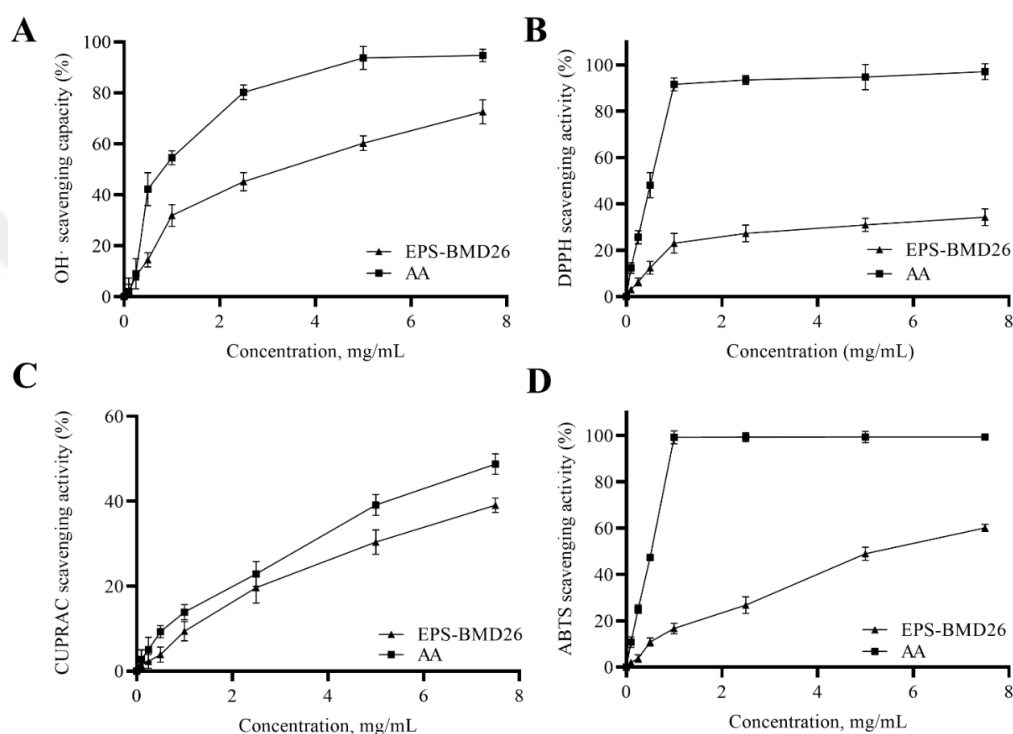


Figure 6.8 : Antioxidant activity of EPS-BMD26 was determined by hydroxyl ($\cdot\text{OH}$) (A), DPPH (B), CUPRAC (C), and ABTS (D) radical scavenging activity tests, with ascorbic acid (AA) as the positive control ($p < 0.05$).

6.4.10 Rheological properties of EPS-BMD26 in aqueous medium

Rheological characteristics of the EPS-BMD26 were investigated in distilled water at different pH conditions (4.0 and 7.0), temperatures (30, 40, and 50 °C), and combinations of these (Figure 6.9A-D). As shear rates increased, the viscosity of EPS-BMD26 decreased, confirming its shear thinning and pseudoplastic behavior (Figure 6.9A). This could be related to the rheological hydrodynamic force resulting in the development of some breaks within the EPS-BMD26 (Kavita et al., 2014). Since the viscosity was higher at pH 4, EPS-BMD26 from *R. glutinis* could be utilized as hydrocolloids to improve the texture of acidic foods such as yogurt (pH 4.0- 4.5) (Nambiar et al., 2018). Due to their easy pumpability and pleasant mouthfeel, these

shear-thinning fluids could also be utilized in food and pharmaceutical settings (W. Li et al., 2021). When it comes to temperature effect on viscosity (Figure 6.9B), the viscosity was detected to be slightly higher at 30 °C, and elevated temperature levels (40 and 50 °C) adversely affected the viscosity. The heat-induced alteration of the tertiary structure in EPS-BMD26 may provide an explanation for this circumstance (Nambiar et al., 2018). The combinations of two different pH values at three different temperature points are given in Figure 6.9C-D. It can be seen that EPS-BMD26 displayed greater pseudoplastic behavior at 30 °C when pH was 4.0. The viscous behavior of EPSs was associated with their glycosidic bond types, monosaccharides compositions, functional groups, salts, molecular weight, pH, temperature, and ionic strength (Ayyash, Abu-Jdayil, Itsaranuwat, et al., 2020; Nambiar et al., 2018). The shear thinning and pseudoplastic (non-Newtonian) behavior was reported in some microbial EPS's aqueous solutions, for example, yeasts including *Rhodotorula glutinis* (Cho et al., 2001) and *L. starkeyi* (W. Li et al., 2021); and bacteria including *Lactobacillus plantarum* HM47 (Nambiar et al., 2018), *Lactobacillus plantarum* C70 (Ayyash, Abu-Jdayil, Itsaranuwat, et al., 2020), *Lactobacillus kefir* (Riaz Rajoka et al., 2019) and *Weissella cibaria* (Vasanthakumari et al., 2015).

