ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

VALORIZATION OF INDUSTRIAL FOOD WASTES BY PRODUCTION OF PHENOLIC ANTIOXIDANTS VIA FERMENTATION WITH NEWLY ISOLATED *ASPERGILLUS* SPP.

Ph.D. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

ENDÜSTRİYEL GIDA ATIKLARININ YENİ İZOLE EDİLMİŞ *ASPERGİLLUS* SPP. İLE FERMANTASYONU YOLUYLA FENOLİK ANTİOKSİDANLARIN ÜRETİMİNDE DEĞERLENDİRİLMESİ

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ARALIK 2019

Zehra GÜLSÜNOĞLU, a Ph.D. student of ITU Graduate School of Science Engineering and Technology student ID 506132504, successfully defended the thesis/dissertation entitled "VALORIZATION OF INDUSTRIAL FOOD WASTES BY PRODUCTION OF PHENOLIC ANTIOXIDANTS VIA FERMENTATION WITH NEWLY ISOLATED *ASPERGILLUS* SPP.", 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 family,

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Zehra GÜLSÜNOĞLU
(Food Engineer, M.Sc.)

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ABBREVIATIONS

ANOVA : Analysis of variance
ATCC : American Type Culture Collection
BHA : Butylated hydroxyanisol
BHT : Butylated hydroxytoluene
BLAST : Basic Local Alignment Search Tool
CCD : Central Composite Design
CE : Catechin Equivalent
CMC : Carboxymethylcellulose sodium salt
CUPRAC : Cupric ion reducing antioxidant capacity
CY20S : Czapek yeast agar with 20% sucrose
CYA : Czapek Yeast Agar
CZ : Czapek Dox Agar
DAD : Diode Array Detector
DG18 : Dichloran Glycerol (18%)
DNS : 3,5-Dinitrosalicylic acid
DPPH : 2,2-Diphenyl-1-picrylhydrazyl
DRBC : Dichloran Rose Bengal Chloramphenicol
DM : Dry Matter
FA : Formic Acid
FC : Folin-Ciocalteau
GAE : Gallic Acid Equivalent
HHDP : Hexahydroxydiphenoyl
HO• : Hydroxyl radical
HPLC : High Performance Liquid Chromatography
ICP-AES : Inductively Coupled Plasma Atomic Emission Spectrometer
ITS : Internal Transcribed Spacer
LC : Liquid Chromatography
ME : Malt Extract
MEA : Malt Extract Agar
MS : Mass Spectrometry

NCBI : National Center for Biotechnology Information
OTA : Ochratoxin A
PBD : Plackett-Burman Design
PDA : Photodioade Array Detector
QPCR : Quantitative Polymerase Chain Reaction
Rhodanine : 2-Thioxo-4-thiazolidinone
RO• : Alkoxyl radical
ROO• : Peroxyl radical
ROS : Reactive Oxygen Species
RP : Reversed Phase
RSM : Response Surface Methodology
SSF : Solid State Fermentation
TBHQ : Tertiary Butylhydroquinone
TE : Trolox® Equivalent
TEAC : Trolox® Equivalent Antioxidant Capacity
TFA : Trifluoroacetic acid
TFC : Total Flavonoid Content
TPC : Total Phenolic Content
Trolox® : 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
YES : Yeast Extract Sucrose Supplement Media

SYMBOLS

$\mathbf{A_s}$	**:** Absorbance of standard
$\mathbf{A_t}$	**:** Absorbance of test solution
C	**:** Concentration
D	**:** Dilution factor
eV	**:** Electronvolt
M	**:** Molarity
m/z	**:** Mass charge ratio
N	**:** Normality
$\mathbf{R^2}$	**:** Regression coefficient
$\mathbf{V_t}$	**:** Volume of test solution
$\mathbf{V_0}$	**:** Volume
w	**:** Weight of sample
$\boldsymbol{\lambda_{max}}$	**:** Wavelenght
µ	**:** Micro

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VALORIZATION OF INDUSTRIAL FOOD WASTES BY PRODUCTION OF PHENOLIC ANTIOXIDANTS VIA FERMENTATION WITH NEWLY ISOLATED *ASPERGILLUS* SPP.

SUMMARY

Food industry generates high amount of fruit and vegetable by-products which can be highly biodegradable and causing environmental pollution. However, by-products of fruit and vegetables have great amount of nutritional and functional compounds that are not utilized but disposed or used as animal feed. To valorize these by-products some new biotechnological processes can be developed to obtain high-value products like enzymes and bioactive components. Fermentation is one of the most popular technology due to its being cost-effective and environmentally friendly. Especially, fungal fermentation can yield various enzymes and bioactive components. In this thesis, local food sources were evaluated to isolate new *Aspergillus* spp. and their hydrolytic enzyme production ability was screened for the release of phenolics from plant wastes. For this purpose, different agro-industrial wastes were used as a substrate for fungal fermentation. To enhance the production of phenolics, fermentation conditions were optimized by Plackett-Burman Design (PBD) and then response surface methodology (RSM). Obtained phenolic extracts from fermented wastes were added to enhance the phenolic content and antioxidant activity of a model food.

The first part of the thesis is focused on the isolation and identification of *Aspergillus* section *Nigri* members from different rotten fruits and vegetables such as carrot, apple, fig, grapes (dry and fresh), corn, apricot (dry and fresh), garlic, onion, and dates. Eight black mold colonies were isolated for molecular and morphological identification. Six different aspergilli were selected from grape and date and named as *Aspergillus tubingensis* ZDM1 and ZGM5, *Aspergillus niger* ZDM2 and ZDM3, *Aspergillus japonicus* ZGM4 and *Aspergillus aculeatus* ZGM6 according to the ITS sequences.

In the second part, selected *Aspergillus* spp. were screened to determine their ability to produce industrially important hydrolytic enzymes including cellulase, tannase and pectinase using specific carbon sources for each enzyme both on solid and in liquid media. According to the screening results, isolated *Aspergillus* spp. had the ability to produce cellulase, tannase and pectinase enzymes on plates and also in liquid media. The highest activity of cellulase was obtained by *A. japonicus* ZGM4 and *A. aculeatus* ZGM6 as 40 and 35 U/g dry biomass, respectively. All isolates exhibited high level of tannase activity in the range of 150–343 U/g dry biomass after 24 h of incubation. The highest pectinase activity was found to yield at a level of 130 and 117 U/g dry biomass for *A. tubingensis* ZGM5 and *A. aculeatus* ZGM6, respectively. These results showed that newly isolated *Aspergillus* spp. were potential hydrolytic enzyme producers. In the light of these results, four *Aspergillus* spp. (*A. niger* ZDM2, *A. tubingensis* ZDM1, *A. japonicus* ZGM4 and *A. aculeatus* ZGM6) were selected from six isolates for further fermentation processes according to the enzymes they secreted.

In the third part, the potential of selected agroindustrial by-products including apple peel, apple pomace, pomegranate peel, pomegranate seed, chestnut shell and black

carrot pomace were evaluated for determining their potential as natural resources for food components and antioxidants. The gross composition of wastes was determined by applying the methods for protein, reducing sugar, ash, mineral (K, Ca, Mg, Na, Cu, Fe, Mn, Zn), condensed tannin and free glucose. Pomegranate seed contained the highest amount of protein while pomegranate peel and black carrot pomace had the highest amount of minerals among the wastes. Pomegranate peel had the highest amount of reducing sugar, however apple pomace had the highest glucose content. Black carrot pomace had the highest overall mineral content, and pomegranate peel showed the highest level of Ca and K. Chestnut peel was the richest in condensed tannin among the wastes. Soluble and insoluble-bound phenolics and flavonoids in wastes were determined. Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity by 2,2-diphenly-1-picrylhydrazyl (DPPH) and cupric reducing antioxidant activity (CUPRAC) assays of wastes were measured. Profile of phenolics were established by RP-HPLC analysis. The highest TPC and antioxidant activity were determined in soluble fraction of pomegranate peel due to significant amount of punicalagin derivatives. Pomegranate peel and seed had the most phenolics and flavonoids in soluble form while other wastes had more than 45% of total phenolics in insoluble-bound form. Chestnut shell showed more antioxidant activity in insoluble-bound fraction compared to that of its soluble fraction. Profile of soluble and bound phenolics of each waste was composed of a wide variety of phenolic compounds. TPC and TFC of wastes were positively correlated with their antioxidant activity.

In the fourth part, apple peel was used as a substrate to obtain phenolic compounds by fermentation with *Aspergillus* spp. due to its higher content of insoluble-bound phenolics compared to that of soluble ones. Fermentation with *Aspergillus* spp. augmented TPC and antioxidant activity of apple peel. *A. niger* ZDM2 and *A. tubingensis* ZDM1 were found to yield the highest level of TPC and TFC. Fermentation with these species increased antioxidant activity of apple peel by 3-4 fold. Phenolics of apple peels were identified by LC-MS/MS and quantified by HPLC-PDA. Quercetin and its glycosides were dominant in unfermented apple peel and their concentrations were reduced by mold fermentation. After fermentation, a tentatively identified isomer of taxifolin was detected in fermented apple peel by *A. aculeatus* ZGM6 and *A. japonicus* ZGM4, whereas isomers of eriodictyol and catechin were identified in apple peel fermented by *A. niger* ZDM2 and *A. tubingensis* ZDM1. According to the these results, *A. niger* ZDM2 was chosen for further optimization studies of fermentation conditions to enhance the TPC and antioxidant activity of apple peel. Firstly, nine factors (sodium nitrate, urea, peptone, ammonium chloride, yeast extract, glucose, sucrose, lactose and fermentation time) were screened by Plackett-Burman design (PBD) to determine the fermentation conditions then central composite design (CCD) was performed to determine the level of significant factors. Concentrations of urea and lactose and fermentation time were found significant factors on the production of phenolic compounds from apple peel. Optimum fermentation conditions were 5.9 g/L urea, 11.5 g/L lactose, 198 h of fermentation time. According to the optimization results, TPC and DPPH activity of fermented apple peel was increased 6 and 10 fold, respectively.

In the fifth part, the valorization of the chestnut shell (shell: inner pellicle and peel) was performed for the production of ellagic acid by fungal fermentation. Selected four *Aspergillus* spp. (*A. aculeatus* ZGM6, *A. japonicus* ZGM4, *A. niger* ZDM2, *A. tubingensis* ZDM1) were used to assess their ellagic acid production capacity. *A. japonicus* ZGM4, the best producer with the ellagic acid concentration of 2.04±0.31

mg ellagic acid/g dry matter, was used as test microorganism to determine the optimum fermentation conditions. CCD was utilized to optimize three significant factors including concentrations of lactose and yeast extract and fermentation time. The CCD resulted in the production of 2.72±0.21 mg ellagic acid/g dry matter in the optimized medium with 11.5 g/L lactose, 5.9 g/L yeast extract after 140 h of fermentation. Optimized fermentation conditions generated 6-fold increase in yield of ellagic acid in fermented chestnut shell compared to that of unfermented chestnut shell.

In the final part, fermented apple peel and chestnut shell phenolic extracts were added into yoghurt as functional ingredients. Yoghurt fortified with unfermented apple peel and chestnut shell extract increased the TPC as 2.2 and 3.1-fold, respectively. On the other hand, yoghurt fortified with fermented apple peel and chestnut shell extract increased the TPC as 5.8 and 6.3-fold compared to the plain yoghurt, respectively. The highest DPPH activity was measured in yoghurt sample with fermented chestnut shell followed by the sample with fermented apple peel and those with unfermented wastes.

ENDÜSTRİYEL GIDA ATIKLARININ YENİ İZOLE EDİLMİŞ *ASPERGİLLUS* SPP. İLE FERMANTASYONU YOLUYLA FENOLİK ANTİOKSİDANLARIN ÜRETİMİNDE DEĞERLENDİRİLMESİ

ÖZET

Gıda endüstrisinde üretimden çok miktarda katı ve sıvı atık açığa çıkmaktadır. Gıda endüstrisi atıklarının önemli bir bölümünü meyve ve sebze atıkları oluşturmaktadır. Bu atıkların çevre kirliliğine sebep olmalarının yanısıra düşük ekonomik değeri olan ürünlere (hayvan yemi, gübre vb.) dönüştürüldüklerinde değerli biyokütle ve besin maddelerinin kaybı söz konusudur. Bu nedenle değerlendirilmeleri ekolojik ve ekonomik fayda sağlamaktadır. Meyve ve sebze atıkları beslenme açısından önemli diyet lifi, vitaminler, pektin, yağ asitleri ve antioksidanlar gibi birçok bileşen içermektedir. Bu bileşenlerin atıklardan eldesi yoluyla biyoaktif ürünler üretmek mümkündür. Fermantasyon, biyoteknolojik olarak mikrobiyal enzimlerin ve biyoaktif ürünlerin üretiminde düşük maliyetli ve çevre dostu olması sebebiyle tercih edilen teknolojilerden biridir. Gıda atıklarının azaltılması ve değerlendirilmesi gıda alanında yapılan güncel araştırma konularından biridir. Bu tez çalışmasında, yerel kaynaklardan yeni *Aspergillus* türleri izole edilmiştir. İzole edilen bu küflerin hidrolitik enzim üretme kabiliyetleri incelenmiş ve bazı endüstriyel gıda atıklarından fenolik maddelerin elde edilmesi amacıyla kullanılmıştır. Bu doğrultuda, izole edilen küflerin hidrolitik enzim üretme potansiyelleri hem katı hem de sıvı besiyeri ortamında değerlendirilmiştir. Fenolik madde üretimi için en uygun gıda atıklarının belirlenmesi amacıyla, atıkların hem kimyasal kompozisyonu hem de fenolik madde içeriği belirlenmiştir. Atıkların izole edilen küflerle fermantasyonu sonucu en yüksek sonuç veren küfler için gerekli fermantasyon koşulları, Plackett-Burman deney tasarım (PBD) metodu ve sonrasında yanıt yüzey metodolojisi (RSM) kullanılarak optimize edilmiştir. Elde edilen fenolikçe zengin fermente atıklar, model bir gıdada fonksiyonel bileşen olarak kullanılmıştır.

Tezin ilk bölümünde, *Aspergillus* section *Nigri* üyesi küflerin izolasyonu için çürümüş havuç, elma, incir, üzüm, mısır, kayısı, sarımsak, soğan gibi farklı meyve ve sebzeler kullanılmıştır. Besiyeri üzerinde gelişme gösteren sekiz adet siyah küfün moleküler ve morfolojik tanımlamaları yapılmıştır. Üzüm ve hurmadan izole edilmiş altı *Aspergillus* türü, *Aspergillus tubingensis* ZDM1 ve ZGM5, *Aspergillus niger* ZDM2 ve ZDM3, *Aspergillus japonicus* ZGM4 ve *Aspergillus aculeatus* ZGM6, ITS gen dizilimi kullanılarak adlandırılmış ve daha sonraki aşamalarda kullanılmıştır.

Tezin ikinci kısmında, seçilmiş *Aspergillus* türlerinin hidrolitik enzimleri (selülaz, tannaz ve pektinaz) üretme potansiyelleri hem katı hem de sıvı besiyerinde incelenmiştir. *Aspergillus* izolatları hem katı hem de sıvı ortamda incelenen enzimleri üretmiştir. En yüksek selülaz aktivitesi *A. japonicus* ZGM4 ve *A. aculeatus* ZGM6 tarafından sırasıyla 40 ve 35 U/g kuru biyokütle olarak belirlenmiştir. Bütün izolatlar, 24 saatlik inkübasyondan sonra 150-343 U/g kuru biyokütle aralığında değişen tannaz aktivitesi göstermiştir. En yüksek pektinaz aktivitesi sırasıyla 130 ve 117 U/g kuru

biyokütle aktivite gösteren *A. tubingensis* ZGM5 ve *A. aculeatus* ZGM6 türlerinde bulunmuştur. Elde edilen bulgulara göre, dört *Aspergillus* türü (*A. niger* ZDM2, *A. tubingensis* ZDM1, *A. japonicus* ZGM4 ve *A. aculeatus* ZGM6), yüksek hidrolitik enzim üretmeleri sebebiyle daha sonraki aşamalarda yapılacak olan fermantasyon çalışmaları için seçilmiştir.

Tezin üçüncü bölümünde, elma kabuğu ve posası, nar kabuğu ve çekirdeği, kestane kabuğu ve kara havuç posasının fermantasyonda kullanım olanaklarının belirlenmesi için içerdikleri gıda bileşenleri tespit edilmiştir. Kimyasal bileşimlerini belirlemek amacıyla protein, indirgen şeker, serbest glukoz, kül, mineral (K, Ca, Mg, Na, Cu, Fe, Mn, Zn) ve tanen miktarları ölçülmüştür. Atıklardan hem serbest hem de çözünmez-bağlı formdaki fenolik bileşenler sırasıyla metanolik ve alkali ekstraksiyon yöntemleri ile ekstrakte edilmiş ve toplam fenolik madde (TPC) ve flavonoid (TFC) içeriği ve antioksidan aktiviteleri (DPPH, CUPRAC) belirlenmiştir. Ayrıca atıklardaki fenolik maddelerin tanımlama ve miktar tayinleri RP-HPLC ile gerçekleştirilmiştir. Protein nar çekirdeğinde en fazla bulunurken, en fazla mineral miktarı nar kabuğu ve kara havuç posasında bulunmuştur. İndirgen şeker miktarı en fazla nar kabuğunda bulunurken, glukoz en fazla elma posasında bulunmuştur. Kara havuç posası mineraller açısından zenginken nar kabuğunun yüksek miktarda kalsiyum ve potasyum içerdiği tespit edilmiştir. Kestane kabuğu, diğer atıklarla karşılaştırıldığında tanen içeriği en yüksek olan atıktır. Atıkların fenolik bileşen miktarına bakıldığında, nar kabuğunun en yüksek TPC ve antioksidan aktiviteye sahip olduğu ve bunun yüksek punicalagin içeriği ile ilişkili olduğu sonucuna varılmıştır. Fenolik maddelerin çoğunluğu nar kabuğu ve çekirdeğinde serbest formda bulunurken diğer atıklardaki fenolik maddelerinin %45'inden daha fazlasının bağlı formda olduğu saptanmıştır. Kestane kabuğundan elde edilen bağlı fenolik ekstraktı serbest fenolik ekstraktına göre daha fazla antioksidan aktivite göstermiştir. Atıkların serbest ve bağlı formda bulunan çeşitli fenolikleri içerdiği saptanmıştır. Atıkların TPC ve TFC değerleri ile antioksidan aktiviteleri arasında pozitif bir korelasyon tespit edilmiştir. Elma ve kestane kabukları yüksek bağlı fenolik madde içerikleri dolayısıyla daha sonraki fermantasyon çalışmaları için seçilmiştir.

Tezin dördüncü bölümünde, elma kabuğu ile hazırlanan besi ortamında dört farklı küf (*A. aculeatus* ZGM6, *A. japonicus* ZGM4, *A. niger* ZDM2, *A. tubingensis* ZDM1) ile fermantasyon işlemi gerçekleştirilmiştir. Bu amaçla elma kabuğu ile hazırlanan besi ortamında 7 gün boyunca katı yüzey fermantasyonu gerçekleştirilmiş ve belirli aralıklarda örnekler alınarak TPC ve antioksidan aktivitedeki değişim incelenmiştir. *Aspergillus* spp. ile fermantasyon sonucunda elma kabuğundaki TPC ve antioksidan aktivitenin arttığı görülmüştür. En fazla artış *A. niger* ZDM2 ve *A. tubingensis* ZDM1 türleriyle fermente edilen elmalarda tespit edilmiştir. Bu türler ile fermente edilen elma kabuklarının antioksidan aktivitesinde fermente edilmemiş elma kabuğuyla kıyaslandığında CUPRAC ve DPPH yöntemlerinde sırasıyla 3 ve 4 kat artış saptanmıştır. Fermente edilmemiş elma kabuğunda bulunan fenolikler LC-MS/MS ile tanımlanmış ve HPLC-PDA ile miktar analizi yapılmıştır. Elma kabuğunda baskın olarak kuersetin ve glikozitlerinin bulunduğu ve fermantasyon sonucunda konsantrasyonlarında azalma meydana geldiği saptanmıştır. *A. aculeatus* ZGM6 ve *A. japonicus* ZGM4 ile fermente edilen elma kabuklarında baskın olan fenolik maddenin taksifolin izomeri olduğu, *A. niger* ZDM2 ve *A. tubingensis* ZDM1 ile fermente edilen elmalardaki baskın fenoliklerin ise eriodiktiol ve kateşin izomerleri olduğu görülmüştür. Bu sonuçlara göre, fermente edilmiş elma kabuğunda en yüksek TPC ve antioksidan aktivite artışı gösteren *A. niger* ZDM2 fermantasyon koşullarının

optimizasyon çalışmalarında kullanılmıştır. İlk olarak, fermantasyonda etkili olan faktörleri belirlemek için dokuz faktör (sodyum nitrat, üre, pepton, amonyum klorür, maya ekstraktı, glukoz, sakkaroz, laktoz ve fermantasyon süresi) PBD metodu ile incelenmiştir. PBD ile belirlenen etkili faktörlerin (üre ve laktoz konsantrasyonları ve fermantasyon süresi) optimum seviyeleri CCD ile RSM uygulanarak saptanmıştır. Optimum fermantasyon koşulları, 5,9 g/L üre, 11,5 g/L laktoz ve 198 saat fermantasyon süresi olarak bulunmuştur. Elma kabuğu optimum koşullar uygulanarak fermente edildiğinde TPC ve antioksidan aktivite (DPPH) değerleri sırasıyla 6 ve 10 kat artmıştır.

Beşinci bölümde, kestane kabuğu dört izolat kullanılarak fermente edilmiş ve ellajik asit üretimi incelenmiştir. LC-MS/MS ile tanımlanan ellajik asidin konsantrasyonu HPLC-PDA detektörü ile belirlenmiştir. Yapılan çalışmalar sonucunda, en yüksek ellajik asit konsantrasyonu (2,04±0,31 mg ellajik asit/g kuru madde) *A. japonicus* ZGM4 ile fermente edilmiş kestane kabuklarında bulunmuştur ve bu izolat fermantasyon koşullarının optimizasyonunda kullanılmıştır. Bu amaçla ilk olarak fermantasyon koşullarını belirlemek amacıyla dokuz faktör (sodyum nitrat, üre, pepton, amonyum klorür, maya ekstraktı, glukoz, sakkaroz, laktoz ve fermantasyon süresi) PBD metodu ile incelenmiş ve üretilen ellajik asit miktarı üzerinde etkili olarak bulunan faktörler CCD kullanılarak RSM ile optimize edilmiştir. Laktoz ve maya ekstraktı konsantrasyonları ve fermantasyon süresi fermantasyonda etkili faktörler olarak bulunmuştur. Optimum fermantasyon koşullarında (11,5 g/L laktoz, 5,9 g/L maya ekstraktı ve 140 saat fermantasyon süresi) ellajik asit miktarı 2,72±0,21 mg ellajik asit/g kuru madde olarak bulunmuştur. Optimizasyon çalışmalarıyla, ellajik asit miktarında fermente edilmemiş kestane kabuğunda bulunan ellajik asit miktarına oranla yaklaşık 6 kat artış sağlanmıştır.

Tez çalışmasının son bölümünde, fermente edilmiş ve edilmemiş elma ve kestane kabukları yoğurda ilave edilerek, yoğurdun TPC ve antioksidan aktivitesinin arttırılması hedeflenmiştir. Hazırlanan yoğurt örneklerinin TPC ve DPPH aktiviteleri hazırlanmalarının hemen ardından ve 3 gün depolanmadan sonra ölçülmüştür. Sade yoğurdun TPC ve DPPH aktivitesi sırasıyla 98,2 mg GAE/100 kuru madde ve 83,8 TE/100 g kuru madde olarak hesaplanmıştır. Fermente olmayan elma ve kestane kabuğu ekstraktı ile zenginleştirilen yoğurt örneklerinde TPC sırasıyla 2 ve 3 kat artarken, fermente elma ve kestane kabuğu ekstraktı ilave edilen yoğurtlarda ise 6 kat artış sağlanmıştır. En yüksek DPPH aktivitesi fermente kestane kabuğu ekstraktı ilave edilmiş yoğurt örneklerinde saptanırken ve bunu fermente elma kabuğu eklenmiş örnekler takip etmiştir.

1. INTRODUCTION

Large amounts of food waste arise during production, preparation and consumption of foods. Thereby, reducing or valorization of food waste is an important issue from ecological and economic point of view. Food wastes can create environmental pollution and loss of natural resources if discarded. Most of these residues have a nutritional potential therefore they are used as feed. Some residues such as agroindustrial by-products can be used for production of fiber and food ingredients. In recent studies, bioactive compounds such as organic acids, phenolic compounds, proteins, vitamins and enzymes have been extracted from these by-products (Graminha et al, 2008). Production or enhancement of bioactive compounds by fermentation of food waste can be an alternative to chemical synthesis methods.

Phenolics are commonly found in plant-based foods like fruits, vegetables, cereals, olive, legumes, nuts, coffee and tea that could be used as natural food ingredients and nutraceuticals (Shahidi and Yeo, 2016). Simple phenolic acids and flavonoids are the most common phenolic compounds and they are present in soluble free, soluble conjugated and insoluble or bound forms in plants. Processing of plants to separate edible parts yield high amount of waste that could have potential for production of bioactive compounds. Phenolics in the bound form are covalently bound to structural components of cell wall such as pectin, cellulose, hemicellulose, lignin, arabinoxylan and structural proteins. It is difficult to release of bound phenolics from structural components with conventional solvent extraction; therefore, acid and alkali-assisted extraction of phenolics from plant wastes has been generally applied. However, usage of these techniques causes several drawbacks which limit their applications in practice. They can cause degradation of phenolic compounds and create safety hazards in final products. Another technique is to use cell-wall degrading enzymes to break down the cell wall matrix resulting in the release of bound phenolic compounds. The main limitation for the application of enzymes is their high cost (Gonzales et al, 2016). Microbial fermentation can also be way to release bound phenolics from plant wastes.

However, in the literature, there are limited number of detailed studies about the antioxidant production via microbial fermentation of plant wastes.

Fermentation has traditionally presented a low cost, straightforward practice to preserve food and to yield foodstuff with high nutritional value (Verotta et al, 2018). Microorganisms have been used for the production of some foods (dairy, fish and meat products) and alcoholic beverages. In addition, several products of microbial fermentation are also incorporated into food as additives and supplements including antioxidants, flavors, colorants, preservatives and sweeteners. There is a great interest in the production and use of natural food additives derived from microorganisms, since they are more desirable than the chemically synthesized ones by consumers (Couto and Sanroman, 2006).

Bioprocesses by microorganisms has been explored to improve extraction of phenolic compounds from agroindustrial by-products and their antioxidant activity. Microorganisms can degrade cell-wall matrix and/or bioconvert of released compounds by their enzymes including cellulase, tannase, pectinase, β-glucosidase, naringinase, α-rhamnosidase and hesperidinase (Huynh et al, 2018). Fermentation of agroindustrial by-products could be a promising strategy for releasing or producing of phenolic compounds.

1.1 Purpose of Thesis

The objective of this thesis was to produce phenolic antioxidants by fermentation with *Aspergillus* spp. from agroindustrial wastes. For this purpose, several steps has been carried out: 1) Black *Aspergilli* was isolated and identified from rotten fruit and vegetables; 2) The hydrolytic enzyme production ability of isolated *Aspergillus* spp. was investigated; 3) The chemical composition and phenolic content of agroindustrial by-products were determined to select waste with potential; 4) *Aspergillus* spp. were screened to determine the best isolate for phenolic production on wastes by solid-state fermentation; 5) Fermentation conditions were optimized to obtain the highest concentration of phenolic compounds from wastes; 6) Produced phenolic extracts from fermented wastes were used for fortification of yoghurt samples in terms of phenolic compounds and antioxidant activity.

2. LITERATURE REVIEW

2.1 Phenolic Compounds and Their Antioxidant Acitivity

2.1.1 Structure and classification of phenolic compounds

Phenolics are a class of organic compounds possessing one or more aromatic rings with one or more hydroxyl groups produced mainly through the shikimic, malonic, and mevalonic acid pathways, as well as the methylerythritol phosphate pathway. They are distributed in the plant and synthesized as secondary metabolites for protection against UV radiation, competitive warfare against viruses, bacteria, insects and other plants as well as responsible from smell, color and flavor of plant products. They are proven as potent natural antioxidants with different functional properties such as anti-inflammatory, anticancer, antimicrobial, antiallergic, antiviral, antithrombotic and hepatoprotective activities (Kumar and Goel, 2019; Rosa et al, 2019; Tsimogiannis and Oreopoulou, 2019).

Phenolic compounds can be divided into 4 general groups: Phenolic acids (gallic, protochatechuic, caffeic, and rosmarinic acids), flavonoids (quercetin and catechin), tannins and the less common stilbenes and lignans. The flavonoids are described based on whether C_3 bridge is open or forms a third heterocyclic ring (ring C). Thus, the flavonoid family are divided into six groups: Flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins, according to the oxidation state of the central C ring. Some of the most common flavonoids include quercetin (onion, broccoli, apple), catechin (tea), naringenin (grapefruit), cyanidin-glycoside (berry fruits), daidzein and genistein (soybean). Figure 2.1 shows examples of the most common naturally occuring flavonoids (Tsimogiannis and Oreopoulou, 2019).

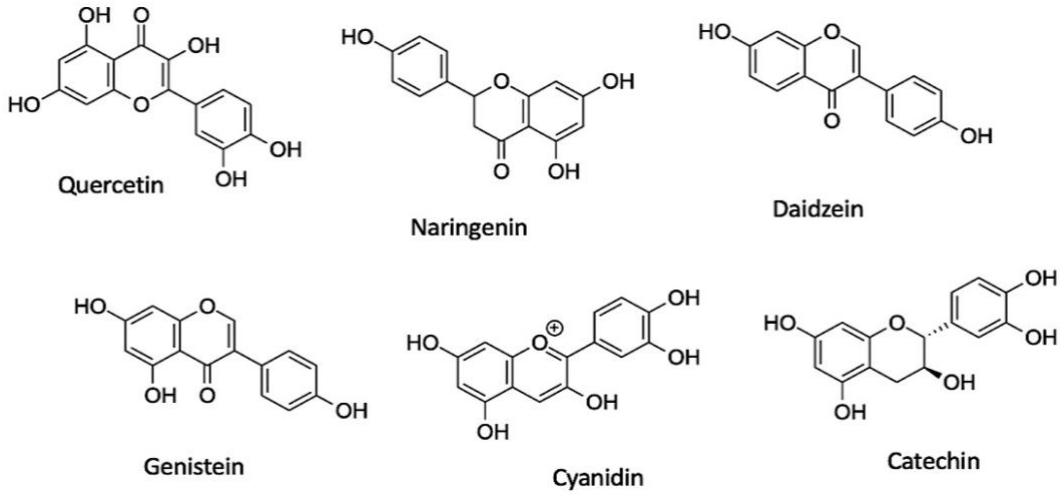


Figure 2.1 : Chemical structure of some flavonoids.

Phenolic acids generally describe the phenolic compounds having one carboxylic acid group. Phenolic acids can be divided into two subgroups according to their structure: The hydroxybenzoic and hydroxycinnamic acids (Figure 2.2). Hydroxycinnamic acids, derived from cinnamic acid, present in foods often as simple esters with quinic acid or glucose. On the other hand, hydroxybenzoic acids possess a common structure of C_6-C_1 and derived from benzoic acid. They are found in soluble form (conjugated with sugars or organic acids) or bound with cell wall fraction such as lignin. The most commonly found hydroxybenzoic acids include gallic, protocatechuic, vanillic and syringic acids, while hydroxycinnamic acids are caffeic, ferulic, *p*-coumaric and sinapic acids (Dai and Mumper, 2010; Kumar and Goel, 2019).

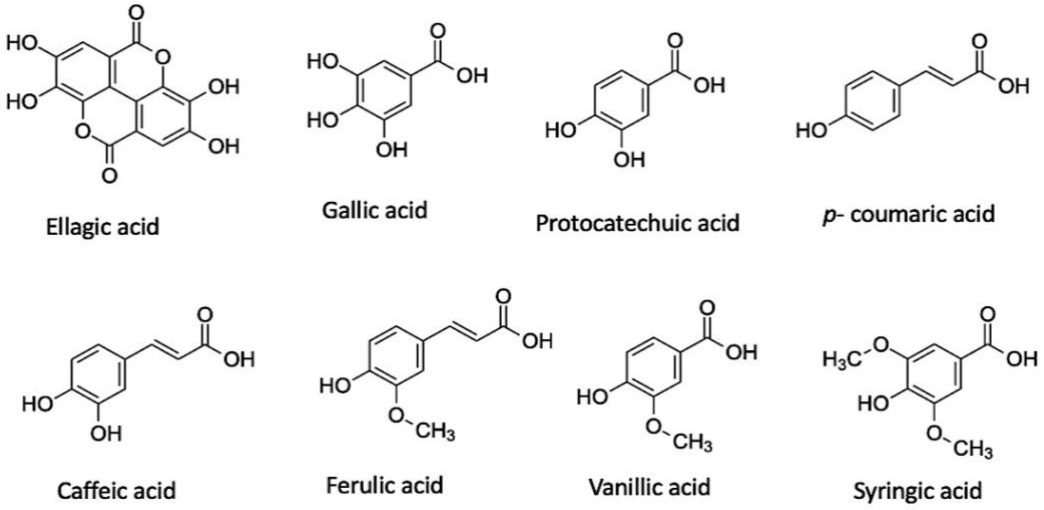


Figure 2.2 : Chemical structure of some phenolic acids.

Tannins, the relatively high molecular weight compounds, which constitute another major group of polyphenols are usually divided into two groups: Hydrolyzable tannins and condensed tannins. Hydrolysable tannins are compounds containing a central core of glucose esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, also called ellagitannins. Hydrolyzable tannins, including tannic acid, can be acid- or enzymatically hydrolyzed to yield glucose and gallic acid. Condensed tannins are oligomers or polymers of flavan-3-ol linked through an interflavan carbon bond and they are not hydrolyzed under physiological digestive conditions, but hydrolyzed by severe acid or alkaline treatment. They are also refered to as proanthocyanidins because they are decomposed to anthocyanidins through acid catalyzed oxidation reaction (Dai and Mumper, 2010; Swanson, 2003).

2.1.2 Antioxidant properties

Oxidation is essential for many living organisms for the production of energy, necessary for biological processes. Oxygen-centered free radicals, also known as reactive oxygen species (ROS), including superoxide, hydrogen peroxide, hydroxyl (HO.), peroxyl (ROO.) and alkoxyl (RO.) radicals, are produced *in vivo* during oxidation. They play important roles in cell signalling, apoptosis, gene expression and ion transportation. However, when these ROS are generated in excess or cellular defences are deficient, biomolecules including protein, lipid and nucleic acids can be damaged by oxidative stress processes. Therefore, oxidative damage play an important role in the degenerative or pathological diseases in humans, such as aging, cancer, heart diseases, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, cataract and inflammation (Babbar et al, 2011; Dai and Mumper, 2010).

Most living organisms possess enzymatic and non-enzymatic antioxidant defence and repair systems that protect them against oxidative stress. However, these native antioxidative systems are generally not enough to protect the living organisms from oxidative damage. Ingestion of antioxidative supplements, or foods containing antioxidants, may reduce the oxidative damage in the human body (Li et al., 2012; Wang et al, 2006).

Phenolics have different biological functions such as cell wall reinforcement, antimicrobial activity, plant growth regulator, defense signaling in plants and scavengers of reactive oxygen species. Phenolic compounds exhibiting a wide range

of beneficial health effects due to their ideal structure for free radical scavenging activities. Antioxidant properties of phenolic compounds can be mediated by the following mechanisms: Scavenging radical species, suppressing radical formation by inhibition of some enzymes or chelating trace metals involved in free radical production, up-regulating or protecting antioxidant defence (Dai and Mumper, 2010). Phenolic compounds inactivate free radicals according to the hydrogen atom transfer and to the single electron transfer mechanisms. Another antioxidant mechanism arises from the possibility that transition metals ions may be chelated by phenols leading to stable complexes (Leopoldini et al, 2011).

Many synthetic compounds, such as butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroquinone (TBHQ), are commonly used as antioxidant in processed foods. However, synthetic antioxidants are being restricted due to their possible toxic effects such as liver damage and carcinogenicity. Due to these health safety concerns, utilization of naturally occuring antioxidants have gained increasing demand. They could improve food quality, terminate free radical chain reactions in biological systems and provide additional health benefits to consumers (Arora and Chandra, 2011).

2.2 Phenolic Compounds from Agro-Industrial By-Products

One-third of food are wasted around 1.3 billion tons as a result of human consumption in the world per year according to the Food and Agriculture Organization of the United Nations (FAO, 2011). This includes 45% of all fruits and vegetables which have the potential to be a valuable co-product. Processing of fruits and vegetables leaves behind a substantial amount of residues in the form of peels, seeds and pomace. These residues have a high amount of valuable food components such as fiber, vitamins, protein, pigments, minerals, hydrocolloids, phenolics and other bioactive components. Canning and freezing processes of fruits and vegetables generate 6 million metric tons of leaves, stalks and stems annually (Laufenberg et al, 2003).

2.2.1 Phenolic profile of apple wastes

Apple and apple products are one of the most commonly consumed fruit products all over the world. Apple is mostly processed into jam, juice and jelly. Apple by-products including peel and pomace are waste from the apple processing industry and they constitute approximately 25% of the original fruit. This is a great volume of residue to be disposed which is highly biodegradable causing serious environmental pollution. Valorization processes can be applied to produce high-value compounds from apple waste instead of using it as animal food or discarding as waste (Berovic and Ostroversnik, 1997).

Apple pomace is a heterogeneous mixture consisting of peel, core, seed, stem and soft tissue. It is an excellent substrate for bioprocesses in point of its high-water content and composition containing polysaccharides such as cellulose, hemicellulose, and lignin. It is rich in galacturonic acid, arabinose, galactose with minor amounts of rhamnose, xylose and glucose, as well as small amounts of minerals, proteins and vitamins. These wastes are rich in lignocellulosic material, especially apple pomace is a natural source of pectic substances. It has been suggested that apple by-products can be used for the production of high value compounds such as enzyme, protein, organic acids, ethanol, aroma compounds, natural antioxidants and pectin (Dongowski and Sembries, 2001; Göğüş et al, 2015).

Fibers are largely composed of complex carbohydrates that are somewhat resistant to digestion. Fruit and vegetable fibers have better quality than other dietary fibers due to the presence of associated bioactive compounds, such as flavonoids, polyphenols and carotenes (Figuerola et al. 2005). Apples are good sources of fibre with a well-balanced proportion between soluble and insoluble fraction. The total fiber content of apple peel and pulp varies from 33.4 to 51.9% (Albuquerque et al, 2006; Sato et al, 2010; Sudha et al, 2007) and includes cellulose (6.8 to 23.2%), lignin (6.4 to 23.5%), pectin (3.5% to 25.0%), and hemicellulose (4.1 to 6.2%) (Kennedy 1999; Shalini and Gupta, 2010).

Apple contains several bioactive phenolic compounds in the flesh part as mainly 5-*O*-caffeoylquinic acid, procyanidin B2 and (−)-epicatechin. Apple peel has been shown to be a good source of polyphenols. Major phenolic compounds isolated and identified in apple peel are catechins, hydroxycinnamates, phloretin glycosides, quercetin glycosides and procyanidins (Marks et al, 2007). The by-products of apple processing

such as pomace and peel might be possibly used as a source of phenolic compounds (Dhillon et al, 2012; Gullon et al, 2008; Hernández-Carranza et al, 2016; Madrera et al, 2015). Lee et al. (2017) identified hydroxybenzoic acids, hydroxycinnamic acids, hydroxyphenylacetic acid and hydroxyphenylpropanoic acid in apple pomace.

2.2.2 Phenolic profile of pomegranate wastes

Pomegranate is an important fruit crop adaptable to a wide range of agro-climatic conditions. The production and consumption of pomegranate has sharply increased with the increased awareness of people about its superior therapeutic properties. It is an antioxidant-rich fruit containing many important bioactive compounds known to provide health benefits. This fruit is consumed directly as fresh seeds as well as fresh juice which can also be used in beverages for jellies, and flavoring and coloring agents. The edible part of the fruit contains considerable amount of acids, sugars, vitamins, polysaccharides, polyphenols and important minerals (Maskan, 2006). Pomegranate seeds are rich source of crude fiber (35.5%) and pectin (6.0%) (El-Nemr et al, 1990). Total lignin content of pomegranate peel was found as 29.4% (Pathak et al, 2017). In fact, the non-edible parts of fruit and tree (i.e. peel, seeds, flowers, bark, buds and leaves), contain even higher amounts of specific nutritionally valuable and biologically active components as compared to the edible fruit (Akhtar et al, 2015).

Pomegranate peel which accounts for about 30-40% of fruit weight is characterized by the presence of high molecular weight phenolic compounds, ellagitannins, proanthocyanidins, complex polysaccharides, flavonoids and appreciable quantities of microelements that exhibit strong anti-mutagenic, antioxidant, antimicrobial and apoptotic properties. The fruit contains a rich variety of flavonoids, constituting nearly 0.2–1.0% of the fruit weight; approximately 30% of all fruit anthocyanidins are concentrated in the peel portion. The concentration of these compounds depends on the cultivar type and on the various developmental phases of the fruit, and is responsible for the variations in pomegranate peel color (Akhtar et al, 2015).