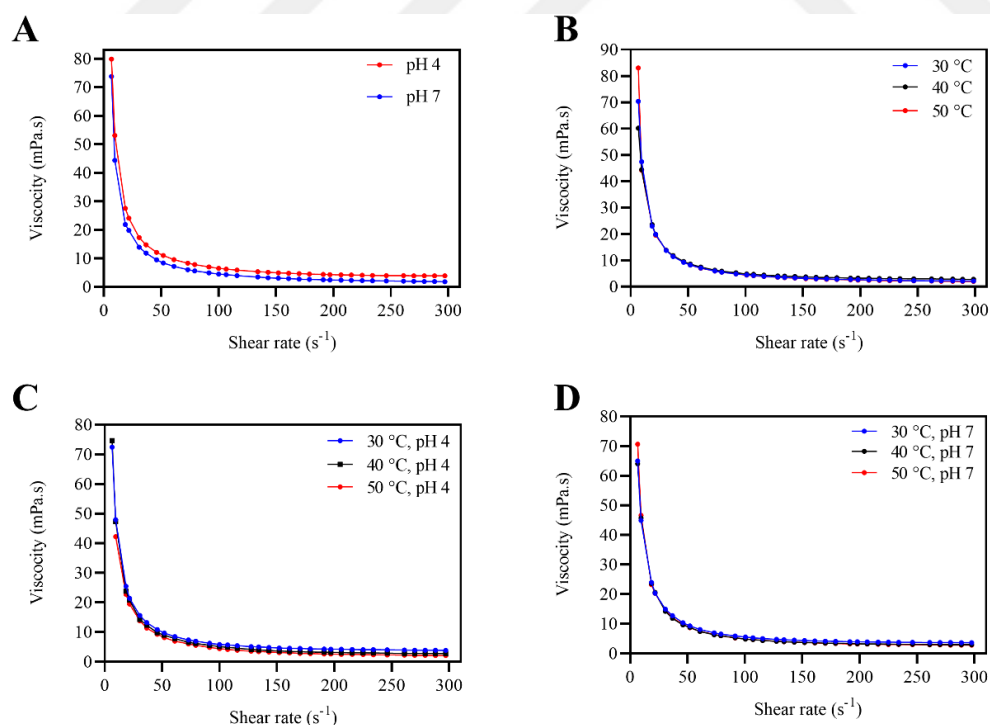


Figure 6.9 : Rheological behavior of EPS-BMD26 from *Rhodotorula glutinis*: effect of shear rate on viscosity in distilled water (A) at pH 4 and 7, (B) at temperatures 30 °C, 40 °C, and 50 °C, (C) at 30 °C, pH 4; 40 °C, pH 4; and 50 °C, pH 4, and (D) 30 °C, pH 7; 40 °C, pH 7; and 50 °C, pH 7.

6.4.11 Antibiofilm activity

EPS-BMD26 showed a dose-dependent antibiofilm activity on gram-positive bacteria *S. aureus* ATCC 25923 (Figure 6.10). The highest antibiofilm activity was noted at 1250 ppm, corresponding to 79.5% of antibiofilm inhibition. This action could be related to EPS-BMD26's ability to disrupt the cell membrane integrity of *S. aureus*, resulting in increased cell permeability with an anti-adhesive effect (Vazquez-Rodriguez et al., 2018). Although *R. mucilaginosa* has been shown to have antibiofilm activity against a variety of pathogenic bacteria, this is the first report demonstrating that *R. glutinis*-derived EPS had antibiofilm action against *S. aureus*. Similarly, the antibiofilm activity of *Weissella confosa* was comparable to that of the present findings (~ 74% antibiofilm activity at 1250 ppm) (Lakra et al., 2020). When compared to *Lactobacillus plantarum*, EPS-BMD26 demonstrated high antibiofilm activity at lower concentrations (5000 ppm) (J. Wang et al., 2015). However, EPS from *Rhodotorula mucilaginosa* had relatively higher antibiofilm activity (81% biofilm inhibition at 1000 ppm) against *S. aureus* (Vazquez-Rodriguez et al., 2018).

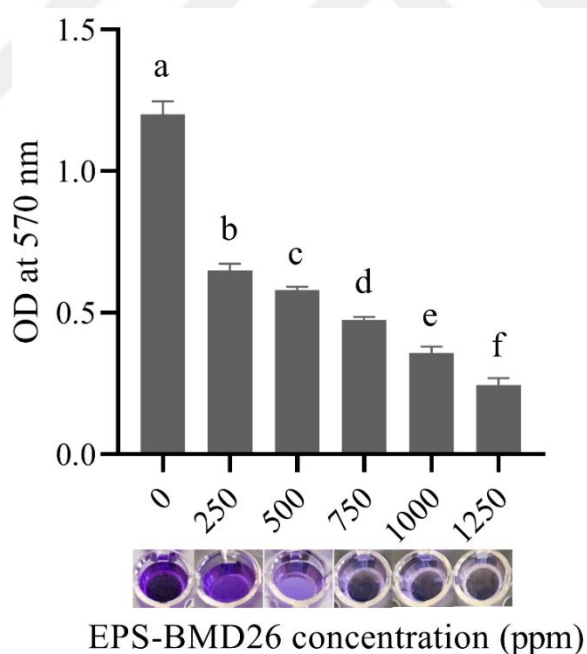


Figure 6.10 : The effect of EPS-BMD26 on biofilm formation of *Staphylococcus aureus* ATCC 25923.

6.5 Conclusions

A novel EPS-BMD26 derived from cold-adapted *R. glutinis* had an average molecular weight of 118 kDa and consisted of glucose and galactose. The EPS-BMD26 had a

higher production yield (31.8 g/L) than other bacteria- and yeast-derived EPSs. Given its rheological, microstructural, and physical properties, EPS-BMD26 could be employed as a bio-hydrocolloid agent in the food, cosmetics, and pharmaceutical industries. EPS-BMD26 was highly thermally stable and did not exhibit degradation up to 326.16°C, making it a promising candidate for thermally processed foods. Furthermore, the EPS demonstrated a moderate dose-dependent antioxidant activity, indicating that it has health benefits. At 1250 ppm, EPS-BMD26 inhibited the biofilm of *S.aureus* by 79.5%, highlighting that it could be used as an antibiofilm adjunct in food and medicine. Further research is needed to investigate the structure-function and dose-activity relationships of microbial EPSs produced by different yeasts under optimal fermentation conditions utilizing agricultural/food waste as the substrate.