Phenolic acids including chlorogenic, caffeic, syringic, sinapic, *p*-coumaric, ferulic, vanillic, ellagic, gallic and cinnamic acid which have been identified in pomegranate peel. Phenolic profiles and their concentrations vary amongst the pomegranate cultivars grown at different geographical conditions. Pomegranate peel is also a potential source of flavonoids such as catechin, epicatechin, quercetin, anthocyanins

and procyanidins. Flavonoid composition changes with the fruit developmental stages and the concentration of flavonols and flavones in pomegranate peel. Pomegranate peel is a rich source of ellagitannins and ellagic acid derivatives such as punicalagin (hexahydroxydiphenoyl (HHDP)-gallagyl-hexoside), punicalin (gallagyl-hexoside), ellagic acid hexoside and ellagic acid pentoside (Singh et al, 2018). Punicalagin was reported as the major soluble phenolic of pomegranate husk which was also identified in pomegranate juice as a result of processing (Aloqbi et al, 2016; Gil et al, 2000). Gallic acid, catechin, epicatechin, ellagic acid and other hydrolyzable tannins were also identified in soluble form in pomegranate peel in these studies.

Pomegranate seed as a by-product of pomegranate processing is about 20% of the whole fruit. Recent studies found that pomegranate seed may have the potential to be a good source of nutrients and antioxidants. The beneficial effects of pomegranate seeds may be related to presence of a variety of biologically active compounds, particularly polyphenols, which have been studied for their antioxidative effects. Significant levels of phenolic content were detected in pomegranate seeds in early studies. 3,4-Dihydroxybenzoic, 4-hydroxybenzoic, syringic, vanillic and ferulic acids were detected in the seed flours of the four tested pomegranate cultivars by Jing et al. (2012). Ambigaipalan et al. (2017) reported phenolic acids, flavonoids, hydrolysable tannins and anthocyanins in soluble form in pomegranate seed. He et al. (2011) also identified caffeic acid, pedunculagin, procyanidin dimer and trimer, catechin, *p*-coumaric acid, quercitrin, kaempferol and ferulic acid in pomegranate seed.

2.2.3 Phenolic profile of chestnut shell

The chestnut is a local crop in Turkey and according to data of Ministry of Agriculture and Forestry (TUIK, 2018) chestnut fruit are produced around 63,000 tone/year. Roughly half of the production is consumed as fresh and the other half is used in food industry to produce several products such as frozen fruit, chestnut pure or flour, chestnut in syrup. Most of the by-products generated from chestnut processing industry are bur and shell. The shell is separated during peeling process in the food factory and represents around 10% of whole fruit and it is generally used as fuel. Chestnut bur generates after fruit harvesting and it remains in the woodland. Based on their phenolic composition, the valorization of this type of lignocellulosic wastes could be an interesting option as sources of natural antioxidants and also would improve its industrial processing both economically and environmentally (Vazquez et al, 2012).

Several studies showed that presence of bioactive molecules in the chestnut waste have some positive effects on human health. The chestnut fruit and its by-products have been studied to determine their composition and antioxidant activity. The antioxidant potential of chestnut leaves (Calliste et al, 2005), antioxidant activity of chestnut flowers, leaf, skins and fruit (Barreira et al, 2008), the phenolic composition of chestnut fruit pericarp and integument (Vasconcelos et al, 2010), the antioxidant properties of chestnut shell (Vazquez et al, 2008), and antioxidant activity of chestnut bur (Vazquez et al, 2012) have been previously evaluated. Squillaci et al. (2018) reported that gallic acid was the most abundant phenolic compound identified in aqueous soluble extract of chestnut shell followed by protocatechuic acid, ellagic acid, epicatechin, catechin, *p*-coumaric acid and scopoletin.

2.2.4 Phenolic profile of black carrot pomace

Black carrot has an attractive bluish-purple color with high levels of anthocyanins which plays a significant role in prevention of diseases such as coronary hypertension, obesity, diabetes, cancer and heart disease. Like many fruit and vegetables, black carrots are seasonal and perishable and difficult to preserve as raw material. Black carrot is an industrial crop mainly processed for the production of anthocyanin-rich concentrate for pigment industry. They are also processed into various products such as juice, concentrate, jam and shalgam. As a result of processing, large amounts of byproducts including peel and pomace are generated (Kamiloglu et al, 2016).

Large scale processing of black carrot generates huge amount of pomace, disposal of which is a major concern as high organic matter and moisture content of pomace makes it highly susceptible to microbial degradation. Despite the extraction of high amounts of polyphenols and anthocyanin in juice, black carrot pomace is still left with significant amounts of residual polyphenolic compounds with high bioactivity, which can be put into use in various industries especially food and nutraceutical industries (Kumar et al, 2019). The major black carrot anthocyanins were detected as cyanidin-based containing different sugars non-acylated or acylated with sinapic, ferulic or coumaric acid (Agcam et al, 2017). Kamiloglu et al. (2016) found five cyanidin derivatives and three chlorogenic acid derivatives, ferulic and caffeic acid in black carrot pomace.

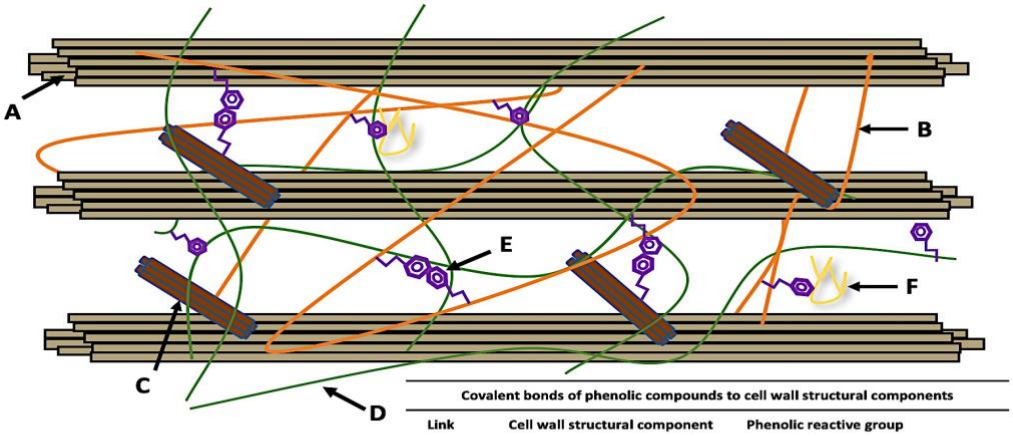
2.3 Bound Phenolics In Foods

Phenolic compounds are ubiquitous in plants, seeds and skins are especially rich sources of phenolics, probably because of the role they play in protecting the fruit and the seed to ensure healthy propagation of the species (Vattem and Shetty, 2002). Flavonoids account for approximately two-third of dietary phenols and they are mostly present as glycosides, and partly as esters, rather than as free compounds. When flavonoids are linked to sugar molecules they are known as flavonoid glycosides, and when they are not they are called aglycones. The antioxidant capacity of flavonoids is directly affected by the degree of glycosylation. Usually, the aglycone forms are more active than the glycoside form (Munoz et al, 2017). Phenolic acids are the second most important group of phenolics, which account for most of the remaining third of the dietary polyphenols, and are mostly found in plants as bound to the cell-wall components. The third class of polyphenols are tannins that mostly present as phenolic polymers (Munoz et al, 2017).

Fruit and vegetables have most of their phenolics in free or soluble conjugated forms. On the other hand, bound phenolics comprise an average of 24% of the total phenolics present in these food matrices (Acosta-Estrada et al, 2014). The major portion of phenolic compounds in grains existed in the bound form (85% in corn, 75% in oats and wheat, and 62% in rice) (Adom and Liu, 2002). Bound phenolics of vegetables, mostly in ester forms, are associated with cell wall components. The distribution of bound phenolic compounds in edible part of some vegetables are as onion (9.7%), spinach (12.5%), broccoli (20%), lettuce (20.8%), cucumber (26.2%), cabbage (32.9%), carrot (37.6%), potato (39.9%) (Chu et al, 2002). Bound phenolic percentage in edible part of some fruits are as apple (8.2%), red grape (9.5%), lemon (19.1%), peach (22.8%), banana (37.9%), and grapefruit (38.1%) (Acosta-Estrada et al, 2014; Chu et al, 2002; Sun et al, 2002; White et al, 2010). Overleaping of bound phenolics causes underestimation of total phenolic contents and antioxidant activities of fruits and vegetables. Although, the presence of soluble phenolic compounds and their antioxidative activity have been reported in agro-industrial wastes, detailed information about the bound phenolics and their antioxidant capacity is limited.

In numerous *in-vitro* antioxidant assays carried out, the bound phenolics have been demonstrated significantly higher antioxidant activity compared to that of soluble phenolics (Acosta-Estrada et al, 2014). Furthermore, bound phenolics may survive under conditions of human stomach and small intestine and reach the colon intact where they were released and exert bioactivity (Chu et al, 2002). In the food industry, waste is produced after separation of the desired component or product from undesired components. The direct disposal of undesired components as a waste to the environment represents an important loss of biomass which could be converted into different products with a higher commercial value. In recent years, extraction of phenolics and other food components from agri-industrial wastes has gained great attention because they could be cheap and safe sources of natural food supplements and ingredients.

Some phenolics exist in conjugated forms mostly with sugars as glycosides and other moieties in plants. Glycoside of phenolic compounds are soluble that can be extracted with a solvent. However, there are also insoluble phenolics bound to the components of cell-wall such as cellulose, hemicellulose (e.g. arabinoxylans), lignin, pectin and structural proteins (Figure 2.3) (Acosta-Estrada et al, 2014; Röpenack et al, 1998). Phenolic acids are linked to the lignin with ether linkages through their hydroxyl groups and structural carbohydrates and proteins are linked with ester linkages through their carboxylic group (Munoz et al, 2017).



Covalent bonds of phenolic compounds to cell wall structural components

Link	Cell wall structural component	Phenolic reactive group
Ether	Lignin	Hydroxyl groups in the aromatic ring
Ester	Structural carbohydrates Proteins	Carboxylic group

Figure 2.3 : Representations of primary cell wall structure of plant material and cross-linking between structural components and phenolic compounds. (A) cellulose, (B) hemicellulose, (C) structural proteins, (D) pectin, (E) phenolic acids, (F) lignin.

In nature, phenolic acids occur mostly in the insoluble or bound forms whereas flavonoids present as glycosides with a single or multiple sugar moieties linked through an OH group (O-glycosides) or through carbon–carbon bonds (C-glycosides) (Kalinowska et al, 2014). This conjugation occurs via the hydroxyl groups of the phenolics reduces their ability to function as good antioxidants since availability of free hydroxyl groups on the phenolic rings is important for resonance stabilization of free radicals. Lowered antioxidant capacity has direct implications on decreasing health functionality when these phenolics are ingested via food or nutraceuticals. Therefore, if phenolics are released from their glycosides or other conjugates then the antioxidant and thus health functionality of these phytochemicals could be improved (Vattem and Shetty, 2002).

2.4 Release of Bound Phenolics

Phenolic compounds are commonly extracted by organic solvents (liquid-liquid/solid-liquid) from plant materials employing various conventional extraction methods such as maceration, Soxhlet extraction, supercritical fluid extraction, high hydrostatic pressure extraction, ultrasound and microwave-assisted extraction etc. However, most of the phenolic compounds cannot be extracted efficiently using these conventional extraction methods alone as they mainly exist as insoluble bound or conjugated with cell wall components through ester, ether or glycosidic bonds. Complete release of bound phenolics is possible by acid or alkali hydrolysis. Hot acidic conditions lead to degradation of cinnamic acid derivatives, *p*-coumaric acid, caffeic acid and ferulic acids. Enzymatic extraction of phenolics by different cell wall degrading enzymes such as pectinases, cellulases, tannase, amylases, hemicellulases and glucanases are also utilized to release phenolic compounds from cell wall complexes (Bhanja Dey et al, 2016; Chamorro et al, 2012; Kalinowska et al, 2014). These enzymes play a role in disrupting the cell wall and helping the polyphenol extraction.

Enzymatic extraction process is not cost effective as it requires multiple steps including multi-enzymes production and then application for phenolic extraction. Extraction/production of bioactive compounds by fermentation is an interesting alternative, since it is able to provide cost-effective and environmentally friendly by precluding any toxicity associated with the organic solvents (Martins et al, 2011).

Submerged and solid-state fermentation (SSF) are two cultivation systems for the exploitation of microorganisms. In submerged systems, microorganisms are cultivated in a liquid growth medium containing essential nutrients. SSF process is generally defined as microbial growth on moist solid substrate in absence or near absence of free liquid. SSF has gained attention due to the several advantages over submerged fermentation system, including high volumetric productivity, relatively higher concentration of the products, less effluent generation, simpler technique and lower cost. SSF resembles the natural habitat for several microorganisms (Couto and Sanroman, 2006; Shabbiri et al, 2012). In fermentation, various extracellular enzymes can be produced and simultaneously utilized for the release of phenolics from the matrix of medium in a single process, along with bioproduction of new phenolic compounds by secondary metabolism of microorganisms.

2.4.1 Production and extraction of bound phenolics by fermentation

The recovery of bioactive compounds particularly phenolics from agro-food by-products has attracted increasing attention in the past years, and studies have been conducted to valorize these residues. The valorization by fermentation process of wastes can provide economical benefits (Giraffa and Carminati, 2012). Fermentation of food matrices by microbial cultures enables the liberation of polyphenols, such as flavonoids and other bound phenolics, from storage cells, which leads to their higher extractability and/or oxidation into other products/metabolites. In addition, fermentation process using suitable substrates or appropriate nutrients and with the right microbial species can be targeted and developed to increase the synthesis of bioactive microbial metabolites with diverse functional benefits (Munoz et al, 2017).

The selection of suitable substrates for SSF has been mainly centred around agro-industrial residues due to their potential advantages for filamentous fungi, which are capable of penetrating into the hardest substrate. In a SSF, the solid substrate both supplies the nutrients to the microbial culture growing in it and serves as an anchorage for the cells. The major factors that affect microbial synthesis in a SSF system include: Selection of suitable substrate and microorganism, pretreatment of the substrate, particle size of the substrate, water content and water activity of the substrate, relative humidity, type and size of inoculum, control of temperature of fermenting matter/removal of metabolic heat, period of cultivation, maintenance of uniformity in the environment of SSF system, and the gaseous atmosphere, i.e. oxygen consumption

rate and carbon dioxide evolution rate (Couto and Sanroman, 2006; Pandey et al, 1999). SSF can be used for the production of some industrially important phenolics, for the improvement of antioxidant potentials of solid substrates by increasing total phenolic content as well as for the enhancement of bioavailability of the phenolic compounds. Besides increasing the antioxidant activity of certain foods, bioconversion of phenolic compounds by SSF may also promote other bioactivities which could improve human health (Bhanja Dey et al, 2016).

Agricultural or forestry refuses including cereal and vegetable wastes such as straw, bagasse, stover, cobs, husks, among others, are lignocellulosic materials are composed mainly cellulose, hemicellulose and lignin. The lignin fraction in these materials contains numerous phenolic components, mainly phenolic acids such as ferulic, *p*-coumaric, syringic, vanillic and *p*-hydroxybenzoic, which can be recovered by SSF. Filamentous fungi have ability to degrade lignin since they produce large amount of enzymes necessary to break down this structure. As fungi grow on these agro-industrial wastes they utilize the polysaccharides after lignin degradation in order to grow and reproduce. This has effect on increasing the nutritional value of the agro-industrial substrates which are generally low (Martins et al, 2011; Verotta et al, 2018).

The growing interest in the exploitation of flavonoids and phenolic acids from processed plant residues, has encouraged research on the fermentation processes to various by-products. Zheng and Shetty (2000) investigated the release of phenolic aglycons from cranberry pomace with β-glucosidase produced by *Lentinus edodes* under SSF. They reported that β-glucosidase produced by *L. edodes* could be used for phenolic extraction from cranberry pomace and flavor enrichment applications in fruit and wine industries. Jamal et al. (2011) studied the production of phenolic compounds from palm oil mill effluent by submerged fermentation using *Aspergillus niger*. They reported that the antioxidant activity of fermented extract was higher than that of the commercial synthetic antioxidant, BHT.

Vattem and Shetty (2002) observed an increase in the total extractable phenolic compounds in cranberry pomace via *Rhizophus oligosporus* fermentation. Both total phenolics and antioxidant capacity correlated well with the increase in the β-glucosidase activity showing that the enzyme may play an important role in the release of phenolic aglycones from cranberry pomace. Gonzales et al. (2016) investigated the incubation of flavonoid aglycones, naringenin and quercetin with *Rhizopus*

azygosporus resulted in the production of flavonoid glucosyl-, hydroxyl- and sulfo-conjugated derivatives. They found that naringenin was completely metabolized into eriodictyol sulfate and eriodictyol glucoside, whereas quercetin was partially metabolized into quercetin glucoside, diglucoside, sulfate and glucosyl-sulfate due to the action of β-glucosidase activity and also glucosylation or conjugation of flavonoids during fungal fermentation.

2.4.2 Biotransformation mechanisms of phenolic compounds by fermentation

When phenolic compounds occur as glycosides, esters or polymers, their antioxidant potential is reduced. For this reason, biotransformation during food processing or in the gastrointestinal tract plays an essential role in phenolic bioavailability. Bound forms of phenolic compounds can be released by microorganisms and gastrointestinal enzymes and absorbed in the gastrointestinal tract. In addition, there are several food processes that release of bound phenolics. These processes are fermentation and malting, as well as thermomechanical processes, such as extrusion, cooking and alkaline hydrolysis. Food bioprocesses, such as fermentation or enzymatic hydrolysis, could be an attractive method for enhancing functional activity of phenolic antioxidants by releasing free phenolics from their glycosides or other conjugates. Fermentation is preferred instead of the utilization of commercial enzymes to enhance the nutraceutical value of foods because it is relatively cheap (Munoz et al, 2017).

Microbial enzymes, such as glucosidase, amylase, cellulase, chitinase, inulinase, phytase, xylanase, tannase, esterase, invertase or lipase can break down plant cell walls or starch. These enzymes play a role in disintegrating the plant cell wall matrix and consequently facilitate phenolic extraction. The increasing in antioxidative activity of plant based foods can be explained by structural changes in phytochemicals as a result of fermentation (Munoz et al, 2017).

The biotransformation of phenolic compounds can be different by the action of different microorganisms. Current enzyme-mediated bioconversion reactions using whole-cell systems are esterification, decarboxylation, amination, halogenation, and hydroxylation. Esterification can be catalyzed by lipases and esterases, decarboxylation can be performed by whole-cell (mold, yeasts and bacteria) and enzymatic processes based on the reaction of phenolic decarboxylase. Several transaminases were employed in the amination of phenolic acid derivatives and also

these derivatives can be converted to halogenated compounds such as vanillin derivatives. Various enzymes like hydroxylase, monooxygenase was employed to hydroxylate phenolic compounds such as hydroxybenzoic acid, ferulic acid, and *p*-coumaric acid (Tinikul et al, 2018).

Phenolic degradation pathways are found in aerobic and anaerobic microorganisms. In O_2-dependent aromatic ring-cleavage, typical pathways begin with first addition of a hydroxyl group by a monooxygenase to form a dihydroxy product that can be cleaved by a dioxygenase. The next ring-breakage usually involves a non-heme dioxygenase which cleaves between the two hydroxyl groups or adjacent to the hydroxyl groups (Tinikul et al, 2018). Taira et al. (2018) proposed the phenolic biotransformation mechanism in *Aspergillus luchuensis* that involves a two-step process, i.e. the CoA ligases followed by a two-carbon elimination of ferulic acid produce vanilin and acetyl-CoA. Also, another vanilin production pathway derived from the vanillin glucoside was proposed. The following dehydrogenation of vanillin produced vanillic acid.

2.5 Microorganisms Used for The Production of Enzymes

A large number of microorganisms, including bacteria, yeast and mold produce different groups of enzymes. Generally, hydrolytic enzymes, e.g. cellulase, xylanases, pectinases, etc. are produced by fungal cultures, since such enzymes are used in nature by fungi for their growth (Pandey et al, 1999). The biodegradation potential of fungi is due to their rich supply of hydrolytic enzymes. Whole-cell *Aspergillus* are often used as catalysts for biodegradation of phenolics. Significant benefits of the biodegradation of phenolics are its simplicty and low costs of their production. Another benefit is presence of multiple enzymes whose concerted action may be more efficient than use of a single enzyme.

SSF holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source. In addition to the conventional applications in food and fermentation industries, microbial enzymes have attained significant role in biotransformations involving organic media, mainly for bioactive compounds (Pandey et al, 1999). Treatment with enzymes to enhance bioaccesibility and bioavailability of polyphenolic compounds, has been employed to release them from their matrices.

Besides pectinases, cellulases and glucanases, tannin acyl hydrolases (tannases) have a potential role in increasing the antioxidant capacity of the final product by hydrolyzing despite bonds and ester linkages of hydrolysable tannins or galloyl esters (Martins et al, 2016).

Table 2.1 summarizes some of the most recent studies by SSF with different microorganisms, produced phenolic compounds and solid supports employed. The enzyme β-glucosidase catalyzes the hydrolysis of glycosidic linkages. The enzyme is capable of hydrolyzing phenolic glycosides and releasing extractable free aglycones potentially having high antioxidant activity, therefore making them very useful for applications in food and beverage industries. Qi et al. (2009) showed that β-glucosidase which produced by *Aspergillus* spp. isolated from soil, can hydrolyze glycosides and increase free isoflavones in soy products.

Table 2.1 : Recent studies of solid-state fermentation using different microorganisms, enzymes and solid substrates for phenolic production.

Substrate	Microorganism	Enzymes	Phenolic compound	Reference
Black soybean	*Rhizopus oligosporus*, *Rhizopus oryzae*	β-glucosidase	Daidzein, genistein	Cheng et al. (2013)
Cranberry pomace	*R. oligosporus*	β-glucosidase	Ellagic acid	Vattem and Shetty (2002)
Cranberry pomace	*Lentinus edodes*	β-glucosidase	Gallic acid, chlorogenic acid, *p*-hydroxybenzoic acid and *p*-coumaric acid	Zheng and Shetty (2000)
Caesalpinia digyna seed cover	*R. oryzae*	Tannase	Gallic acid	Kar and Banerjee (2000)
Green coconut husk	*Phanerochaete chrysosporium*	Lignolytic enzymes	Vanillin	Barbosa et al. (2008)
Grape pomace and wheat bran	*A. niger* 3T5B8	β-glucosidase, xylanase, polygalacturonase	Phenolic compounds	Teles et al. (2017)
Oat	*Aspergillus oryzae, A. niger*	Cellulose and xylan-degrading enzymes	Caffeic acid, ferulic acid	Cai et al. (2012)
Oryza sativa	*Aspergillus awamori* *A. oryzae*	α-amilaz	Phenolic compounds	Sadh et al. (2017)
Rice-koji	*Aspergillus luchuensis*	β-glucosidase	Vanillin, ferulic, vanillic acid	Taira et al. (2018)
Pomegranate husk	*A. niger*	Tannase	Ellagic acid	Aguilar et al. (2008)

Table 2.1 (continued): Recent studies of solid-state fermentation using different microorganisms, enzymes and solid substrates for phenolic production.

Substrate	Microorganism	Enzymes	Phenolic compound	Reference
Pomegranate seed and husk	*Aspergillus niger* GH1 and PSH	Ellagitannase	Ellagic acid	Robledo et al. (2008)
Soy whey	*Lactobacillus plantarum*	β-glucosidase	Daidzein, genistein	Xiao et al. (2015)
Soybean	*Bacillus pumilus* HY1	β-glucosidase and esterase	Flavanols, gallic acid	Cho et al. (2009)
Soybean flour	*A. niger, Aspergillus niveus, A. awamori*	β-glucosidase	Phenolic acids and flavonoids	Georgetti et al. (2009)
Soy germ	Lactic acid bacteria	β-glucosidase	Phenolic acids, flavonoids, saponins, phytosterols and tocopherols	Hubert et al. (2008)
Soybean seeds	*A. oryzae, Rhizophus oryzae, Bacillus subtilis*	Glucosidase	Phenolic acids and flavonoids	Duenas et al. (2012)
Soybean tempeh	*Rhizopus azygosporus* ATCC 48108	β-glucosidase	Eriodictyol sulfate and glucoside, quercetin glucoside, diglucoside, sulfate and glucosylsulfate	Gonzales et al. (2016)
Tar and creosote bush	*A. niger* GH1	Tannase	Gallic acid, pyrocatechol	Ventura et al. (2009)
Valonea tannins	*A. niger and Candida utilis*	Tannase, polyphenol oxidase	Ellagic acid	Shi et al. (2005)
Wheat bran	Lactic acid bacteria and yeast	Ferulic acid esterase and xylanase	Ferulic acid, caffeic acid, sinapic acid	Savolainen et al. (2014)

2.5.1 Cellulase

Cellulase is the general term for a group of enzymes consisting of endo-(1,4)-β-D-glucanase (EC 3.2.1.4), exo-(1,4)-β-glucanase (EC 3.2.1.91) and β-D-glucosidase (EC 3.2.1.21). The exoglucanase acts on the ends of the cellulose chain and releases β-cellobiose as the final product; endoglucanase randomly attacks the *O*-glycosidic bonds, resulting in glucan chains of different lengths; and the β-glycosidases act specifically on the β-cellobiose disaccharides and produce glucose. These enzymes are used in feed, fuel and chemical industries for the processing of lignocellulosic materials (Botella et al, 2005; Kuhad et al, 2011).

Cellulose is a fibrous, tough, water-insoluble substance, which is found in the protective cell walls of plants, particularly in stalks, stems, trunks and all woody portions (Osullivan, 1997). Many plant cell walls of commercial importance are made up of three layers: Middle lamella, primary cell wall and secondary cell wall. The secondary cell wall is further subdivided into three sub-layers and these sub-layers present in the cell wall have two phases: Microfibrilar and matrix phase. The microfibrilar phase (crystalline phase) is composed of microfibrils of cellulose and the matrix phase (noncrystalline phase) is composed of a variety of polysaccharides (pectins and hemicelluloses), proteins and phenolic compounds (lignin, ferulic acid, coumaric acid, and others) (Festucci-Buselli et al, 2007).

There are a large variety of microorganisms involved in cellulase production including aerobic and anaerobic bacteria, white rot and soft rot fungi and anaerobic fungi. In filamentous fungi, actinomycetes and aerobic bacteria, are mostly secreted cellulases as free molecules. Most of the cellulases exploited for industrial applications are obtained from filamentous fungi such as *Trichoderma, Penicillium, Aspergillus, Fusarium, Humicola, Phanerochaete,* etc. (Kuhad et al, 2011; Singhania et al, 2010). *Trichoderma reesei* is the most efficient producer of endo- and exo-glucanases, but *Aspergillus* spp. are more efficient than *Trichoderma reesei* to excrete of β-glucosidase (Bansal et al, 2012).

In food industry, cellulases have been used for releasing of antioxidants from fruit and vegetable pomace; improvement of yields in starch and protein extraction; improving maceration, pressing, and color extraction of fruits and vegetables; clarification of fruit juices; improving texture and quality of bakery products; improved viscosity of fruit

purees; improving texture, flavor, aroma, and volatile properties of fruits and vegetables; controlling bitterness of citrus fruits. Cellulases can also be used to increase efficiency of malting and mashing; improving of pressing and color extraction from grapes; to improve of aroma, clarification process and filtration rate of vines; improving of fermentation and quality of beer; improved viscosity and filterability of wort in beer production (Dhillon et al, 2012; Kuhad et al, 2011).

2.5.2 Tannase

Tannase (tannin acyl hydrolyase, E.C.3.1.1.20) is an important enzyme and extensively used in many industrial applications. The enzyme tannase is an inducible enzyme and it catalyzes the hydrolysis of ester linkages in hydrolysable tannins, such as tannic acid, methyl gallate, ethyl gallate, n-propyl gallate and isoamyl gallate, to release glucose and gallic acid (Sariözlü and Kivanç, 2009; Lal and Gardner, 2012). It has been reported that tannase is responsible for gallotannin and ellagitannin hydrolysis producing gallic acid and ellagic acid, respectively (Batra and Saxena, 2005; Lal and Gardner, 2012; Muslim et al, 2015).

Tannins are naturally occurring water-soluble polyphenols with a molecular weight of 0.5–3 kDa (Bhardwaj et al, 2003). Tannins are the second most abundant group of plant phenolic compounds. One of the major characteristics of tannins is their ability to form strong complexes with protein and other macromolecules such as starch, cellulose, and minerals (Marco et al, 2009). Tannins are phenolic compounds, which are divided into two main groups: Hydrolyzable tannins (gallotannins, ellagitannins) and condensed tannins (Mahapatra et al, 2005). Tannins are present in many different parts of plants, such as pine (*Pinusdensiflora*), eucalyptus (*Eucalyptus camaldulensis*), cinnamon (*Cinnamomum cassia*) and oak (*Quercus rubra*) tree galls, peels of pomegranate (*Punicagranatum*) fruits, fruits of date *(Phoenix dactylifera)* tree, black cumin (*Nigella sativa*), grape (*Vitis vinifera*) as well as in many tropical plants (Muslim et al, 2015).

Tannase producers have been identified among bacteria, fungi, yeasts and plant sources. Tannase can be obtained from tannin-rich plant sources, such as myrobalan (*Terminalia chebula*) fruits, divi divi (*Caesalpinia cariaria*) pods, dhawa (*Anogeissus latifolia*), konnam leaves (*Cassia wstula*), badul (*Acacia arabica*), avarum (*Cassia auriculata*) trees and gilo seeds (*Caesalpinia digyna*) (Trevino-Cueto et al, 2007).

However, the most important amount of industrial tannase is obtained from microbial production, as these enzymes are more stable than plant sources (Podrigues et al, 2007). Many researches proved that fungi are major producers of tannase, in which *Aspergillus* and *Penicillium* spp. was found to be most promising (Mehta et al, 2013). Ascacio-Valdes et al. (2016) reported that punicalin, gallagic acid and ellagic acid were obtain from punicalagin by the action of ellagitannase enzyme produced by *Aspergillus niger* GH1 during fermentation. Shi et al. (2005) found that the combination of tannase and polyphenol oxidase enzymes can be used for degradation of valonea tannin and accumulation of ellagic acid during fermentation of *A. niger* and *Candida utilis*.

Other important commercial applications of tannases are clarification of beer, wines and fruit juices, beer chill proofing, manufacture of coffee flavored drinks, manufacture of instant tea, de-tannification of food, preparation of food preservatives, treatment of green tea to inhibit the carcinogenic and mutagenic effects of N-nitrosamines, stabilization of malt polyphenols, improved color stability and additional organoleptic properties (Lal and Gardner, 2012; Marco et al, 2009; Sariözlü and Kivanç, 2009; Seth and Chand, 2000). Tannases have also been used for the cleavage of polyphenols present in the cell wall of plants which is essential for plant cell wall digestibility (Costa et al, 2014).

2.5.3 Pectinases

Pectinases are a heterogeneous group of related enzymes that hydrolyze the pectic substances, present mostly in plants. The pectinase group is formed from five enzymes which are pectinesterase (EC 3.1.1.11), endopolygalacturonase (EC 3.2.1.15), exopolygalacturonase (EC 3.2.1.67), pectin lyase (EC 4.2.2.10) and pectate lyase (EC 4.2.2.2) (Botella et al, 2005). Pectinases are enzymes involved in the degradation of the plant cell wall. Hence, these enzymes are applied to provide a good alternative to chemical processing (Venkatesh and Umesh-Kumar, 2005).

Pectinases or pectinolytic enzymes which are produced by bacteria, yeasts, fungi, insects, nematodes and plants. The filamentous fungi are most often used in the commercial production of pectinases, especially *Aspergillus* spp. Pectinases produced by *A. niger* are the most extensively used in industry. This fungus produces many enzymes which are active on homogalacturonan, including pectin methyl- and acetyl-

esterase, endopolygalacturonase, exopolygalacturonase, pectate lyase and pectin lyase (Polizeli et al, 2013; Venkatesh and Umesh-Kumar, 2005).

Over the years, pectinases have been used in several industrial processes. Pectinase enzymes from fungal origin, are used as processing aids for extraction, clarification and maceration purposes (Angayarkanni et al, 2002). Pectinases are now an integral part of food and textile industries such as maceration of tea leaves; extraction of olive oil, wine production, animal feed, processing of cotton fabrics, textile industry, cellulose and paper industries as well as in various biotechnological applications, functional foods and pharmaceuticals (Banu et al, 2010; Polizeli et al, 2013).

Pectinases together with other cell wall degrading enzymes enhance oil extraction yield from lemon peel and olive fruits. They can be used for the production of carrot puree and neutraceutically important oligouronides and monosaccharides pectins (Venkatesh and Umesh-Kumar, 2005).

2.6 Hypothesis

Currently, plant wastes contain large amounts of phenolic antioxidants especially in insoluble-bound form that are not converted into a product. These phenolic antioxidants can be used in manufacture of high-value food ingredients and nutraceuticals. Insoluble-bound phenolics present in plant wastes can be released and bioconverted by fermentation with microorganisms. Molds, especially black aspergilli have the ability to synthesize a variety of hydrolytic enzymes. Black aspergilli were isolated from local sources and used in fermentation for the aim of recovery and/or bioconversion of phenolics from various plant wastes with antioxidant activity.

3. MATERIALS AND METHODS

3.1 Materials

Dichloran glycerol (18%) agar (DG18), dichloran rose bengal chloramphenicol (DRBC) agar, malt extract agar (MEA), malt extract (ME), peptone, gliserin, NaCl, Tween 80, yeast extract, sucrose, lactose, urea and agar agar were purchased from Merck Chemicals (Darmstadt, Germany). Analytical grade KCl, $MgSO_4.7H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4.7H_2O$, K_2HPO_4, Na_2CO_3, $CuCl_2$, NH_4Cl, $NaNO_3$, KH_2PO_4, $(NH_4)_2SO_4$, NaOH, NaCl, KOH, HCl, acetic acid (CH_3COOH), NaK-tartrate.$4H_2O$, sodium acetate ($NaC_2H_3O_2$), anhydrous glucose and Congo red were purchased from Sigma-Aldrich (Steinheim, Germany). Carboxymethylcellulose sodium salt (CMC), tannic acid, pectin, 3,5-dinitrosalicylic acid (DNS), 2-thioxo-4-thiazolidinone (rhodanine), gallic acid, galacturonic acid, vanillin, catechin, methyl red, bromocresol green, boric acid, Folin-Ciocalteu's phenol reagent, trifluoroacetic acid (TFA), formic acid (FA), acetonitrile (HPLC grade) and methanol (HPLC grade) and all HPLC standards were purchased from Sigma-Aldrich (Steinheim, Germany).

Apple peel, apple pomace, pomegranate peel, pomegranate seed and black carrot pomace were donated by Targid Ind. Corp. (Mersin, Turkey). The company used a mixture of different cultivars of each species from various regions of Turkey for processing. Apple peel and apple pomace were collected after separate productions of clear juice and apple concentrate, respectively. Pomegranate peel was obtained after peel removal stage and pomegranate seed was from press-out stage of clear juice process. Black carrot pomace was collected from concentrated juice production process. Chestnut shell (a mixture of the outer brown peel and the inner pellicle) was supplied from a local producer (Kafkas Confectionery, Bursa, Turkey).

3.2 Isolation and Purification of Fungal Isolates

Rotten fruit and vegetables (carrot, apple, fig, grapes, corn, apricot, garlic, onion, and dates) were collected from local outdoor markets in Turkey. Molds from infected parts of fruits or vegetables were transferred onto the plates containing MEA, DG18, and DRBC agar after aseptic homogenization in peptone water or directly depending on the microbial load. Incubations were performed at 30 ºC for 7 days. Cultures were purified by sub-culturing the spores from black colonies on fresh MEA medium. The inoculated plates were incubated at 30 ºC for 7 days again. Isolated molds were firstly identified according to their macroscopic and microscopic features, then molecular identification was performed.

3.3 Identification of *Aspergillus* spp.

3.3.1 Morphological identification

Macromorphological features of each isolate was examined by measuring the colony color, size and zonation by naked eye, while micromorphological features such as type of the conidial heads, ornamentation of conidia, vesicle, conidiophores, presence of exudates and sclerotia were studied under light microscope (Nikon Eclipse, Melville, NY, USA). To determine the morphological features of molds, Czapek yeast agar with 20% sucrose (CY20S), Czapek Dox agar (CZ), Czapek yeast agar (CYA) and MEA were used. Media compositions are given in Table 3.1.

Table 3.1 : Compositions of culture media for morphological identification.

Media	**Constituents** (Klich (2002))
Czapek concentrate (g/100 mL)	30 g $NaNO_3$, 5 g KCl, 5 g $MgSO_4.7H_2O$, 0.1 g $FeSO_4.7H_2O$, 0.1 g $ZnSO_4.7H_2O$, 0.05 g $CuSO_4.5H_2O$
CY20S (g/L)	1 g K_2HPO_4, 10 mL Czapek concentrate, 5 g yeast extract, 200 g sucrose, 15 g agar
CZ (g/L)	10 mL Czapek concentrate, 1 g K_2HPO_4, 30 g sucrose, 17.5 g agar
CYA (g/L)	1 g K_2HPO_4, 10 mL Czapek concentrate, 5 g yeast extract, 30 g sucrose, 15 g agar

The media were sterilized by autoclaving at 121 °C for 15 min. The isolates were inoculated at three points, equidistant from the center and incubated in the dark for seven days (Klich, 2002). CYA incubated at 25 °C was called as CYA25, when incubated at 37 °C it was called CYA37. CY20S, MEA and CZ were incubated at 25 °C. Colonies from MEA plates were used for micromorphological identification. The species characterization was determined following the key characters by Klich (2002) and Raper and Fennell (1965).

3.3.2 Molecular identification

DNA was extracted with Biospeedy Fungal DNA kit (Bioeksen, Istanbul, Turkey) according to the instructions of the manufacturer. Molecular identification was performed by amplifying internal transcribed spacer region (ITS) using ITS1-5.8S rRNA and ITS4 (5'TCCTCCGCTTATTGATATGC3') as forward and ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3') as reverse primers for real-time polymerase chain reaction (QPCR) (Schoch et al, 2012). QPCR amplification was performed under the following conditions: Initial denaturation at 95 ºC for 10 min, 45 cycles at 95 ºC for 15 s, 53 ºC for 20 s and final extension at 72 ºC for 40 s. QPCR products were purified using PCR Purification Kit (Bioeksen, Istanbul, Turkey) following the manufacturer's instruction. DNA sequences were analyzed with Sanger Dideoxy Sequence Termination Method using ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster city, CA, USA). Sequences for ITS region were compared with the sequences available in National Center for Biotechnology Information (NCBI) using online basic local alignment search tool (BLAST) (Schoch et al, 2012).

3.4 Preparation of Mold Suspension

All mold isolates were grown on MEA slants (containing 3% malt extract, 0.3% soy peptone and 1.5% agar) at 30 ºC for 3 days. Mold suspensions were prepared from three day-old cultures (app. 10^7 spores/mL) by adding 5 mL sterile distilled water containing 0.05% Tween 80 to the slants. Aqueous solution with dislodged mold spores were transferred to tubes aseptically and the tubes were centrifuged (Hermle Z300K, Wehingen, Germany) to separate micelles from molds at 2000×g, 10 ºC for 20

minutes. Mold spores were washed two times using peptone water. Obtained mold suspension was used for fermentation and related analyses.

3.5 Enumeration of *Aspergillus* spp.

Spores were counted using Thoma slide and concentration was adjusted to 10^7 spores/mL. Molds were also enumerated on Sabouraud dextrose agar by using the pour plate technique to determine the number of spores in the inoculum. Sabouraud agar was used as culture medium containing 10 g mycological peptone, 40 g glucose anhydrous, and 15 g agar/L of water. Serial dilutions were prepared with physological water (0.85% NaCl). Plates were incubated at 30 °C for 72 h.

3.6 Ochratoxin A (OTA) Analyses by HPLC

Yeast extract sucrose supplement media (YES) and CYA were used for OTA production of *Aspergillus* spp. tested in this study. YES media was prepared by using the following constituents (g/L): 4 g yeast extract, 20 g sucrose, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 15 g agar. All isolates were three-point inoculated into YES and CYA media and incubated at 30°C for seven days. Each assay was performed in duplicate. OTA was extracted by using a previously described method of Bragulat et al. (2001). Three agar plugs were removed from the inner, middle and outer area of the colony, weighed and mixed with 0.5 mL of methanol for 60 min. The extracts were filtered with a Ultrafree-MC filter (Merck Millipore, Billerica, MA, USA) which had 0.45 µm pore diameter and injected into the high-performance liquid chromatography system (HPLC) (Agilent Technologies, Waldbronn, Germany) with fluorescence detector. OTA was separated on a Zorbax Eclipse XDB-C18 reversed phase (RP) column (150 mm, 4.6 mm, 5 µm). The mobile phase consisted of acetonitrile–water–acetic acid (99:99:2, v/v/v) at a flow rate 1 mL/min. Injection volume was 20 µL and excitation wavelength was 333 nm and emission wavelength was 477 nm. The detection limit of the analysis was 0.1 ng OTA/g of medium.

3.7 Screening of Hydrolytic Enzyme Production of Isolated Molds

3.7.1 Production of enzymes on solid media

To screen the extracellular hydrolytic enzyme activity, 20 µL of mold suspension was inoculated to the middle of the plates containing enzyme specific media as described in Table 3.2. *Aspergillus oryzae* MUCL 14492 was supplied from Mycothèque de l'Université Catholique de Louvain (Louvain-la-Neuve, Belgium) and used as reference culture for analysis of enzyme screening.

Table 3.2 : Media composition for enzyme production on solid media.