7. CONCLUSIONS AND RECOMMENDATIONS

This dissertation aimed to evaluate the biocontrol potential of fruit-isolated antagonistic yeasts in the sustainable bio-management of postharvest fungal diseases in fruits. Further, the possible modes of action of antagonistic yeasts were investigated through *in vitro* and *in vivo* experiments to elucidate their inhibition mechanisms against notorious fungi. In addition, yeast isolates are capable of producing exopolysaccharides (EPSs), and a high-yielding EPS from *Rhodotorula glutinis* was characterized by its microstructural, rheological, bioactive, and physical properties.

For this purpose, *Metschnikowia*, *Hanseniaspora*, *Rhodotorula*, and *Meyerozyma* spp. were isolated from organic fruits or their leaves, including grapes, rosehip, hawthorn, blackberry, and cornelian cherries. First, eleven distinct yeast cultures of *Metschnikowia* sp. belonging to six different species were subjected to *in vitro* preliminary screening against *Alternaria alternata*, *Botrytis cinerea*, *Fusarium oxysporum*, *Penicillium digitatum*, and *Penicillium expansum*. The highest inhibition was observed against *Penicillium* spp. Then, further studies were conducted on lemons, which were chosen as a model fruit for *Penicillium* spp. The iron depletion, biofilm formation, and secretion of cell-wall degrading enzymes (chitinase, protease, cellulase, gelatinase, and β -1,3 glucanase) with volatile organic compounds were recognized as the major biocontrol mechanisms of selected *Metschnikowia* yeasts. Among the tested yeasts, *M. aff. fructicola* showed the highest biocontrolling efficacy on lemons, and other *Metschnikowia* yeasts also reduced disease incidence and lesion diameters in comparison to the control. This study showed that different *Metschnikowia* species have different levels of biocontrol efficacy, and their commercialization holds immense potential for the development of yeast-based biocontrol agents that can be used in place of synthetic pesticides.

Second, the compatible combinations of *Hanseniaspora uvarum* 38-KMD, *Meyerozyma guilliermondii* 7-BYM, and *Metschnikowia aff. pulcherrima* 32-AMM were employed to inhibit *Penicillium digitatum* on mandarin fruit while elucidating their underlying mechanisms for controlling green mould. These three yeasts were

selected based on their *in vitro* mycelial growth inhibition activity (> 73%) against *P. digitatum*. This study demonstrated that the postharvest diseases in fruits could be bio-managed sustainably using customized yeast consortia. However, induction of host resistance was not included in this research, which needs further investigation to determine the precise mechanisms of action of yeasts.

Thirdly, grape-isolated and pectinase-negative antagonistic yeasts (*M. aff. fructicola* 1-UDM, *Metschnikowia pulcherrima* 34-UEM, and *H. uvarum* 3-UDM) were tested on detached grape berries against *Botrytis cinerea*, a causative agent of grey mould disease. Then, their possible antifungal mechanisms of action were investigated. The findings revealed that lytic enzyme activity, suppression of spore germination, biofilm activity, iron depletion, diffusible metabolites, wound-site colonization, mycocin production, and volatile organic compounds (VOCs) production all played key roles in antifungal modes of action. As a result, *M. aff. fructicola* had the highest biocontrolling efficacy, while *H. uvarum* VOCs significantly inhibited the grey mould and showed a promise to be used as a biofumigant. The biocontrolling effect of VOCs alone, however, was insufficient, as seen by significantly greater DI and LD values. Furthermore, all yeasts showed significant mycocinogenic activity on *S. cerevisiae* NCYC 1006 with the formation of blue zones around the yeast colonies. Grape-isolated yeasts were specifically selected to be tested on grapes rather than other fruit-derived yeasts because they showed great colonization ability in wounds of grapes with their outstanding ecological niche adaptability. Future research should identify VOCs from *H. uvarum* in order to better understand the mechanisms of action of yeast antifungals. These VOCs could function as potent biofumigants without requiring direct physical contact with the food commodities.

Finally, a novel exopolysaccharide (EPS-BMD26) was produced from *Rhodotorula glutinis*, a blackberry isolate. The findings pointed out that EPS-BMD26 was a heteropolysaccharide of glucose, and galactose. The average molecular weight of a novel EPS-BMD26 produced from cold-adapted *R. glutinis* was 118 kDa. EPS-BMD26 demonstrated high thermal stability (326.16 °C), which indicated that it could be a suitable ingredient in the thermally-processed foods. The aqueous solution of EPS-BMD26 exhibited a pseudoplastic behavior (non-Newtonian), high-water holding capacity (190 % ± 0.22), and water-solubility (60.6% ± 8.3), which indicated its potential to be used as a bio-hydrocolloid agent in the food, cosmetics, and

pharmaceutical industries. Its porous texture with fissures confirmed its suitability as a stabilizer or thickening agent. The antioxidant activity of EPS-BMD26 was found to be moderate and dose-dependent, indicating the presence of bioactive compounds with potential health benefits. With its high antibiofilm activity (79.5% of antibiofilm inhibition at 1250 ppm) on *Staphylococcus aureus* ATCC 25923, it was suggested that it could be employed as an antibiofilm adjuvant in food and medicine.

According to the general results of the studies, it is critical to determine which antagonistic yeast and/or combinations will be used in which fruit, against which fungi, in what condition and at what level in order to develop sustainable solutions. Although yeasts show promise in the biocontrolling of fruits, it is not totally clear how they work against pathogenic fungi. Therefore, the antagonistic mechanisms of yeasts should be thoroughly investigated to develop tailored yeast-based bio-products by rationally combining the antagonistic consortia.