Enzyme	Composition	Incubation conditions	Reference
Cellulase	10 g/L CMC, 6.5 g/L $NaNO_3$, 6.5 g/L K_2HPO_4, 3.0 g/L $MgSO_4.7H_2O$, 6.5 g/L KCl, 0.3 g/L yeast extract, 0.65 g/L glucose, 17.5 g/L agar	30 °C for 72 h	Peciulyte (2007)
Tannase	10 g/L tannic acid, 6 g/L $NaNO_3$, 0.52 g/L KCl, 0.52 g/L $MgSO_4 \cdot 7H_2O$, 1.52 g/L KH_2PO_4, 0.01 g/L $CuSO_4 \cdot 5H_2O$, 0.01 g/L $ZnSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 30 g/L agar	30 °C for 48 h	Bradoo et al. (1996)
Pectinase	10 g/L pectin, 0.5 g/L urea, 0.5 g/L $(NH_4)_2SO_4$, 20 g/L KH_2PO_4, 0.05 g/L $MgSO_4 \cdot 7H_2O$, 20 g/L agar	30 °C for 72 h	Zheng et al. (2011)

Media with CMC, tannic acid and pectin were used for determination of cellulase, tannase and pectinase activity, respectively. After the given incubation period, enzyme producing isolates were detected by observation of the clear zone around the margins of the colony. Congo red (0.02% w/v) was directly added to the medium for determination of pectinase activity since the medium could not be distinguished from the clear zone. Congo red (1%, w/v) was used to dye the medium for cellulase activity for 15 min and excess dye was removed by washing the media with 1 M NaCl solution for 20 min. The media prepared with tannic acid had a purple color which became colorless after hydrolysis by tannase. All screening analyses were conducted in triplicates for each enzyme and mold.

3.7.2 Production of enzymes in liquid media

Mold suspension with 10^7 spores/mL were added to liquid medium which contains the same constituents given in Table 3.2 without agar and incubation took place at 30 ºC in a shaking incubator at 200 rpm. Fermentation was carried out in 100 mL Erlenmayer flasks containing 25 mL of growth medium after sterilization at 121 ºC for 15 min. After interval days (day 0, 1, 2, 3, 4, 7), a flask was taken and the biomass was separated from the liquid media using Whatman filter paper (Grade 1). The filtrate was used as the crude extracellular enzyme source and stored at -18 ºC for further analyses.

Mycelial biomass collected on the Whatman filter paper (Grade 1), was dried to a constant weight at 105 ºC for 24 h to determine the biomass yield. Change in pH of the filtrate was measured using pH meter (Consort C830, Cohasset, MA, USA). All trials were performed in triplicate.

3.7.2.1 Cellulase activity

Cellulase activity was determined by mixing 1 mL of 1% (w/v) CMC (prepared in 50 mM Na-acetate buffer pH 5.1) with 1 mL of crude extracellular enzyme source and this mixture was incubated at 50 °C for 15 min. The release of reducing groups was determined by the DNS method (Miller, 1959). After the incubation time, the reaction was stopped by the addition of 3.0 mL of DNS solution (1 g DNS, 16 g NaOH, 402.7 g NaK-tartrate.$4H_2O$ in 1 L distilled water) and the contents were boiled for 5 min and transferred into an ice-water bath to cool it quickly. The color developed was read at 540 nm. The amount of reducing sugar liberated was quantified using glucose as standard. One unit of cellulase activity is defined as the amount of enzyme that liberates 1 µmol of glucose equivalents per minute under the assay conditions (Sridevi and Charya, 2011).

3.7.2.2 Pectinase activity

To determine the pectinase activity, 2 mL of 1% pectin in 0.2 M acetate buffer (pH 4.8) and 0.5 mL crude enzyme solution were mixed and kept at 50 ºC for 30 min. The release of reducing groups was determined by the DNS method (Miller, 1959). Tubes were removed from water bath, 2.5 mL DNS solution was added, the solution was boiled for 5 min and cooled quickly in an ice-water bath. Finally, the absorbance was measured at 540 nm and the corresponding D-galacturonic acid content was

determined from the standard calibration curve. One unit of pectinase activity is defined as the amount of enzyme that liberates 1 µmol of D-galacturonic acid equivalents per minute under the assay conditions (Debing et al, 2006).

3.7.2.3 Tannase activity

Tannase activity was examined by mixing of 50 µL of the fermentation extracts and 1 mL of a 0.3 mM solution of tannic acid (in 0.1 M sodium acetate buffer, pH 5.1). After incubation at 30 ºC for 30 min, the reaction was stopped by addition of 0.2 mL 2 M HCl. To 100 µL of reaction mixture, 150 µL of rhodanine solution (0.667% (w/v) in methanol) was added and the mixture was vortexed. After 5 min, 2.25 mL of aqueous 0.5 M KOH solution was added and 20 min afterwards, the absorbance was read at 520 nm. Reactions were carried out at room temperature. The standard curve was drawn using gallic acid as standard. One unit of tannase activity was expressed in µmol of gallic acid released per milliliter of fermentation extract per minute under the assay conditions (Lagemaat and Pyle, 2001).

3.8 Determination of Physicochemical Composition of Wastes

3.8.1 Preparation of waste sample

All waste samples were dried separately using a freeze drier (Labconco Freezone 2.5 Plus, Kansas City, MO, USA) for 24 h at 0.63 mBar at -48 °C. Dried samples were ground finely using a stainless-steel grinder (IKA A11 Basic, Königswinter, Germany) and then the powdered samples were stored at -20 °C for use in subsequent analysis.

3.8.2 Moisture analysis

The moisture content was determined gravimetrically as described in the method of AOAC (2000). The moisture content was measured by placing 3 g of wastes into pre-weighed pans and drying at 105 °C to a constant mass.

3.8.3 Total protein content

Total protein content (TP) was determined by Kjeldahl method (AOAC, 2000), with some modifications. To each tube containing 4 g sample, 12 g K_2SO_4, 0.3 g $CuSO_4.5H_2O$, several boiling stones and ten drops of parafin were added to prevent foaming. Then under fume, 25 mL of H_2SO_4 was added into the tubes. For burning of

samples, temperature was increased gradually to 450 °C. After burning all samples, tubes were cooled and distilation was performed. Distillation was carried out by adding 100 mL of NaOH (40% w/v) and distillate was collected in an Erlenmayer flask which contained 50 mL of boric acid (4% w/v) mixed with methyl red and bromocresol green (5:1). Titration was done with 0.05 N HCl and TP was calculated with equation 3.1:

$$\% TP = \frac{(V_t - V_0) \times N \times 14 \times 6.25 \times 100}{w \times 1000} \quad (3.1)$$

Where; N: normality of HCl, w: mass of the sample (g), V_t: volume, in mL, of HCl used in the sample titration, V_0: volume, in mL, of HCl used in the blank titration, 6.25: protein convertion factor, 14: molar mass of nitrogen. The TP was expressed as g protein /100 g dry matter (dm).

3.8.4 Ash and mineral contents

Total ash content of wastes was determined by gravimetric method as described in the AOAC (2000). Dried samples (3 g) was placed into a pre-weighed crucible. The samples were carbonized and transferred into a muffle furnace and ignited at 550 °C overnight until ashing was completed. The crucibles were removed from furnace and placed in desicator and then weighed.

The remaining ash was dissolved with nitric acid/water solution (1:1 ratio) and filtered with Whatman filter paper (Grade 1). This solution was used for mineral analysis. Elements K, Ca, Mg, Na and trace elements Cu, Fe and Zn were determined by Inductively Coupled Plasma Atomic Emission-Spectrometer (ICP-AES) (Varian model Vista-MPX, Palo Alto, CA, USA). Operating conditions of the instrument were as follows: Plasma power, 1400 W, plasma flow, 15 L/min, auxiliary gas flow, 1.50 L/min, nebuliser pressure 200 kPa. The wavelength was set to 766.5 nm for potassium, 422.7 nm for calcium, 383.8 nm magnesium, 588.9 nm for sodium, 327.4 nm for cooper, 239.6 nm for iron and 472.2 nm for zinc. ICP multi-element standard solution IV (Merck, Darmstadt, Germany) containing 1000 ppm of the 23 elements in diluted nitric acid was used as reference during the analysis. Concentration of each mineral contained in test solutions was calculated from the standard curve prepared.

3.8.5 Reducing sugar content

Total content of reducing sugar in samples was determined with DNS method (Miller, 1959). Each waste (0.5 g) was weighed into test tubes and then 10 mL distilled water was added. After shaking vigoruosly, tubes were held for 24 h at room temperature. After this period, tubes were centrifuged at 4000 rpm for 5 minutes. To determine the reducing sugar content, 0.5 mL supernatant of samples and 2 mL DNS solution was mixed and boiled for 5 min. After boiling step, tubes were cooled immediately and the absorbance was read at 540 nm using a spectrophotometer.

3.8.6 Free glucose content

Free glucose content in each waste was measured according to Englyst et al. (1992) using glucose oxidase/peroxidase assay kit (GOPOD-format K-GLUC 09/14, Megazyme International Ireland Ltd., Wicklow, Ireland) and calculated using the equation 3.2:

$$\%Glucose = \frac{A_t \times V_t \times C \times D \times 100}{A_s \times w} \quad (3.2)$$

Where; A_t is absorbance of test solutions, V_t is total volume of test solutions, C is concentration (in mg glucose/mL) of standard, D is a dilution factor, A_s is absorbance of standard, w is weight (in mg) of sample taken for analysis.

3.9 Extraction of Soluble-Free and Insoluble-Bound Phenolic Compounds

Soluble phenolic compounds were extracted by using the method described by Gonzales et al. (2014) with slight modifications. Approximately 2 g freeze dried sample of each waste was homogenized with 15 mL of 80% methanol in water using ultraturrax (IKA, Wilmington, NC, USA) at 10000 rpm for 45 seconds. Tubes were placed on ice for 15 minutes, then the mixture was centrifuged at 2500×g for 10 minutes at 4 °C. Residue was re-extracted with 10 mL of 80% methanol following the same procedure and the volume was completed to 25 mL with 80% methanol. These extracts will be subsequently referred to as soluble phenolic extracts throughout the text. Analysis yielded only soluble free phenolics not soluble esterified ones which require hydrolysis (Ambigaipalan et al, 2016). The residues were dried overnight at room temperature and analyzed for insoluble-bound phenolic compounds.

Insoluble-bound phenolic compounds extracted by alkaline hydrolysis method as described by Gonzales et al. (2014). Briefly, 0.1 g of dried residue was hydrolyzed using 2 mL of 2 N NaOH in a screw-capped test tube in a water bath sonicated with the probe of an ultrasonic processor at 100% amplitude (Hielscher UP400S, Hamm, Germany) at 60 °C for 30 minutes. The samples were then neutralized with 2 N HCl and extracted with 100% methanol containing 0.1% formic acid using vortex for 2 minutes. The tubes were centrifuged at 2500×g at 4 °C for 10 min. Supernatants were collected and extraction was repeated two more times. All supernatants were pooled into the same flask and volume was completed to 20 mL with 90% methanol. The phenolic content from these extracts will be subsequently referred to as bound phenolics throughout the text.

3.10 Determination of Total Phenolic Content (TPC)

The TPC was determined according to the method Folin-Ciocalteau (FC) described by Singleton et al. (1999). Gallic acid was used as a standard in a concentration range between 0 and 50 mg/L. To 200 µL of sample or standard, 1.5 mL of FC reagent (0.2 N) was added, and the contents were vortexed. After 6 min of incubation, 1.2 mL of Na_2CO_3 (7.5%) solution was added and the mixture was incubated for 90 min at room temperature in dark. The absorbance was measured at 760 nm. The results were expressed in mg gallic acid equivalents (GAE) per g of dm based on the obtained standard.

3.11 Determination of Total Flavonoid Content (TFC)

The TFC was determined by colorimetric method described by Dewanto et al. (2002). Sample or standard at a volume of 1 mL was added into test tube and then following solutions were added: 0.3 mL 5% $NaNO_2$ (at t=0 min), 0.3 mL 10% $AlCl_3$ (at t=5 min) and 2 mL 1M NaOH (at t=6 min). Finally, 2.4 mL distilled water was added and the absorbance was measured spectrophotometrically at 510 nm. Catechin was used as standard and the results were expressed as mg catechin equivalents (CE) per g dm.

3.12 Determination of Condensed Tannins

Insoluble-bound and soluble-free condensed tannins were determined using the vanilin/HCl method described by Price et al. (1978). A set of tubes was prepared by adding 1 mL of sample or standard and 5 mL of vanilin reagent (50:50 mixture of 5% vanillin and 24% HCl, w/v) into the tubes and incubated for 20 min in a water bath at 30 °C. Control tubes were prepared by adding 1 mL of sample or standards followed by 12% HCl and incubated under same conditions. The absorbance was measured spectrophotometrically at 500 nm. The corrected absorbance was calculated by substracting the absorbance of the control sample from that of vanillin containing sample. The condensed tannin was expressed as mg CE per g dm.

3.13 Quantification of Phenolic Compounds by HPLC-PDA

The phenolic profile of each waste was determined by HPLC (Waters 2695, W600 Waters, Milford, MA, USA) using the method of Bino et al. (2005). Soluble and bound extracts were passed through 0.45 µm membrane filters and injected into HPLC system with photodiode array (PDA) detector. Phenolic compounds were separated on a supelcosil LC-18 25cm×4.60mm, 5-µm column (Sigma-Aldrich, Steinheim, Germany). The mobile phase consisted of solvent A (Milli-Q water with 0.1% (v/v) TFA) and solvent B (acetonitrile with 0.1% (v/v) TFA) with a flow rate of 1 mL/min and 10 µL injection volume. A linear gradient program was applied as 95% solvent A and 5% solvent B at 0 min, 65% solvent A and 35% solvent B at 45 min, 25% solvent A and 75% solvent B at 47 min and returning to the initial conditions at 54 min. Detection was carried out at 280 nm for ellagic acid, gallic acid, catechin, protocatechuic acid, 320 nm for *p*-coumaric acid, ferulic acid, chlorogenic acid, 360 nm for quercetin, and 520 nm for malvinidin, cyanidin and peonidin. Quantification of phenolic compounds except punicalagin was done by using available standards. Punicalagin was identified with UV-spectra from the literature (Mininel et al, 2014) and concentrations of punicalagin derivatives were calculated as ellagic acid equivalent. All phenolic compounds are expressed as mg per 100 g dm.

3.14 Identification of Phenolic Compounds by LC-MS/MS

Two separate HPLC instruments housed in different locations were used for this part of the study. The first instrument utilized LC having diode array detector (DAD) coupled to a Thermo TSQ Vantage (San Jose, CA, USA) quadrupole mass spectrometer. The second instrument used accurate mass detection, but did not have DAD capabilities with the LC system. In both cases, LC separation was carried out a using an Agilent poroshell 120 PFP (2.1x100mm, 2.7µm) column. Mobile phase solvent A consisted of Milli-Q water with 0.1% (v/v) FA and solvent B acetonitrile with 0.1% (v/v) FA. The liquid flow rate was 350 µL/min and a 5 µL injection volume was used. Injections carried out on the first instrument identified retention times for the peaks of interest as determined by the DAD spectra and corresponding nominal *m/z* values (accurate to 1 decimal place). Injections carried out on the second instrument were used for accurate mass analysis and structure elucidation of the *m/z* values (at given retention times) of interest. This second instrument, a Thermo TSQ Q-Exactive was used to determine the *m/z* values to 4 decimal places. Full scan data was acquired in both modes using a mass resolution of 140,000 (@*m/z* 200). Tandem mass spectra of *m/z* values of interest were acquired in subsequent runs using a data dependent acquisition and an inclusion list. Data were acquired using normalized collision energies of 15, 35, and 55 eV.

3.15 Measurement of Antioxidant Capacity

3.15.1 DPPH free-radical scavenging activity

DPPH free-radical scavenging activity was determined by the method described by Rai et al. (2006). DPPH (2 mL, 0.1 mM) solution in methanol was added to 100 µL extract, vortex mixed for 10 s and left in the dark (30 min) at room temperature. The absorbance was measured at 517 nm using a spectrophotometer. Trolox was used as standard and the results were expressed as mg trolox equivalents (TE) per g dm.

3.15.2 Copper reducing antioxidant power assay (CUPRAC)

The CUPRAC method was performed with the method described by Apak et al. (2005). A hundred μL of sample extract was mixed with 1 mL of $CuCl_2$ solution (0.01 mM), 1 mL of neocuproine (Nc) solution (7.5 mM), 1 mL of ammonium acetate buffer (pH=7.0, 1.0 M) and 1 mL of distilled water. After 30 minutes, absorbance was measured at 450 nm against reagent blank. Results were expressed in terms of mg TE per g dm.

3.16 Solid-State Fermentation (SSF) by *Aspergillus* spp.

The SSF was carried out in 100 mL Erlenmeyer flask with 5 g of selected waste. Concentrated salt solutions to provide following final concentrations ($NaNO_3$, 6g/L; KH_2PO_4, 1.52 g/L; KCl, 0.5 g/L; $MgSO_4.7H_2O$, 0.5 g/L; $ZnSO_4.H_2O$, 0.01 g/L; $CuSO_4.5H_2O$, 0.01 g/L; $FeSO_4.H_2O$, 0.01 g/L) were added to the fermentation media. Each Erlenmeyer flask was inoculated with 1 mL of the spore suspension (10^7cfu/mL) and incubated at 30 °C up to 7 days. Spore suspension was prepared according to the described method in section 3.4. A sample without inoculum was also included as control of fermentation by natural flora. After interval days (0, 3 and 7 day), an Erlenmeyer flask was taken and the fermented medium was dried in a freeze drier with the conditions given in Section 3.8.1. Dried samples were ground and stored at -20 ºC for further analysis. Fermentation process was carried out in triplicate.

3.17 Extraction of Phenolic Compounds from Fermented and Unfermented Wastes

Phenolic compounds in unfermented and fermented wastes were extracted by the method of Ajila et al. (2011). In brief, 10 mL of methanol/water (80/20; v/v) was added to 1 g of sample, and placed in an ultrasonic bath (Azakli, Turkey) at 40 ºC for 30 min. Sample mixture was centrifuged at 9000×g for 10 min at 4 ºC and the supernatant was collected. Re-extraction with the same procedure was performed after the addition of 10 mL methanol/water (80/20; v/v) to the pellet. Then, both supernatants were pooled and filtered through filter paper (Whatman No.1, Springfield Mill, England). Obtained extracts were analyzed for TPC, DPPH, CUPRAC, identification and quantification of phenolic compounds by LC-MS/MS and HPLC-PDA as described previously.

3.18 Optimization Studies of Fermentation Media for Apple Peel

3.18.1 Screening of SSF conditions by Plackett-Burman Design (PBD)

Aspergillus niger ZDM2 was chosen as a test microorganism due to the highest phenolic production on apple peel according to the experimental results. SSF was carried out in 100 mL Erlenmeyer flask with 5 g apple peel powder. The pH of fermentation medium was 5.6 at the beginning. Fermentation medium was moistened with 25 mL of water with added salts (as described in Section 3.16). The effects of different carbon sources (glucose, sucrose and lactose), nitrogen sources (sodium nitrate, urea, peptone, ammonium chloride, yeast extract) and incubation time on the phenolic production were assessed by PBD. The PBD was employed in this study to correlate dependent and independent variables using the following polynomial model:

$$Y = A_0 + A_1X_1 + A_2X_2 + \cdots + A_nX_n \quad (3.3)$$

Where Y is the response, A_0 the constant and A_1 to A_n are the coefficients of the response values. The independent and dependent variables evaluated in this study are listed in Table 3.3. Each indepented variable was studied at two levels, namely high (+1) and low (-1) that upper and lower limits of the range covered.

Table 3.3 : Coded factors and their concentrations used in the PBD.

	Code	Low level	High level
Independent factors			
Sodium nitrate (g/L)	*A*	2	10
Urea (g/L)	*B*	2	10
Peptone (g/L)	*C*	2	10
Ammonium chloride (g/L)	*D*	2	10
Yeast extract (g/L)	*E*	2	10
Glucose (g/L)	*F*	1	10
Sucrose (g/L)	*G*	1	10
Lactose (g/L)	*H*	1	10
Incubation time (h)	*J*	48	168
Dependent factor			
TPC (mg GAE/100 g dm)	Y_1		

Fifteen experiments were conducted according to the experimental design given in Table 3.4. MINITAB statistical software (Minitab 18, Minitab Inc., Coventry, UK) was used to generate and analyze the data. The carbon and nitrogen sources of fermentation medium were changed according to the experimental design. After interval days, an Erlenmeyer flask was taken and the content was dried in freeze drier as described in Section 3.8.1. Dried fermented samples were ground and stored at -20 ºC for further analysis. Extraction of phenolic compounds was carried out using the method mentioned in Section 3.17.

Table 3.4 : Experimental runs generated by PBD to determine effects of nine independent variables on TPC.

Run	*Experimental value*								
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*J*
1	+1	-1	+1	-1	-1	-1	+1	+1	+1
2	+1	+1	-1	+1	+1	-1	+1	-1	-1
3	+1	+1	+1	-1	+1	+1	-1	+1	-1
4	-1	+1	+1	-1	+1	-1	-1	-1	+1
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	+1	-1	-1	-1	+1	+1	+1	-1	+1
9	+1	-1	+1	+1	-1	+1	-1	-1	-1
10	-1	+1	-1	-1	-1	+1	+1	+1	-1
11	-1	+1	+1	+1	-1	+1	+1	-1	+1
12	+1	+1	-1	+1	-1	-1	-1	+1	+1
13	-1	-1	-1	-1	-1	-1	-1	-1	-1
14	-1	-1	+1	+1	+1	-1	+1	+1	-1
15	-1	-1	-1	+1	+1	+1	-1	+1	+1

All experiments were carried out in duplicate and the average of the TPC was taken as responses. From one-way analysis of variance (ANOVA), variables that have a significant impact on enhancement of TPC were determined (P<0.05). Selected significant factors were used further for optimization studies.

3.18.2 Optimization of fermentation conditions by Response Surface Methodology (RSM)

Significant factors from PBD were further optimized using Central composite design (CCD) to optimize the fermentation conditions for enhancing production of phenolic compounds. The key independent variables were determined as incubation time, urea and lactose for TPC according to the PBD. Fermentation trials were carried out by adding different levels of urea as nitrogen source and lactose as carbon source for varying fermentation time as shown in Table 3.5. Three selected independent variables were studied at five different levels coded as $-\alpha$, -1, 0, 1, and $+\alpha$. The value for alpha (1.633) was chosen to fulfill the rotatibility in the design. According to the CCD matrix, a total of 17 experiments were designed including 3 replications at the center point for estimation of the pure error sum of squares.

Table 3.5 : CCD experimental design for optimization of production of phenolics.

Run	Coded variables*			Real variables		
	X_1	X_2	X_3	X_1 (h)	X_2 (g/L)	X_3 (g/L)
1	$-\alpha$	0	0	42	7.5	3.5
2	0	0	$-\alpha$	120	7.5	1.1
3	0	0	$+\alpha$	120	7.5	5.9
4	$+\alpha$	0	0	198	7.5	3.5
5	0	$-\alpha$	0	120	3.4	3.5
6	0	$+\alpha$	0	120	11.5	3.5
7	0	0	0	120	7.5	3.5
8	+1	+1	-1	168	10	2
9	+1	-1	-1	168	5	2
10	-1	-1	-1	72	5	2
11	0	0	0	120	7.5	3.5
12	-1	-1	+1	72	5	5
13	+1	-1	+1	168	5	5
14	0	0	0	120	7.5	3.5
15	-1	+1	+1	72	10	5
16	+1	+1	+1	168	10	5
17	-1	+1	+1	72	10	5

*X_1, X_2, and X_3 represent fermentation time, concentrations of lactose and urea, respectively.

The statistical analysis of data was performed by using the ANOVA. The relationship between independent variables and the response was expressed by a second-order polynomial model (3.4):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad (3.4)$$

Where Y is the predicted response, b_0 is the intercept term, b_1, b_2, and b_3 coefficients of the linear terms, b_{11}, b_{22}, and b_{33} the coefficients of the squared terms, b_{12}, b_{13}, and b_{23} are the coefficients of the interaction terms, X_1, X_2, and X_3 are coded independent variables.

Optimization analysis was conducted to find fermentation conditions that yield maximum TPC and DPPH activity. Validation of the results of the optimization analysis were done by measuring the TPC and DPPH activity after fermentation in the medium with optimum urea and lactose concentrations for optimum incubation time. Means of TPC and DPPH activity from triplicate experiments were compared with those predicted by the regression models.

3.19 Optimization Studies of Fermentation Media for Chestnut Shell

3.19.1 Screening of SSF conditions by PBD

The isolate that produced the maximum level of ellagic acid was used in optimization studies. SSF was conducted in 100 mL Erlenmeyer flask with 5 g of chestnut shell powder. Fermentation medium was moistened with 25 mL of water with added salts (as described in Section 3.16). The pH of medium was 6.2 at the beginning. The effects of carbon sources (glucose, sucrose and lactose), nitrogen sources (sodium nitrate, urea, peptone, ammonium chloride, yeast extract) and fermentation time were analyzed by PBD. A design with 9 factors at 2 levels was developed (Table 3.6). Each variable was represented at two levels, namely, low (-1) and high (+1). Concentration of ellagic acid (mg/g dm) was used as the response variable.

Table 3.6 : Coded factors and their concentrations used in the PBD.

	Code	Low level	High level
Independent factors			
Sodium nitrate (g/L)	*A*	2	10
Urea (g/L)	*B*	2	10
Peptone (g/L)	*C*	2	10
Ammonium chloride (g/L)	*D*	2	10
Yeast extract (g/L)	*E*	2	10
Glucose (g/L)	*F*	1	10
Sucrose (g/L)	*G*	1	10
Lactose (g/L)	*H*	1	10
Fermentation time (h)	*J*	48	168
Dependent factor			
Ellagic acid (mg/g)	Y_1		

To determine the significant factors on the production of ellagic acid, fifteen experiments were conducted according to the experimental design given in Table 3.4. All fermentation experiments were realized with two replications and the average of produced ellagic acid concentration was taken as response. Factors which had significant impact (P<0.05) on ellagic acid production were selected at this stage for further optimization studies.

3.19.2 Enhancement of ellagic acid production using RSM

Effective factors from PBD analysis (fermentation time, yeast extract, lactose), were used for further optimization studies by RSM for improvement of ellagic acid production. Three independent variables were studied at five levels coded as $-\alpha$, -1, 0, 1, and $+\alpha$ according to CCD. A total of 17 experiments were designed (Table 3.7) including 3 replications at the center point for estimation of the pure error sum of squares. Glucose was added to each fermentation medium with a constant concentration of 5 g/L due to its significant effect on ellagic acid production determined by PBD analysis.

Significance of the main effects and interactions were determined by ANOVA. Three-dimensional plots were obtained by keeping one variable constant at the center point and changing the other two variables within the experimental range. The model

equations with the help of contour plots were used to describe the individual and cumulative effects of factors on the response. The relationship between independent variables and the response was described according to a second-order polynomial equation by regression analysis. The fit of the model equation was determined by the coefficient of determination (R_2) and the adjusted coefficient of determination (Adjusted R_2). Experimental validation of the optimum conditions was carried out. Means of concentrations of ellagic acid obtained from triplicate trials at optimum conditions were compared with that predicted by the regression model.

Table 3.7 : CCD experimental design for optimization of production of ellagic acid.

Run	Coded variables*			Real variables		
	X_1	X_2	X_3	X_1 (h)	X_2 (g/L)	X_3 (g/L)
1	$-\alpha$	0	0	25	7.5	3.5
2	0	0	$-\alpha$	84	7.5	1.1
3	0	0	$+\alpha$	84	7.5	5.9
4	$+\alpha$	0	0	143	7.5	3.5
5	0	$-\alpha$	0	84	3.4	3.5
6	0	$+\alpha$	0	84	11.6	3.5
7	0	0	0	84	7.5	3.5
8	+1	+1	-1	120	10	2
9	+1	-1	-1	120	5	2
10	-1	-1	-1	48	5	2
11	0	0	0	84	7.5	3.5
12	-1	-1	+1	48	5	5
13	+1	-1	+1	120	5	5
14	0	0	0	84	7.5	3.5
15	-1	+1	+1	48	10	5
16	+1	+1	+1	120	10	5
17	-1	+1	+1	48	10	2

*X_1, X_2, and X_3 represent fermentation time, lactose and yeast extract concentration, respectively.

3.20 Preparation of Yoghurt Samples Fortified with Fermented and Unfermented Wastes

Phenolic compounds from fermented and unfermented apple peel and chestnut shell samples were extracted by the method given in the Section 3.17. Solvent was evaporated using rotary evaporator (IKA, RV10 Basic, Staufen, Germany) and residual water was removed by freeze drying. Dried phenolic extracts from fermented and unfermented wastes were added into commercial plain yoghurt (Light yoghurt, 1.4%, Sütaş, Bursa) at a level of 1%. Yoghurt samples were analyzed after preparation and 3 days of storage at 4 °C. Yoghurt samples were dried by freeze-drier using the method described in Section 3.8.1. Phenolic compounds were extracted from yoghurt samples by extraction method given in Section 3.17. Total phenolic contents and antioxidant activity of yoghurt samples were measured with the method described in Section 3.10 and Section 3.15.1, respectively.

3.21 Statistical Analysis

Effects of treatments on measured variables were analyzed by ANOVA, and when needed comparison of mean values was performed using Tukey's test at a significance level of 0.05 using the MINITAB 18 statistical software. Screening of factors and optimization studies for fermentation conditions were conducted by PBD and RSM using the same statistical software.

4. RESULTS AND DISCUSSION

4.1 Isolation and Identification of *Aspergillus* spp. Isolated from Grape and Date

4.1.1 Molecular identification of *Aspergillus* spp.

Eight of the black colonies that grew on date and grape with different morphology were isolated and identified by molecular techniques. However, there were not found black colonies in other screened rotten fruit and vegetable. The amplified sequences were compared with the sequences available in NCBI GenBank. Two of the colonies were found to be similar according to molecular identifications and therefore six molds were selected. The GenBank code of the reference cultures, sequence identities and identification of isolates are shown in Table 4.1. After searching for DNA homology using the BLAST program available in the Genbank database showed that isolates ZDM1 and ZGM5 shared 99% similarity with *Aspergillus tubingensis* and isolates ZDM2 and ZDM3 shared 99% similarities with *Aspergillus niger*. Meanwhile, the isolate ZGM4 shared 99% similarity with *Aspergillus japonicus* and the isolate ZGM6 shared 99% similarity with *Aspergillus aculeatus*.

Table 4.1 : Identification of isolates from grape and date on sequences.

Isolate codes	Species	Genbank code[a]	Identities	%
ZDM1	*A. tubingensis*	KP131626	589/594	99
ZDM2	*A. niger*	KT852982	566/568	99
ZDM3	*A. niger*	KT898789	557/558	99
ZGM4	*A. japonicus*	KC128815	560/568	99
ZGM5	*A. tubingensis*	GU595290	574/576	99
ZGM6	*A. aculeatus*	JF439460	546/550	99

[a] These GenBank code are those with the sequences of the present work were compared with the available ones in September 2016.

ITS region is the most common target used for molecular identification of fungi; however, this target does not work equally for all groups of fungi. In this case, ITS sequencing and classical morphological identification can be combined to be sure (Baffi et al, 2012).

4.1.2 Morphological characteristics of *Aspergillus* spp.

Identification of all isolates based on morphological characteristics was confirmed by following the key which reported by Raper and Fennell (1965) and Klich (2002). The isolates belonging to the genus *Aspergillus* section *Nigri* characteristically present dark-brown to black conidia, with uniseriate or biseriate conidiophores, spherical vesicles and hyaline or lightly pigmented hyphae. Colony color, size and zonation were observed by naked eye, while type of conidial heads, shape, size, ornamentation of conidia, vesicle and conidiophores were observed under optical microscope. Colony diameter and characteristics of the tested isolates on given medium after 7 days of incubation are presented in Table 4.2 and colony appearance are shown in Figure 4.1.

Table 4.2 : Macroscopic characteristics of the species of isolated *Aspergillus* spp.

Isolate codes	Colony Diameter (mm)					Production of sclerotia
	CYA 25	MEA	CY20S	CYA 37	CZ	
ZDM1	44-70	44-56	40-70	40-57	44-66	Present
ZDM2	40-70	44-60	40-70	48-60	40-56	Absent
ZDM3	40-70	44-53	44-71	42-64	40-52	Absent
ZGM4	40-72	40-70	44-74	NG*	45-70	Absent
ZGM5	42-70	40-42	46-75	46-73	44-61	Present
ZGM6	45-72	44-72	44-73	NG	44-73	Absent

*Not grown.

Identification of black aspergilli on the basis of micromorphological characteristics can be helpful but it is not easy to distinguish the differences among isolates such as *A. niger* (ZDM2, ZDM3) and *A. tubingensis* (ZDM1, ZGM5) which produced biseriate sterigmata and also *A. japonicus* ZGM4 and *A. aculeatus* ZGM6 which exhibited uniseriate sterigmata. Table 4.3 presents the microscobic features of *Aspergillus* spp. studied in MEA after 7-day incubation.

Table 4.3 : Microscopic characteristics of the isolated *Aspergillus* spp.

Isolate codes	Stipe		Vesicle		Seriation	Conidia		
	Length (µm)	Surface texture	Diameter (µm)	Shape		Shape	Diameter (µm)	Surface texture
ZDM1	600-1700	rf*	45-55	gl	b	gl/el	3-4.5	fr/rf
ZDM2	400-800	sm/fr	40-50	gl	b	gl/el	2-3.5	sm/rf
ZDM3	400-1000	sm/fr	43-72	gl/el	b	gl	3-3.5	rf
ZGM4	650-1300	fr/rf	40-45	gl	u	gl/el	4-5	rf
ZGM5	700-1200	rf	70-90	gl	b	gl	3-4	sm/fr
ZGM6	350-650	sm/fr	63-65	gl	u	gl/el	4-4.5	rf

*Rf: rough, fr: finely roughened, sm: smooth, gl: globose, el: ellipsoidal.

The uniseriate species *A. aculeatus* ZGM6 and *A. japonicus* ZGM4 are morphologically similar, on the other hand *A. aculeatus* ZGM6 presents larger vesicles compared to *A. japonicus* ZGM4. *A. japonicus* ZGM4 can be macroscopically distinguished from *A. aculeatus* ZGM6 by the color of mycelium on CYA at 25 °C. *A. aculeatus* ZGM6 and *A. japonicus* ZGM4 showed no growth on CYA37 medium, while they showed sporulation on CYA25 attained 40-72 mm. *A. japonicus* ZGM4 showed heavy sporulation, while *A. aculeatus* ZGM6 produced light flocculent colonies. Morphological characteristics of *A. aculeatus* ZGM6 and *A. japonicus* ZGM4 produced dark brown and black colonies on CY20S; reverse of colonies were colorless to pale yellow and slightly yellow, respectively. They showed heavy sporulation essentially azonate colonies on CY20S. The colonies of *A. aculeatus* ZGM6 and *A. japonicus* ZGM4 on MEA was attained 40-72 mm, lightly flocculent and centrally abundant sporulation, colonies were black to dark-brown and reverse of colonies were yellow. The colonies of *A. japonicus* ZGM4 on CZ showed light sporulation and zonate colonies with dark brown colors and reverse pale yellow and slightly yellow at center. The colonies of *A. aculeatus* ZGM6 on CZ showed lightly flocculent and colony color was brown and reverse was colorless. *A. aculeatus* ZGM6 had globose conidia head, vesicle measured 40-45 µm in diameter and globose in shape. Stipe had thick walls that measured 350-650×10-13 µm and brown in color. Conidia sizes ranged between 4 and 4.5 µm, rough and globose to ellipsoidal.

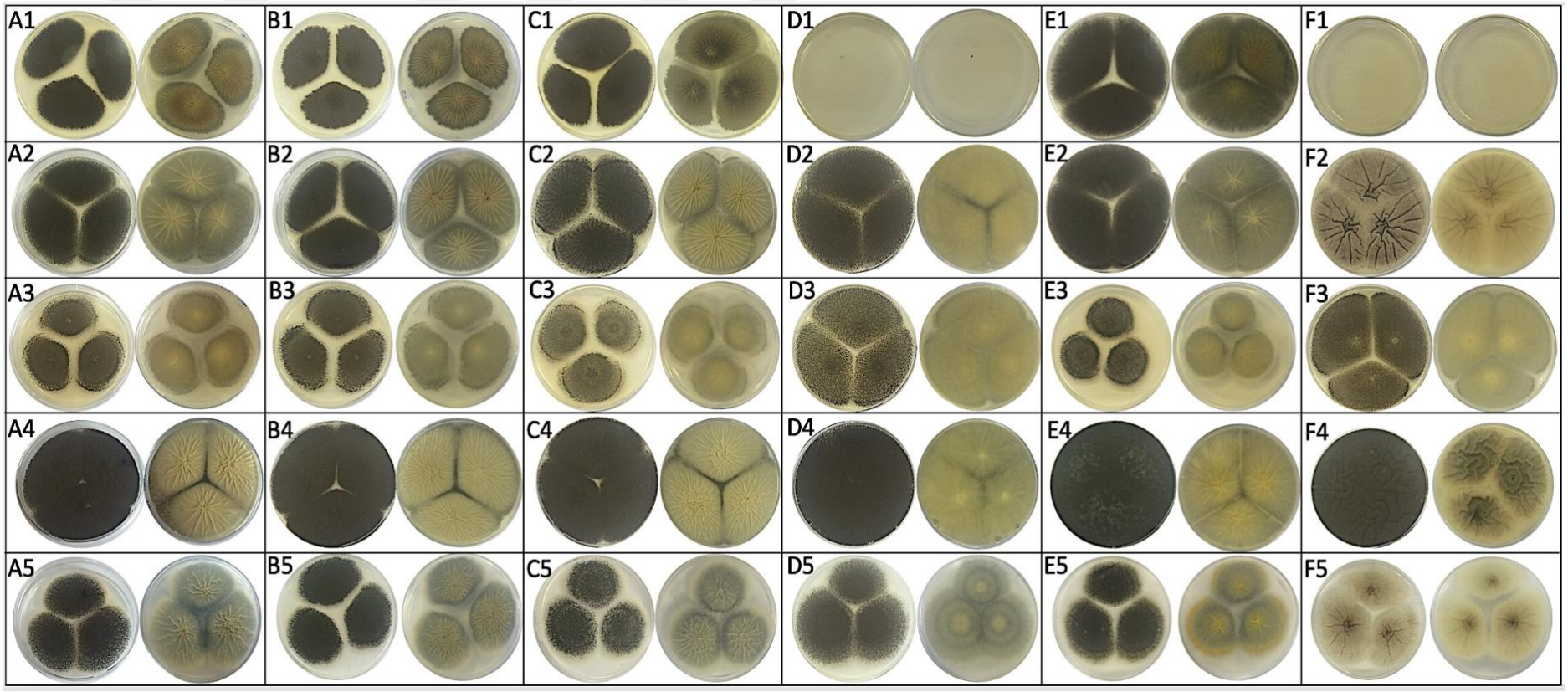


Figure 4.1 : Colony types of three points culture inoculated with *Aspergillus* spp. on CYA25, CYA37, MEA, CY20S and CZ medium after 7 days incubation. The letters mentioned in the figure represented the different *Aspergillus* spp. as A: *A. tubingensis* ZDM1; B: *A. niger* ZDM2; C: *A. niger* ZDM3; D: *A. japonicus* ZGM4; E: *A. tubingensis* ZGM5; F: *A. aculeatus* ZGM6. The numbers mentioned in the figure represented the medium as CYA37 (1), CYA25 (2), MEA (3), CY20S (4) and CZ (5).

Colony characteristics of *A. tubingensis* (ZDM1, ZGM5) and *A. niger* (ZDM2, ZDM3) on MEA, CYA25, CYA37 and CZ agar was not helpful in distinguishing them from each other. Colony colors of *A. tubingensis* (ZDM1, ZGM5) on MEA, CYA25 and CYA37 were black to dark brown, and reverse of colonies were colorless to slightly yellow. Colonies on CZ was attained 45-65 mm, and showed central abundant sporulation, colonies were black to dark-brown and reverse colors were yellow. The colonies of *A. tubingensis* ZGM5 on CY20S produced velvety and essentially azonate colonies; however, *A. tubingensis* ZDM1 showed heavy sporulation and have not any velvety colonies on the same medium. *A. tubingensis* (ZDM1, ZGM5) produced biseriate sterigmata, radiate conidial heads with 100-300 μm in range, rough-walled stipes and average stipes length 600-1700 μm while the width ranged between 13 and 19 μm. Primary series of sterigmata mostly ranged between 10 and 15 μm and secondaries was 5-6 μm. Conidia diameter was 3-4,5 μm with rough to finely roughened surface, mostly globose and slightly ellipsoidal with hyaline echinulations. The vesicle diameter was ranged between 45 to 55 μm. *A. niger* (ZDM2, ZDM3) formed black to dark brown colonies on CYA25 and CYA37, and reverse of colonies was colorless and pale yellow at colony center, while colony colors on MEA were brownish black, and reverse colorless to slightly yellow at center. Conidial heads were biseriate and globose in shape with spherical to globose vesicle that measured 40-70 μm. The stipe measured 400-1300 μm with rough-walls. Conidia diameter was 2-3.5 μm with rough surface, mostly globose and slightly ellipsoidal. *A. tubingensis* (ZDM1, ZGM5) was the only isolate produced sclerotia. Several species in *Aspergillus* section *Nigri* have been reported to produce sclerotia which are important morphological characters for identifying some *Aspergillus* spp. Sclerotial development is considered to be an important prerequisite for sexual development. Sclerotium production may be strain-specific, but it can be induced when molds grow in different standard media or plant parts (Frisvad et al, 2014).

Different type of fungi produce different looking colonies, some colonies may be colored, some colonies are globose in shape, and others are irregular. A specific terminology is used to describe colony types growing on specified media. In Figure 4.2, illustrative microscope images of *Aspergillus* spp. are given.