Yeasts are capable of producing versatile metabolites such as exopolysaccharides. The study's conclusions showed that yeast-derived exopolysaccharide had a relatively higher yield than any known bacteria as well as antioxidant, antibiofilm, and bio-hydrocolloidal potential. Due to their simplicity in extraction, high yield, and lack of toxicity, yeast exopolysaccharides have received a lot of attention.

In light of the findings of the antagonistic yeasts, further studies can be recommended:

- A comprehensive understanding of the action mode at yeast basis is necessary to develop novel yeast-based biofungicides. Therefore, future studies should employ omics approaches, including genomics, transcriptomics, proteomics, metagenomics, and metabolomics to analyse the interactions between fruit (hosts), fungi (pathogens), biocontrol yeast (antagonists), and microbiome (natural microbiota).
- Moving these strategies from lab-scale to large-scale commercial applications is quite challenging. Therefore, the appropriate administration method (such as dipping, spraying, and coating) and the proper concentration of yeast antagonists must be established before commercialization.
- The combination of antagonistic yeasts with different bio-agents, physical processes, conditions or microorganisms is necessary to enhance and stabilize their biocontrol activity.

Fermentation and culture conditions need to be optimized to increase the yield of EPS, which also need to be tested it in various food matrices to see how they affect texture, consistency, bioavailability, antioxidant, and antibacterial activity.



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APPENDICES

APPENDIX A: Supplementary materials for Chapter 3

APPENDIX B: Supplementary materials for Chapter 4

APPENDIX C: Supplementary materials for Chapter 6



APPENDIX A : Supplementary metarials for Chapter 3

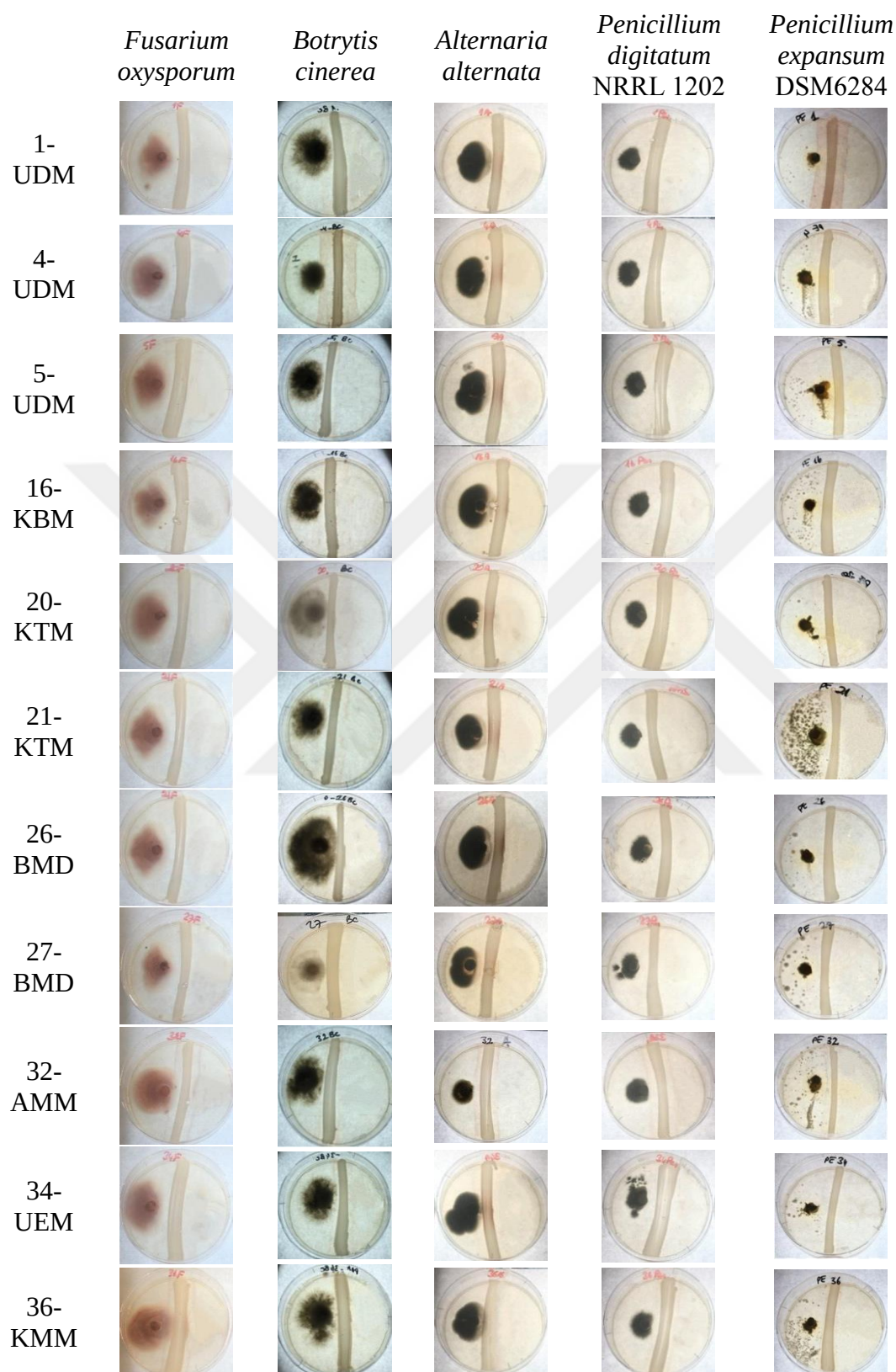


Figure A.1 : *In vitro* mycelial growth inhibition of *F. oxysporum*, *B. cinerea*, *A. alternata*, *P. digitatum* NRRL 1202 and *P. expansum* DSM6284.

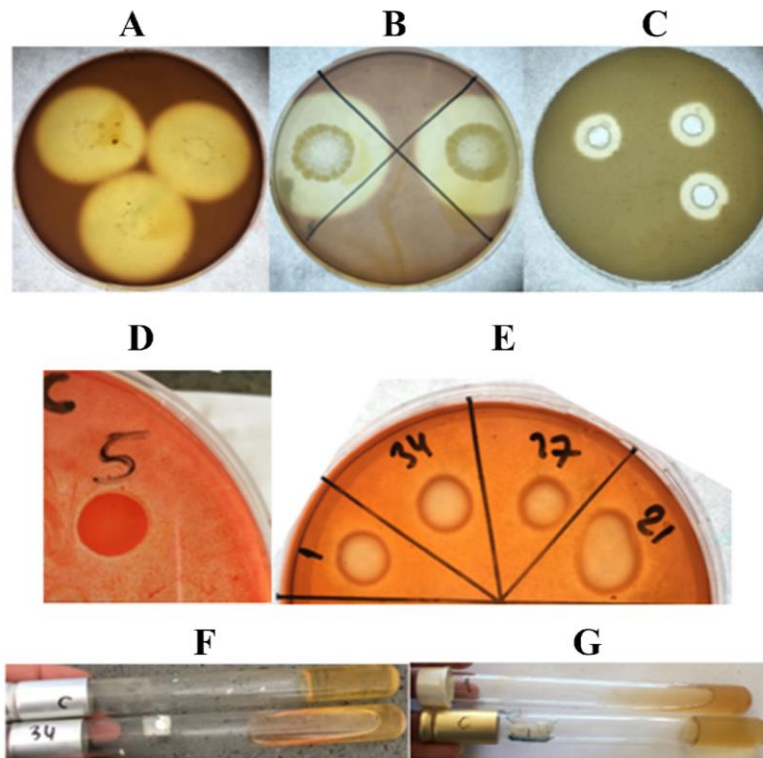


Figure A.2 : Extracellular lytic enzyme activity of 32 (*Metschnikowia* aff. *pulcherrima* P01A016 32-AMM) (A) chitinase, (B) pectinase, (C) protease, while (D) shows cellulase activity, (E) shows β -1,3-glucanase, and, (F) and (G) represents gelatinase activities.

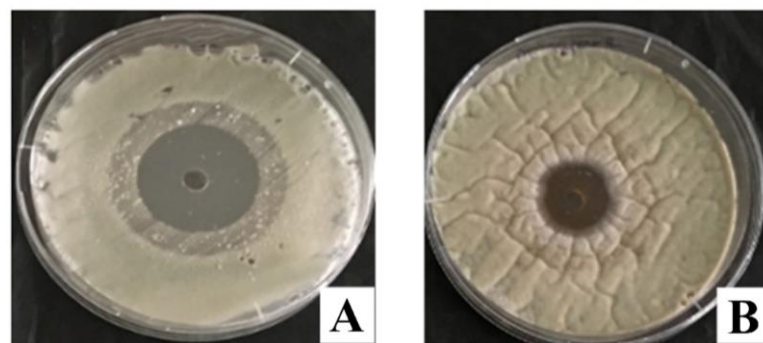


Figure A.3 : Inhibition of (A) *Penicillium digitatum* and (B) *Penicillium expansum* by tropolone.



APPENDIX B : Supplementary metarials for Chapter 4

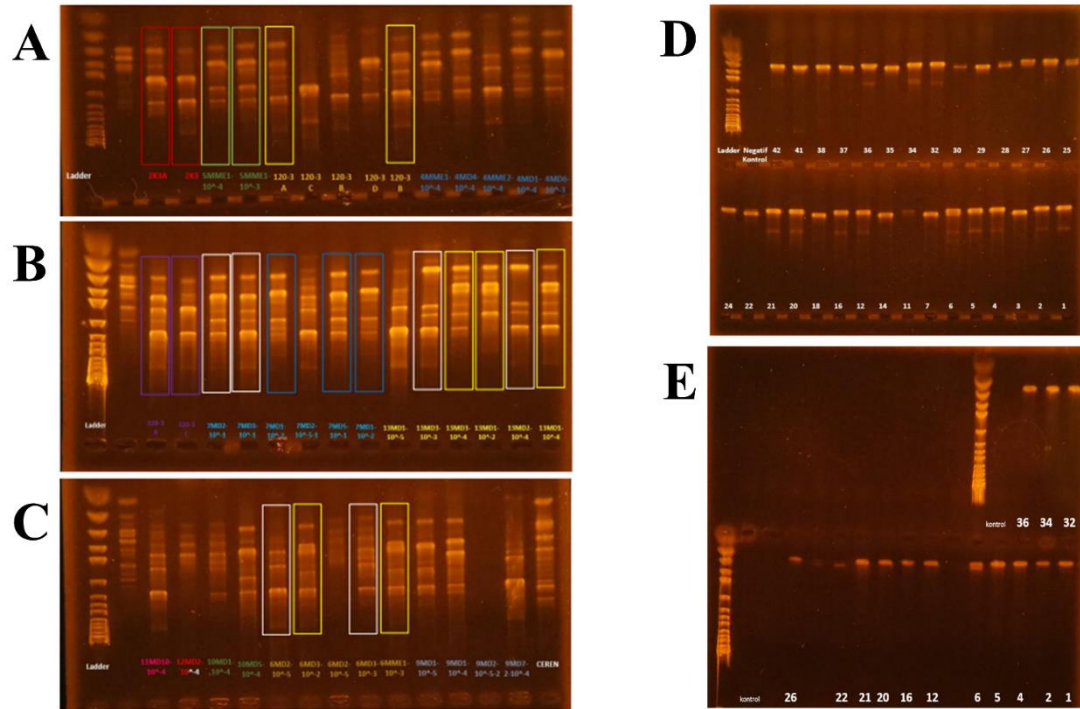


Figure B.1 : A-C) Agarose gel (1%) electrophoresis of the Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) products. D-E) Agarose gel photo documentation of conventional PCR sequencing the D1/D2 domain of the large subunit 26S rRNA E) 10-fold diluted DNA with one specific band.