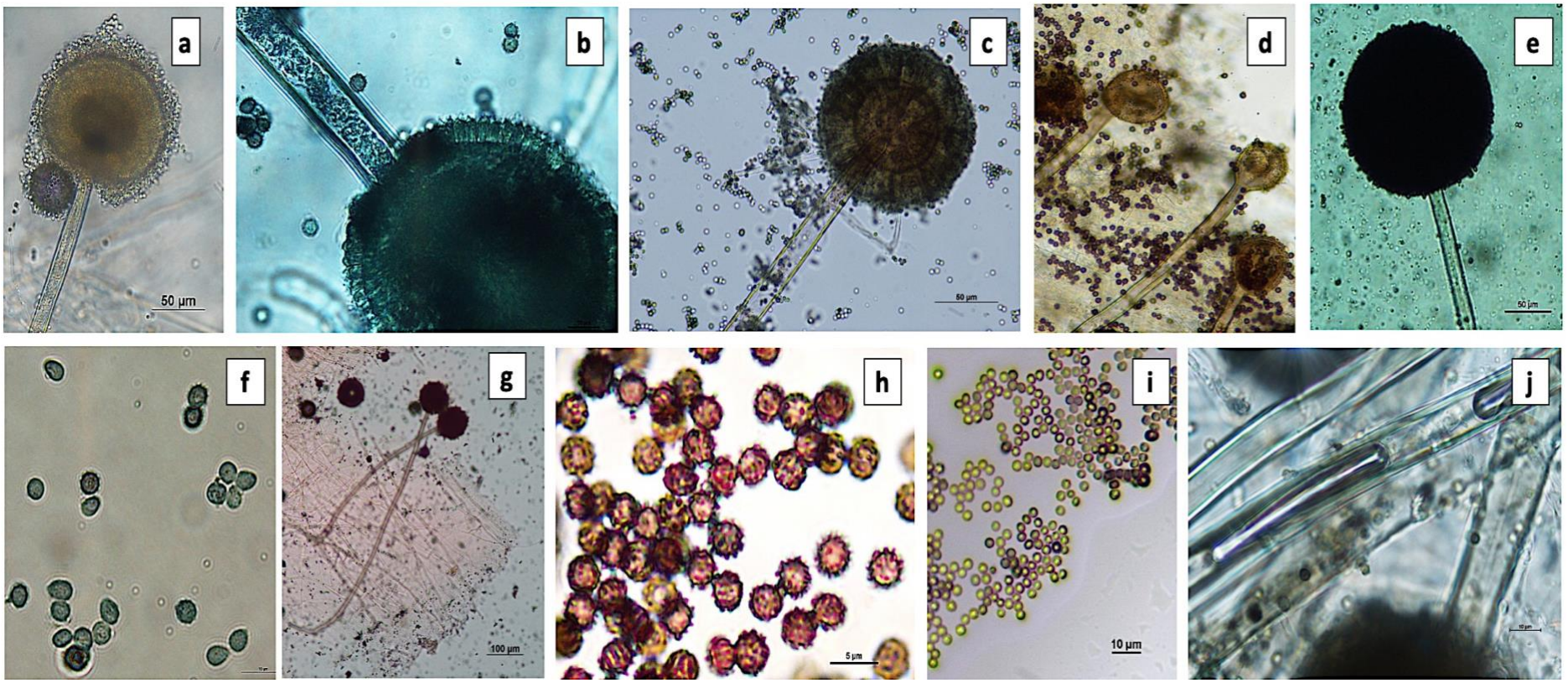


Figure 4.2 : The morphological characteristics of isolated *Aspergillus* spp. (a) slightly subglobose vesicle found in *A. niger*, (b) typical head showing only one series of sterigmata appeared in *A. japonicus*, (c) typical head showing sterigmata in two series and vesicle in globose defined in *A. niger*, (d) elongate vesicle found in *A. japonicus*, (e) globose conidial head formed in *A. tubingensis*, (f) globose to somewhat elliptical conidia found in *A. aculeatus*, (g) stipes (conidiphores) found in *A. niger*, (h) pigmented echinulate conidia appeared in *A. tubingensis*, (i) mostly globose conidia formed in *A. niger*, (j) detail of some stipes found in *A. niger*.

4.1.3 OTA production by *Aspergillus* spp.

OTA production was analyzed after 7 days of incubation at 30 °C tested on CYA and YES media for all *Aspergillus* spp. isolated in this study. None of the isolates showed a peak at a retention time identical to that of standard OTA. According to HPLC results, all isolates did not show any OTA production at given conditions. Among the OTA-producing *Aspergillus* spp. most of them belong to two sections: *A.* section *Circumdati* (*A. orchraceus*) and *A.* section *Nigri* (*A. carbonarius* and *A. niger*) (Wang et al, 2016). Uniseriate species of black aspergilli noted that they are not OTA producer, while biseriate species are OTA producer like *Aspergillus carbonarius*, *A. niger*, *Aspergillus lacticoffeatus*, *Aspergillus sclerotioniger*, *Aspergillus welwitschiae* (Cabanes and Bragulat, 2018). In our study, *A. niger* (ZDM2 and ZDM3) did not show any OTA production and this can be explained with the source of isolation. In the literature, it has been reported that ochratoxigenic potential of *A. niger* was lower than *A. carbonarius* (Freire et al, 2018). Additionally, non-toxigenic *A. niger* has been widely used for production of citric acid and various industrial enzymes (Yu et al, 2004).

4.1.4 Conclusion

In conclusion, morphological characteristics are primary tools in the identification of various *Aspergillus* species, however, molecular identification is also essential in distinguishing these species. To obtain more precise results, morphological and molecular methods could be used for the identification of *Aspergillus* spp. The results of the analysis of the sequences provided in the database showed that ITS works well as a barcode for the majority of species. Analysis of 18S gene for fungal cultures provide suitable phenotypic data that can be used to determine close relationships between species. The closest phylogenetic neighbours according to the 18S gene sequence data for the isolates ZDM1 and ZGM5 were *A. tubingensis*; ZDM2 and ZDM3 were *A. niger*; ZGM4 was *A. japonicus* and ZGM6 was *A. aculeatus*. Identified isolates were used for further enzyme screening analyses and fermentation studies in this thesis. Toxin analyses also showed that none of the isolates did not produce OTA on CYA and YES media.

4.2 Screening of Isolates for Enzyme Production

4.2.1 Screening of isolates for enzyme production on solid medium

The objective of the screening studies was to identify isolates with a high level of cellulase, tannase and pectinase enzyme producing ability. During the initial screening, 3 molds were isolated from grapes and 3 molds were isolated form dates. All isolates were screened by both qualitative (zone of inhibition, Figure 4.3) and quantitative (diameter of clear zones in mm, Table 4.4) activity assays in plates. Plate screening was performed on agar plates containing CMC, tannic acid and pectin as substrates for cellulase, tannase and pectinase enzyme, respectively. The plate screening method was chosen due to its simplicity and speed. When an isolate had an ability to produce the enzyme, the substrate was hydrolyzed, clear zone appeared around the colonies and the activity can be determined easily. In the case of isolate without an ability to produce the corresponding enzyme, growth was restricted and no clear zone observed.

Table 4.4 : Clear zone diameter of *Aspergillus spp.* measured by plate screening method.

Isolates	Cellulase	Tannase	Pectinase
	Clear zone (mm)*		
Aspergillus oryzae MUCL 14492	49.8±4.9$_a$	15.5±0.4$_c$	38.3±1.4$_a$
Aspergillus tubingensis ZDM1	51.8±7.1$_a$	22.7±2.3$_b$	29.6±8.1$_a$
Aspergillus niger ZDM2	53.9±7.7$_a$	22.9±2.0$_b$	36.4±10.4$_a$
Aspergillus niger ZDM3	57.1±6.2$_a$	22.0±2.3$_b$	30.5±6.7$_a$
Aspergillus japonicus ZGM4	49.3±5.9$_a$	30.3±1.0$_a$	28.4±5.5$_a$
Aspergillus tubingensis ZGM5	54.4±8.0$_a$	22.2±2.5$_b$	37.8±7.3$_a$
Aspergillus aculeatus ZGM6	51.0±7.4$_a$	28.9±1.9$_a$	30.7±9.2$_a$

*Mean ± Standard deviation (n=3). Means marked with different letters in the same column are significantly different (P<0.05).

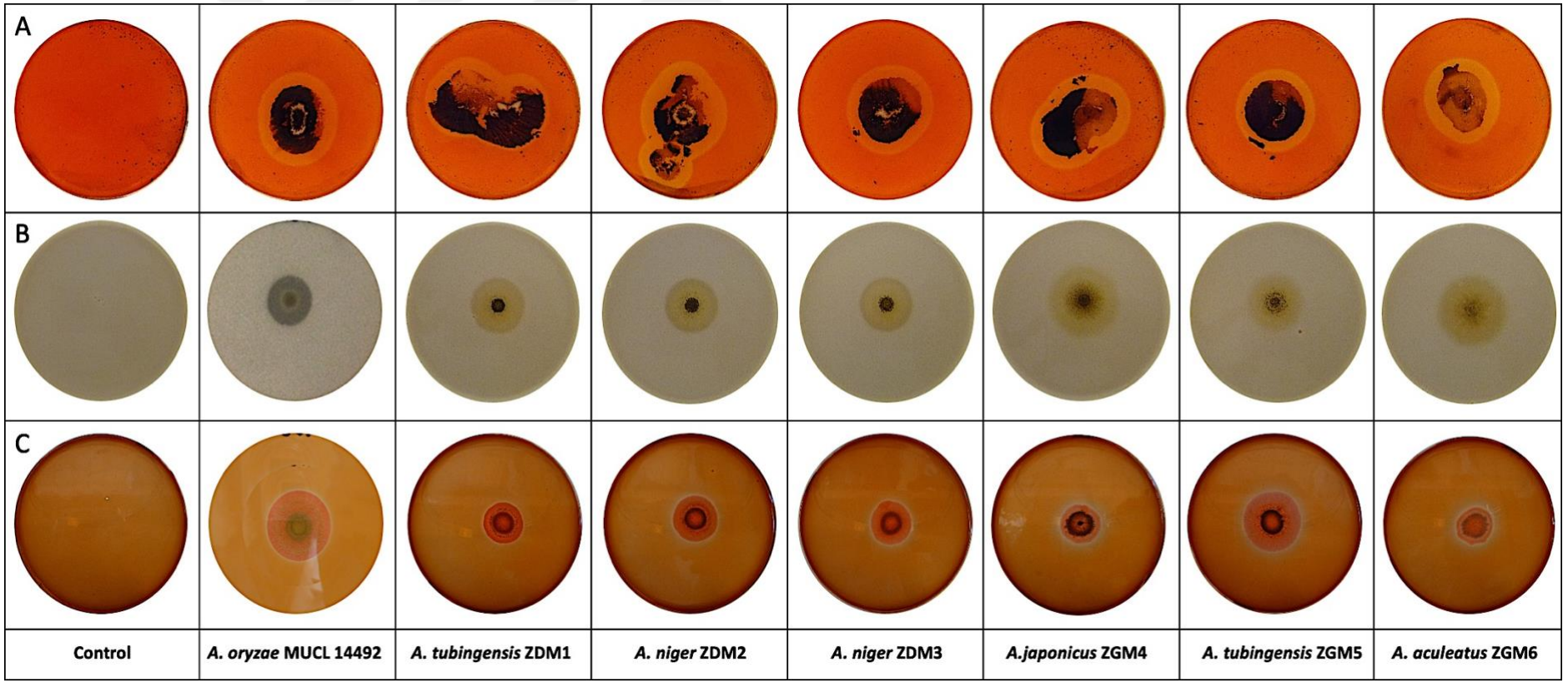


Figure 4.3 : Cellulase (A), tannase (B), and pectinase (C) enzyme activity on plate screening method.

Aspergillus oryzae MUCL 14492 was employed in solid medium as a reference culture. This culture has been extensively used before, particularly to produce cellulase, tannase and pectinolytic enzymes (Heerd et al, 2012; Koseki et al, 2018; Pirota et al, 2016). According to plate screening results, there were no significant differences among isolates and reference culture for cellulase and pectinase production. In the case of the screening of cellulase activity of molds, decoloration was seen better after the dying with Congo red.

The diameter of the clear zones showing cellulase activity ranged between 49 and 57 mm. There were no significant differences in diameter of clear zones produced by molds ($P>0.05$). Extracellular cellulase enzyme was reported by Rathore et al. (2014) for *Aspergillus fumigates* and *A. niger* on the CMC supplemented agar with a clear zone of 12 and 16 mm, respectively.

As a result of hydrolysis of tannic acid by tannase produced from molds, color of the plates turned from purple to colorless. The diameter of the clear zones ranged between 22 and 30 mm. *A. japonicus* ZGM4 and *A. aculeatus* ZGM6 produced the highest activity of tannase compared to those of the other isolates and reference culture showed the lowest tannase activity. Tannase enzyme was reported to be produced by *Aspergillus* spp. on solid media with a clear zone of 13–14 mm (Murugan et al, 2007) which was lower than those found in this study.

All six molds were incubated in pectin-Congo red containing medium after 72 h incubation time to determine the pectinase enzymes acitivity. The diameter of the clear zones ranged from 28 to 38 mm and there was no significant difference between zones produced by molds ($P>0.05$). Our findings were higher than that found by Souza et al. (2003), a clear zone of 19-25 mm, and Hannan et al. (2008), a clear zone of 13 mm. However, similar results reported for *A. aculeatus* with 35–40 mm clear zone by Taskin et al. (2008).

In the literature, some studies reported that the enzyme production on solid medium showed high correlation with those of liquid medium (Ten et al 2004; Murugan et al 2007). However, some studies showed the opposite (Tseng et al 2000). Therefore, the enzyme production ability of molds was examined on both solid and in liquid media.

4.2.2 Screening of isolates for enzyme production in liquid medium

Enzyme production ability of molds were confirmed in standard liquid medium for each enzyme. Similar to the plate screening method, for the liquid screening CMC, tannic acid and pectin were used as the main carbon source for cellulase, tannase and pectinase enzyme activity, respectively.

The enzyme production is generally associated with the growth phase of microorganisms. Therefore, to follow growth during incubation, biomass of all molds was determined. The biomass of molds showed different trend for each enzyme during incubation time. For cellulase and pectinase enzymes, the biomass of all isolates was found as 5.36±1.22 mg/mL and 5.71±0.15 mg/mL, respectively. There was no significant difference between incubation days. The biomass in the case of tannase increased until 48 h of incubation (3.76±0.51 mg/mL) and remained constant until the end of incubation.

4.2.2.1 Cellulase

The initial pH value of the cellulose containing medium was around 7 and the pH value of the medium did not change during incubation time. Cellulase activity of all isolates increased during the incubation time and peak activity was achieved after 96 h (Figure 4.4). *A. niger* ZDM2, *A. japonicus* ZGM4 and *A. aculeatus* ZGM6 produced higher cellulase activity compared to other isolates after 96 h of incubation (Table A.1). Peak enzyme activity of the isolates expressed per volume of medium was ranged between 2–3.8 U/mL, except *A. tubingensis* ZGM5 which produced an activity of 0.56 U/mL. In a study by Gautam et al. (2011), the maximum cellulase activity (1.8 U/mL) was produced by *A. niger* after 96 h of incubation. Imran et al. (2017) also reported that cellulase activity of *A. tubingensis* IMMIS2 increased continuously up to 96 h and then decreased. Enzyme activity fluctuated in some isolates which can be explained by catabolite repression from excess product during degradation of cellulose (Ang et al, 2013).

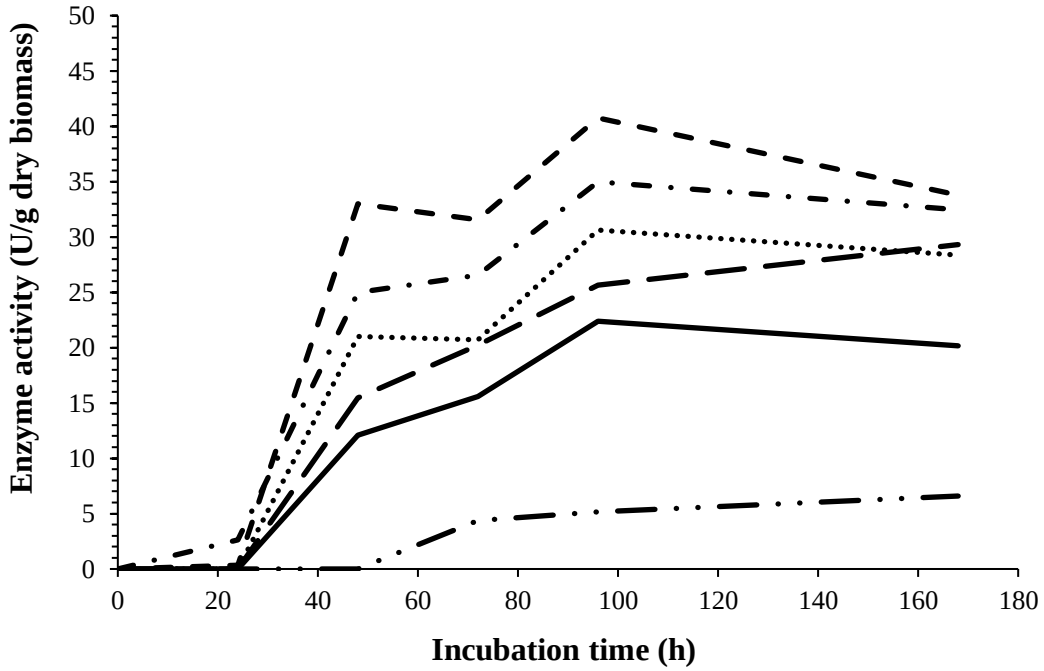


Figure 4.4 : Change in cellulase activity of *Aspergillus* spp. during seven days of incubation. *A. tubingensis* ZGM5 (__ ••) ; *A. tubingensis* ZGM1 (___) ; *A. niger* ZDM3 (__ __) ; *A. niger* ZDM2 (• • •) ; *A. aculeatus* ZGM6 (__ •) ; *A. japonicus* ZGM4 (_ _).

In some studies, cellulase production and enzyme activity were found to be higher in solid state fermentation than those in submerged fermentation (Kumar et al, 2011; Mrudula and Murugammal, 2011; Reddy et al, 2015). In solid state fermentation, growth occurs in absence or nearly absence of free water, thus being close to the natural environment for microbial growth. Because of that, molds can be easily adapted to solid state fermentation. One important advantage in solid state fermentation is that enzyme synthesis is less affected by catabolic repression which appeares to be limiting enzyme production in submerged fermentation (Acuna-Arguelles et al, 1995; Kumar et al, 2011).

4.2.2.2 Tannase

The tannase activity was obtained by *A. tubingensis* ZDM1, *A. niger* ZDM2 and *A. japonicus* ZGM4 and *A. aculeatus* ZGM6 at higher level compared to others after 24 h of incubation (Table A.2). These results were higher compared to those reported in the literature for *Aspergillus* spp. by Banerjee et al. (2007) and Murugan et al. (2007). Lal et al. (2012) reported higher tannase activity than those in this study for *A. niger* isolated from bark of *Acacia nilotica* which contained high level of tannin.

The maximum tannase activity was found at 96 h incubation for *A. fumigatus* reported in the study of Yadav et al. (2008), while Banerjee et al. (2007) showed a maximum tannase production by *A. aculeatus* after 72 h of incubation. In our study, the maximum tannase activity was obtained after 24 h incubation and then a decline was observed for all *Aspergillus* spp. (Figure 4.5). This decline can be explained by the changing of media components due to the fungal growth like accumulation of end-products such as gallic acid and secretion of toxic substances such as catechuic acid, benzoic acid and pyrogallol (Kar and Banerjee, 2000). Previous studies also showed that presence of pyrogallols, gallic acid and gallaldehyde can cause cell disruption and inhibit the tannase production (Srivastava and Kar, 2009).