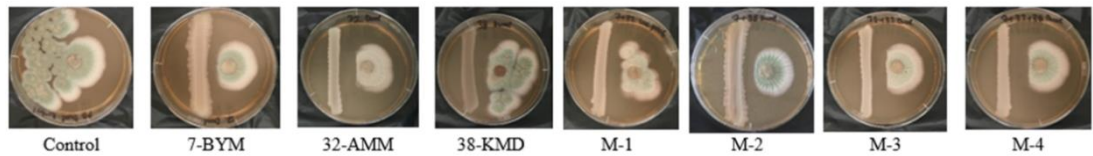


Figure B.2 : *In vitro* mycelial growth inhibition of *Penicillium digitatum* DSM2750 by tested yeast cultures (*Meyerozyma guilliermondii* 7-BYM; *Metschnikowia* aff. *pulcherrima* P01A016 32-AMM and *Hanseniaspora uvarum* 38-KMD), and their mixtures (M-1 = 7-BYM + 32-AMM; M-2 = 7-BYM + 38-KMD; M-3 = 32-AMM + 38-KMD; M-4 = 7-BYM + 32-AMM + 38-KMD). *P. digitatum* DSM2750.

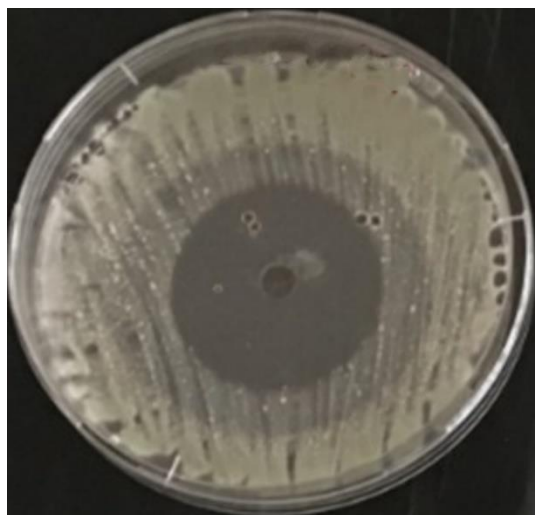


Figure B.3 : Inhibition of *Penicillium digitatum* DSM2750 by tropolone. Five microliters of 1 mg/mL tropolone was pipetted into a hole in the middle of the plate.

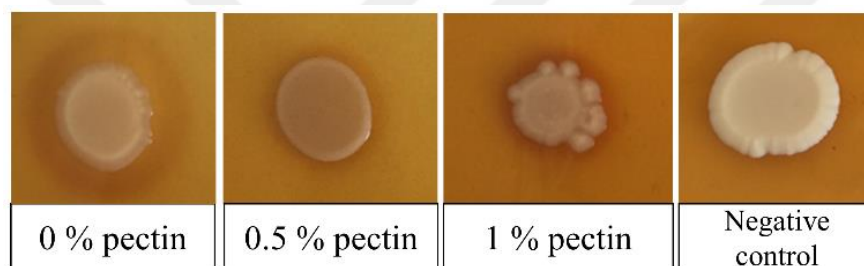


Figure B.4 : Effect of pectin content (0.5 and 1%) on the pigment formation of pulcherrimin produced by *Metschnikowia* aff. *pulcherrima* P01A016 32-AMM. *Metschnikowia chrysoperlae*, a non-pigment-producing yeast, used as a negative control.

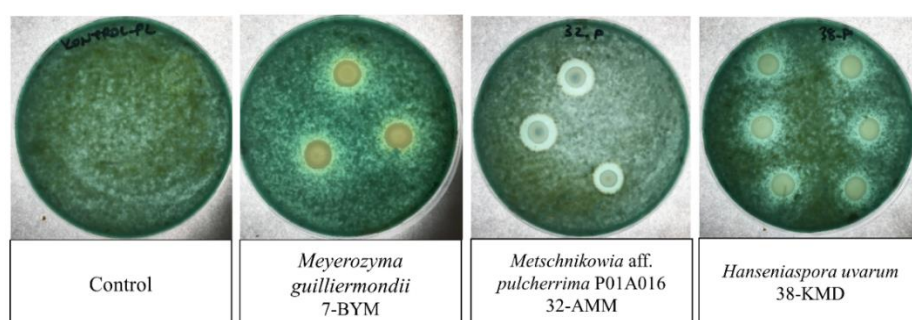


Figure B.5 : Mycocinogenic activity of *Meyerozyma guilliermondii* 7-BYM; *Metschnikowia* aff. *pulcherrima* P01A016 32-AMM, and *Hanseniaspora uvarum* 38-KMD suspensions (1×10^8 cells/mL) against *Penicillium digitatum* (1×10^5 conidia/mL) in yeast extract methylene blue agar (YEPD-MBA) at pH 4.5.

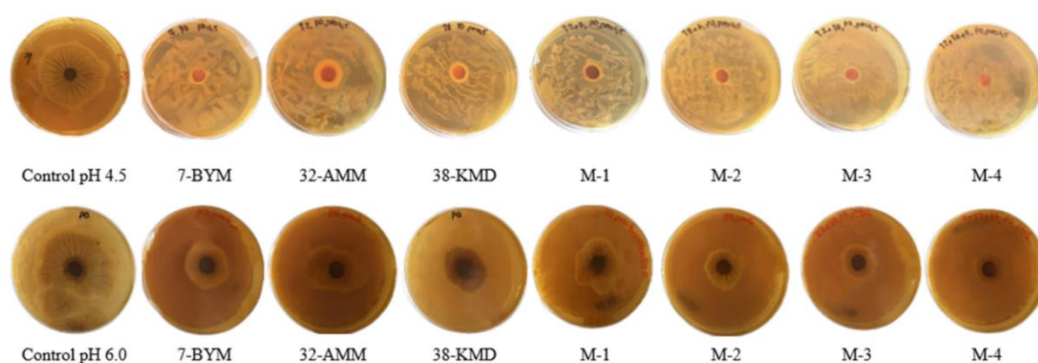


Figure B.6 : The plates displaying the effect of *in vitro* volatile organic compounds of three different yeast cultures (*Meyerozyma guilliermondii* 7-BYM; *Metschnikowia aff. pulcherrima* P01A016 32-AMM and *Hanseniaspora uvarum* 38-KMD) and their mixtures (M-1 = 7-BYM + 32-AMM; M-2 = 7-BYM + 38-KMD; M-3 = 32-AMM + 38-KMD; M-4 = 7-BYM + 32-AMM + 38-KMD) against *P. digitatum* DSM2750 at pH 4.5 and pH 6.0.



Figure B.7 : *In vivo* treatment of *M. aff. pulcherrima* P01A016 32-AMM (1×10^8 cells/mL) on mandarins after 5th day of incubation at 20 °C.

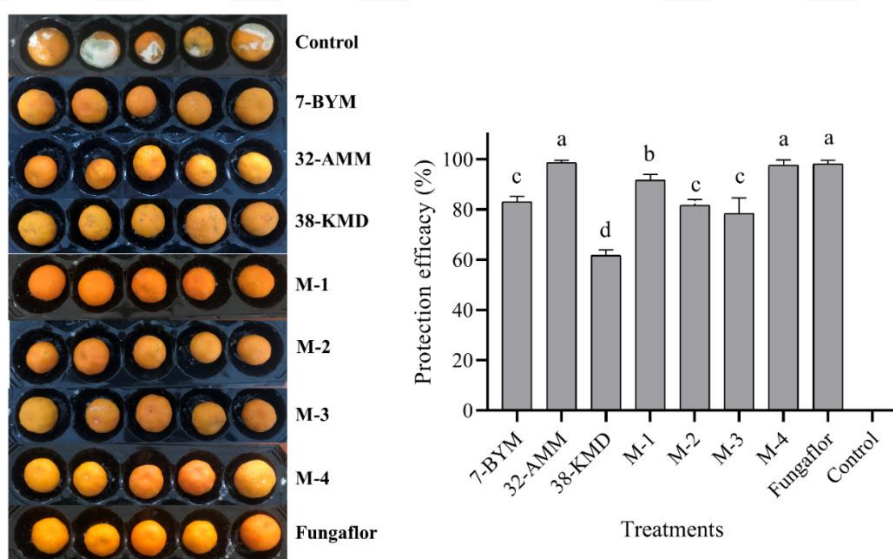


Figure B.8 : *In vivo* biocontrol efficacy of imazalil-based synthetic fungicide Fungaflor® 75 WSG (0.13 g/L), yeasts (*Meyerozyma guilliermondii* 7-BYM; *Metschnikowia aff. pulcherrima* P01A016 32-AMM and *Hanseniaspora uvarum* 38-KMD; 1:1, v/v; 1×10^8 cells/mL) and their mixtures (M-1 = 7-BYM + 32-AMM; M-2 = 7-BYM + 38-KMD; M-3 = 32-AMM + 38-KMD; M-4 = 7-BYM + 32-AMM + 38-KMD; 1:1:1, v/v/v, 1×10^8 cells/mL) against *P. digitatum* DSM2750 (1×10^4 conidia/mL) on the intact mandarin fruits, referring to the histograms on the right side.



APPENDIX C : Supplementary materials for Chapter 6

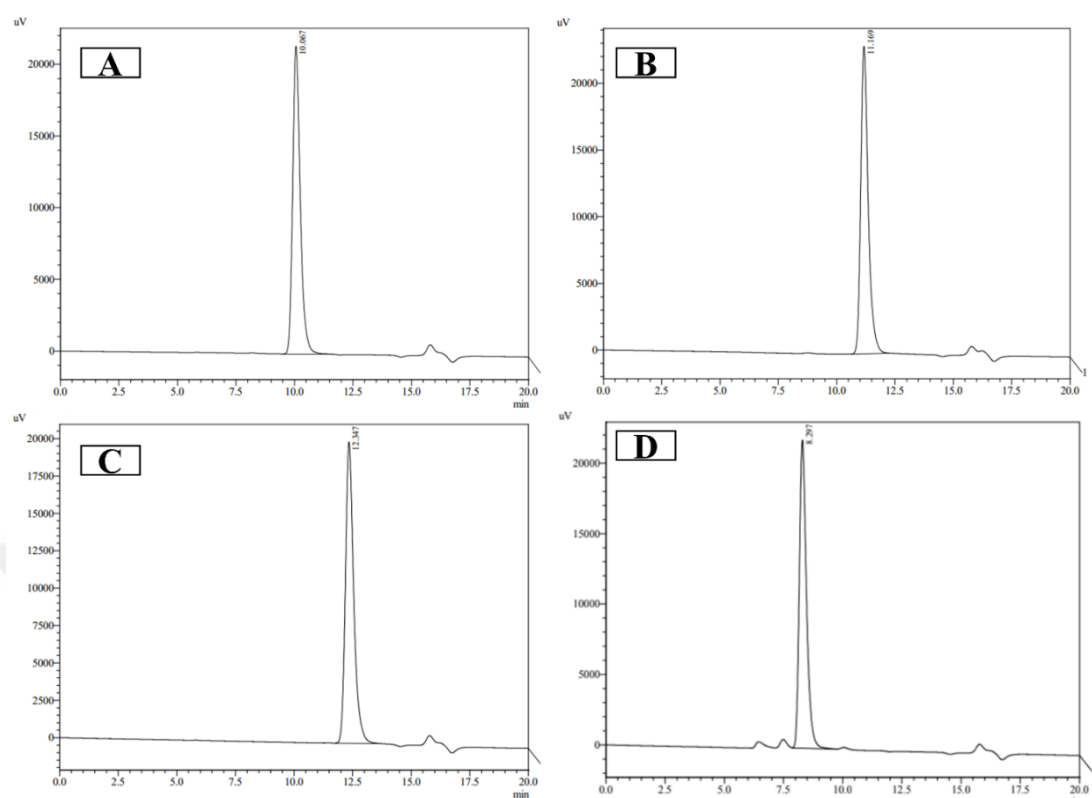


Figure C.1 : HPLC chromatogram of standard sugars tested for EPS-BMD16; a) Glucose, b) Galactose, c) Fructose, and d) Mannose.



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PROFESSIONAL EXPERIENCE AND REWARDS:

- 2019 Visiting Scholar (40,500\$), Award Turkish Council of Higher Education (CoHE) Research Scholarship for Doctoral Studies Abroad (YOK-YUDAB/2019)/ UPenn Medicine, Philadelphia, U.S.A.
- 2019 Visiting Scholar Award (\$4,500), M.I.T./ New England Biolabs Molecular Biology Summer Workshops, Massachusetts, U.S.A.
- 2015 Visiting Scholar (300 €), KTH Royal Institute of Technology (KTH), T.I.M.E European Summer School, “Sustainability and the Global Economy”, School of Industrial Engineering and Management, Department of Energy Technology, Stockholm, Sweden.
- 2014 Short-term Visiting Scholar, Fulda University, Hochschule Fulda, University of Applied Sciences, Fulda, Germany.
- 2012-to present, Research Assistant at Bayburt University, Faculty of Engineering, Department of Food Engineering, Bayburt, Türkiye.

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Öztekin, S.,** Dikmetaş, D. N., Devecioğlu, D., Acar, E. G., & Karbancıoğlu-Güler, F. (2023). Recent insights into the use of antagonistic yeasts for sustainable bio-management of postharvest pathogenic and mycotoxigenic fungi in fruits with their prevention strategies against mycotoxins, *Journal of Agricultural and Food Chemistry*, (Submitted).
- **Öztekin, S.,** & Karbancıoğlu-Güler, F. (2023). A novel exopolysaccharide from cold-adapted yeast *Rhodotorula glutinis*, along with structural, rheological, antioxidant, and antibiofilm properties, (Submitted)

- **Öztekin, S., & Karbancioglu-Guler, F. (2023).** Recruiting grape-isolated antagonistic yeasts for the sustainable bio-management of *Botrytis cinerea* on grapes, (Submitted)
- **Öztekin, S., & Karbancioglu-Guler, F. (2023).** Biological control of green mould on mandarin fruit through the combined use of antagonistic yeasts. *Biological Control*, 10518 <https://doi.org/10.1016/j.biocontrol.2023.105186> (Published).
- **Öztekin, S., & Karbancioglu-Guler, F. (2021).** Bioprospection of *Metschnikowia* sp. isolates as biocontrol agents against postharvest fungal decays on lemons with their potential modes of action. *Postharvest Biology and Technology*, 181, 111634. <https://doi.org/10.1016/j.postharvbio.2021.111634> (Published).
- **Öztekin, S., & Karbancıoğlu-Güler, F. (2021).** Utilization of antagonistic yeasts in grapes to control *Botrytis cinerea* DSM5145, Agriculture Asia Pacific 2021, Agriculture and Organic Farming, February 22-23 (Virtual Oral presentation).
- Yörücü, G., Tulum, K.A., Devocioğlu, D., **Öztekin, S., & Karbancıoğlu-Güler, F. (2020).** Meyve örneklerinden izole edilen mayaların mikosin üretme potansiyellerinin incelenmesi, Türkiye 13. Gıda Kongresi; 21-23 Ekim 2020, Çanakkale (Poster presentation).
- **Öztekin, S., Dertli, E., & Karbancıoğlu-Güler, F. (2018).** Antimicrobial activity of yeasts isolated from fruits, EurasianBiochem, International Eurasian Conference on Biological and Chemical Science, Ankara, 26-27-04.2018 (Poster presentation).

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

International Articles:

- **Oztekin, S., & Karbancioglu-Guler, F. (2022).** Simultaneous detection of ochratoxin a and aflatoxins in industrial and traditional red and *isot* pepper flakes along with dietary exposure risk assessment. *ACS Omega*, 7(36), 31756-31766 <https://doi.org/10.1021/acsomega.2c02236>
- Boaro, A., Ageitos, L., Torres, M. D., Broset, E., **Oztekin, S., & de la Fuente-Nunez, C. (2023).** Structure-function-guided design of synthetic peptides with anti-infective activity derived from wasp venom. *Cell Reports Physical Science*.

Book Chapters:

- **Oztekin, S., Anaya, K., & Yurdunuseven-Yıldız, A. (2023).** Öztekin, S., Anaya, K., & Yurdunuseven-Yıldız, A. (2022). Regulation of Natural Food Additives. In *Natural Additives in Foods* (pp. 343-372). Cham: Springer International Publishing.
- Devocioğlu, D., Dikmetaş, D.N., **Öztekin, S., & Karbancioglu-Guler, F. (2023).** Safety Aspects of non-thermal processing applications for fruit and vegetable processing. In *Non-thermal Processing Technologies for the Fruit and Vegetable Industry* (pp. 223-264). CRC Press.

Projects:

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