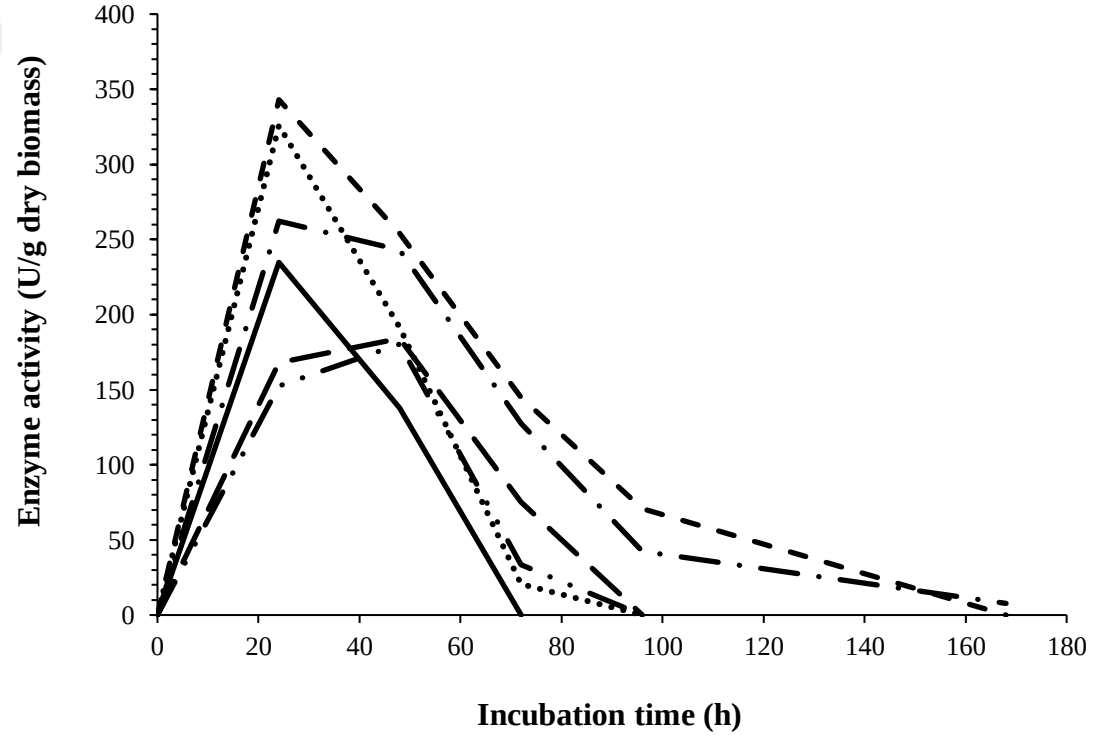


Figure 4.5 : Change of tannase activity of *Aspergillus* spp. during seven days of incubation. *A. tubingensis* ZGM5 (__ • •) ; *A. tubingensis* ZGM1 (___) ; *A. niger* ZDM3 (__ __) ; *A. niger* ZDM2 (• • •) ; *A. aculeatus* ZGM6 (__ •) ; *A. japonicus* ZGM4 (_ _).

The optimum pH of tannase enzyme is around 5.5. Tannase enzyme activity could be affected by pH of medium due to its acidic origin (Banerjee et al, 2007). Initial pH of medium was around 4 and it declined to 3 after 1 day of incubation. The pH of medium started to increase after 2 days of incubation and it reached to 6–7 after 7 days of incubation. This increase can be explained by the consumption of tannic acids or the production of alkaline compounds during the incubation time (Zeni et al, 2011). Lal et

al. (2012) also reported similar trend for tannase activity of *A. niger* with pH change where maximum activity was observed at pH 5.0, and then decreased when the pH of medium reached alkaline values.

4.2.2.3 Pectinase

All isolates produced pectinase, but the incubation time for peak activity was changed according to the isolate (Figure 4.6). *A. tubingensis* ZGM5 showed the maximum pectinase activity (130±66 U/g dry biomass; 25±2 U/mL) (Table A.3). Taskin et al. (2008) reported pectinase production by *Aspergillus* spp. isolated from vineyards with activity in the range of 44–122 U/mL. The difference between these results could be due to the composition of the media and the method used for determination of activity.

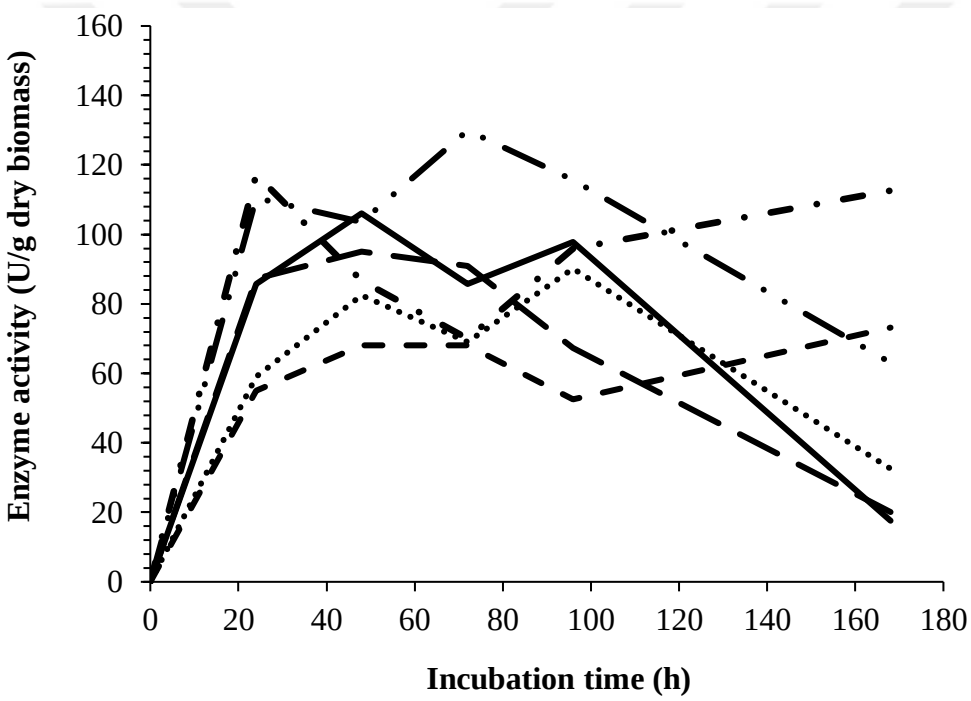


Figure 4.6 : Change of pectinase activity of *Aspergillus* spp. during seven days of incubation. *A. tubingensis* ZGM5 (__ • •) ; *A. tubingensis* ZGM1 (___) ; *A. niger* ZDM3 (__ __) ; *A. niger* ZDM2 (• • •) ; *A. aculeatus* ZGM6 (__ •) ; *A. japonicus* ZGM4 (_ _).

The pH of medium showed fluctuations for pectinase enzyme during incubation time depending on the isolate. The pH of liquid medium used for all isolates decreased from 4.5 to 3–3.5 after 3 days of incubation. An increase in pH to 5.5 was observed for *A. japonicus* ZGM4 and *A. aculeatus* ZGM6 while the pH remained constant for other isolates after 4 days. Mahesh et al. (2016) reported that pH 4 was optimum for pectinase activity by *Aspergillus ibericus*. It increased with pH increase from 3 to 4

and then the activity decreased with further increase in pH. The increase in pH can be explained by the consumption of organic acids by molds due to the lack of carbon source (Botella et al, 2005). The decrease in pH can be related to the release of galacturonic acid to the medium due to the action of pectinase enzymes produced by molds during the incubation time (Zeni et al, 2011).

There was a decline in activity of tannase and pectinase after a few days of incubation for some isolates. This decline might be explained with the hydrolysis of the produced enzymes by isolates due to lack of nutrients in fermentation medium (Botella et al, 2005). In addition, changes in the fermentation conditions such as pH and production of inhibitory substances compared to the initial conditions could affect the activity of enzymes (Gautam et al, 2011; Zeni et al, 2011).

4.2.3 Conclusion

Findings of this study have revealed the potential of hydrolytic enzyme production by newly isolated black *Aspergillus* spp. in standard plate screening and liquid media. All isolates produced tannase with high activity, however *A. japonicus* ZGM4 and *A. aculeatus* ZGM6 were found to be the best isolates for producing tannase. Additionally, *A. tubingensis* ZGM5 can produce tannase and pectinase, while it was not competent to produce cellulase with high activity. Various enzymes such as cellulase and pectinase are often required to disrupt the plant cell wall, thus enhancing the extraction of bioactive components. The results obtained in this section provided perspective to evaluate use of isolated black *Aspergillus* spp. for releasing phenolic compounds from industrial wastes.

4.3 Gross Composition, Phenolic Profile and Antioxidant Activity of Wastes

4.3.1 Gross composition of wastes

The results of the physicochemical analyses of wastes are shown in Table 4.5. Apple peel, apple pomace and pomegranate peel had over 70% moisture content. Pomegranate seed and black carrot pomace had moisture content around 45-60%. The lowest moisture content was measured in chestnut shell. The moisture of wastes except chestnut shell is high enough to maintain microbiological growth.

Table 4.5 : Composition of wastes.

Component (%)	Apple peel	Apple pomace	Pomegranate peel	Pomegranate seed	Chestnut shell	Black carrot pomace
Moisture	72.0	78.2	71.5	58.3	20.6	44.1
Ash	1.7	1.4	3.7	2.2	1.2	4.0
Total protein	5.9	3.4	3.7	13.7	4.3	2.6
Reducing sugar	2.5	10.9	22.5	14.2	6.7	3.8
Free glucose	0.8	75.8	22.3	24.4	2.7	11.3
Condensed tannin	0.3	0.4	0.6	0.2	5.2	0.3

In the six analysed industrial wastes, the protein content ranged from 2.6 to 13.7%. The lowest and highest protein contents were found in black carrot pomace and pomegranate seed, respectively. The protein content found in pomegranate seed (13.7%) was lower than melon seed protein content (16.4%) obtained by Kamel et al. (1985), but higher than found in grape seed (10.1-11.8%) by Fantozzi (1981). The protein content of peels in pomegranate was consistent with the findings of Romelle et al. (2016) and Pathak et al. (2017) who reported that pomegranate peel consititutes 3.46% and 5.1% protein.

In all tested wastes, the greatest concentration of condensed tannin was found in chestnut shell (5.2%) followed by pomegranate peel (0.6%) and the least amount was in pomegranate seed (0.2%). Lees et al. (1995) reported that condensed tannin ranged from 1.4 to 4.3% in peel and 0.2 to 0.6% in pulp of eleven different apple varieties. Vasconcelos et al. (2010) reported the condensed tannin of eight different cultivars of chestnut integument and pericarp where the integument contained 13.8% and pericarp had 2.0% on dry basis.

The ash fraction was determined at a concentration of 1.7% and 1.4% in apple peel and pomace, respectively. In the literature, similar results were reported for apple peel and pulp ranging from 1.4 to 3.5% (Albuquerque et al, 2006; Romelle et al, 2016; Sato et al, 2010). Ash content was 3.7 and 2.2% in pomegranate peel and seed, respectively. Ash content of pomegranate peel was reported as 6.1% (Romelle et al, 2016). The ash content of chestnut shell was 1.2%. Vazquez et al. (2008) also found ash content of

chestnut shell as 0.8%. The highest ash content was present in black carrot pomace because of its high potassium, calcium and sodium content.

The chemical composition of fruits, especially mineral composition, can be changed in different types of fruits as well as within different parts of the same fruits depending on the several factors like variety, maturity, soil type, soil condition and growing region, climate and also storage (Romelle et al, 2016). The findings present in the literature quite vary in a wide range because of the above mentioned differences. Giving information about the main mineral and essential trace elements of wastes is important in terms of minerals play a key role in various physiological functions of the body, especially in the building and regulation processes. The mineral compositions of wastes are represented in Table 4.6. The main minerals in wastes were found as calcium, potassium, sodium and magnesium followed by iron, cooper and zinc. Black carrot pomace had the highest sodium, calcium, magnesium, iron and zinc. Pomegranate peel was rich in potassium, whereas pomegranate seed was rich in copper.

Table 4.6 : Mineral composition of wastes.

Mineral (mg/100 g)	Apple peel	Apple pomace	Pomegranate peel	Pomegranate seed	Chestnut shell	Black carrot pomace
Sodium	13.4±1.7	11.6±3.8	15.3±5.0	20.5±3.0	13.0±2.5	385.1±12.0
Potassium	497.9±30.0	544.0±10.4	1392.7±116.3	720.4±9.1	712.0±9.1	804.2±69.6
Calcium	146.5±10.4	80.6±4.0	412.0±21.5	129.4±4.0	392.6±20.4	492.2±11.2
Magnesium	81.7±7.1	61.2±1.1	55.2±7.1	99.6±4.0	89.8±8.6	160.5±6.9
Iron	7.5±0.6	1.4±0.3	ND	4.2±0.3	2.2±0.3	20.9±2.1
Copper	0.6±0.1	0.1±0.0	ND	1.4±0.1	ND	0.1±0.0
Zinc	0.5±0.4	0.1±0.2	0.1±0.1	1.7±0.2	0.9±0.1	1.9±0.2

The mineral content was previously analysed in different cultivars of chestnut by Pereira-Lorenzo et al. (2006) and the main macroelements were found as potassium, phosphorus, calcium and magnesium with potassium representing the majority. Manganase, iron, zinc and copper were also reported as microminerals. In this study, the macrominerals of chestnut shell were potassium, calcium, magnesium and sodium, and iron was also present.

4.3.2 Total phenolic and flavonoid contents of wastes

The highest TPC was found in pomegranate peel followed by chestnut shell, pomagranate seed, apple peel, black carrot pomace and apple pomace (Figure 4.7a). In respect of the distribution of phenolics, insoluble-bound phenolics were 88, 53, 47, 45, 25 and 16% of TPC of apple peel, black carrot pomace, chestnut shell, apple pomace, pomegranate seed and pomegranate peel, respectively. The TFC varied from 1.0±0.1 to 8.0±0.3 mg CE/g dm in soluble form; 2.1±0.2 to 24.4±4.0 mg CE/g dm in insoluble-bound form. The highest TFC was determined in chestnut shell followed by pomegranate peel (Figure 4.7b). About 88, 86, 54, 54, 49 and 35% of TFC were present in insoluble-bound form in apple peel, chestnut shell, black carrot pomace, apple pomace, pomegranate seed and pomegranate peel, respectively.

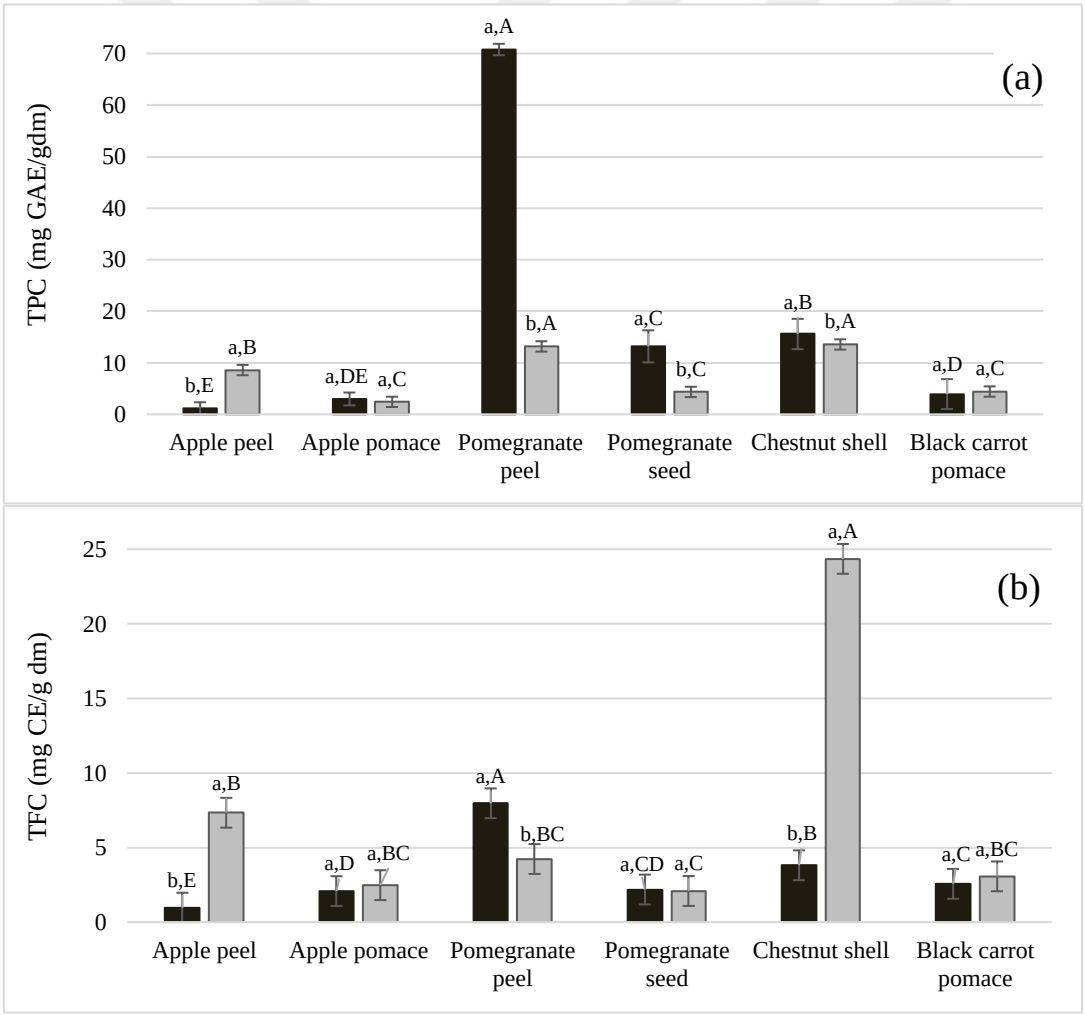


Figure 4.7 : Total phenolic (a) and total flavonoid (b) contents in soluble (black) and bound (gray) extracts of wastes. Means of soluble and insoluble-bound phenolics for each waste marked with different lowercase letters are significantly different (P<0.05). Means of soluble or insoluble-bound phenolics of wastes marked with different uppercase letters are significantly different (P<0.05).

Pomegranate is known to be one of the richest fruits in polyphenols. Particularly, fruit peel contains high amounts of phenolic compounds compared to edible part. Studies have also reported that the phenolic content of pomegranate peels was higher than that found in the pulp (Li et al, 2006). Pomegranate peel contained the highest amount of phenolics in soluble form among the wastes. It also contained higher TPC and TFC in its soluble phenolics extract than those in its bound phenolics extract. TPC content of soluble extract of pomegranate peel was found 70.8±4.0 mg GAE/g dm and 13.2±2.8 mg GAE/g dm in insoluble-bound form and these results were 5.4 and 3-fold higher than TPC of pomegranate seeds, in soluble and insoluble-bound form, respectively. This distribution of TPC and TFC in pomegranate peel shows that the majority of phenolics could be easily extracted. Previous studies also showed that pomegranate peel had higher amount of soluble phenolics than its pulp and seed (Fischer et al, 2011; He et al, 2011). Al-Rawahi et al. (2014) found TPC and TFC of pomegranate peel as 64.2 mg GAE/g dm and 1.4 mg CE/g dm, respectively. Abid et al. (2017) found the highest TPC and TFC at a level of 304.60 mg GAE/g dm and 15.46 mg QE/g dm, respectively. Values found in this study were between in the results reported in these two studies.

The TPC in soluble extract of pomegranate seed was found as 13.2±6.9 mg GAE/g dm and 4.4±3.6 mg GAE/g dm in insoluble-bound form. These results were lower than those reported by He et al. (2011). However higher than the study performed by Ambigaipalan et al. (2017). The difference between the values reported in the literature and values of TPC and TFC from this study could be explained with the variety of pomegranate, sample preparation method and extraction conditions (Wang et al, 2011).

The TFC in insoluble-bound extract of chestnut shell were found 6.4-folds higher than that present in soluble phenolic extract. Chestnut shell contained the highest TPC and TFC in its bound phenolics extract among the wastes. Comandini et al. (2014) evaluated TPC of four different chestnut bark samples, and found values ranged from 239 and 561 mg GAE/g. Barreira et al. (2008) reported that outer skin of chestnut had more phenolic and flavonoid contents than inner skin. They found the TPC of outer skin and inner skin as 510 and 475 mg GAE/g dm, respectively. TFC of outer skin and inner skin were determined as 503 and 330 mg CE/g dm, respectively. The findings indicated by Squillaci et al. (2018) showed TPC and TFC values of a blend of inner

and outer chestnut shell extract was almost 5-folds higher than that of the inner chestnut shell extract.

Black carrot pomace had similar amounts of phenolics in soluble and bound forms. TPC of black carrot was reported as 350 mg/100 g fresh weight by Kaur and Kapoor (2002). Assuming the moisture content of black carrot as 86%, we can conclude that black carrot pomace retained phenolics of fresh black carrot at a level around 16 and 18% in soluble and insoluble-bound form, respectively.

The TFC and TPC of apple peel were present in insoluble-bound form more than in soluble form. The TPC in insoluble-bound form of apple peel was almost 7 times higher than that in soluble form. The phenolic content of apple peel was more than that of apple pulp which composed of more flesh part. Sun et al. (2002) showed that edible parts of apple contained more soluble phenolics than bound ones. In addition, Henriquez et al. (2010) reported that apple peel had higher amount of soluble phenolics than those in pulp and whole fruit.

4.3.3 Antioxidant activity of wastes

The chemical activities of polyphenols in terms of their reducing properties as hydrogen or electron-donating agents predicts their potential for their action as free-radical scavenger. Antioxidant activity of soluble and insoluble-bound phenolic extracts was determined by DPPH and CUPRAC assays (Figure 4.8). The DPPH assay measures the ability of an antioxidant to reduce the stable deep purple DPPH radical and reduction in its color is monitored over a given time. CUPRAC assay is an electron transfer-based antioxidant capacity assay.

Pomegranate peel showed the highest antioxidant activity in soluble form with a decreasing order of chestnut shell > pomegranate seed > black carrot pomace > apple pomace > apple peel. Chestnut shell showed the highest antioxidant activity in bound form with a decreasing order of pomegranate peel > apple peel > black carrot pomace > pomegranate seed > apple pomace. Similar tendencies were observed in antioxidant activities measured by DPPH and CUPRAC methods.

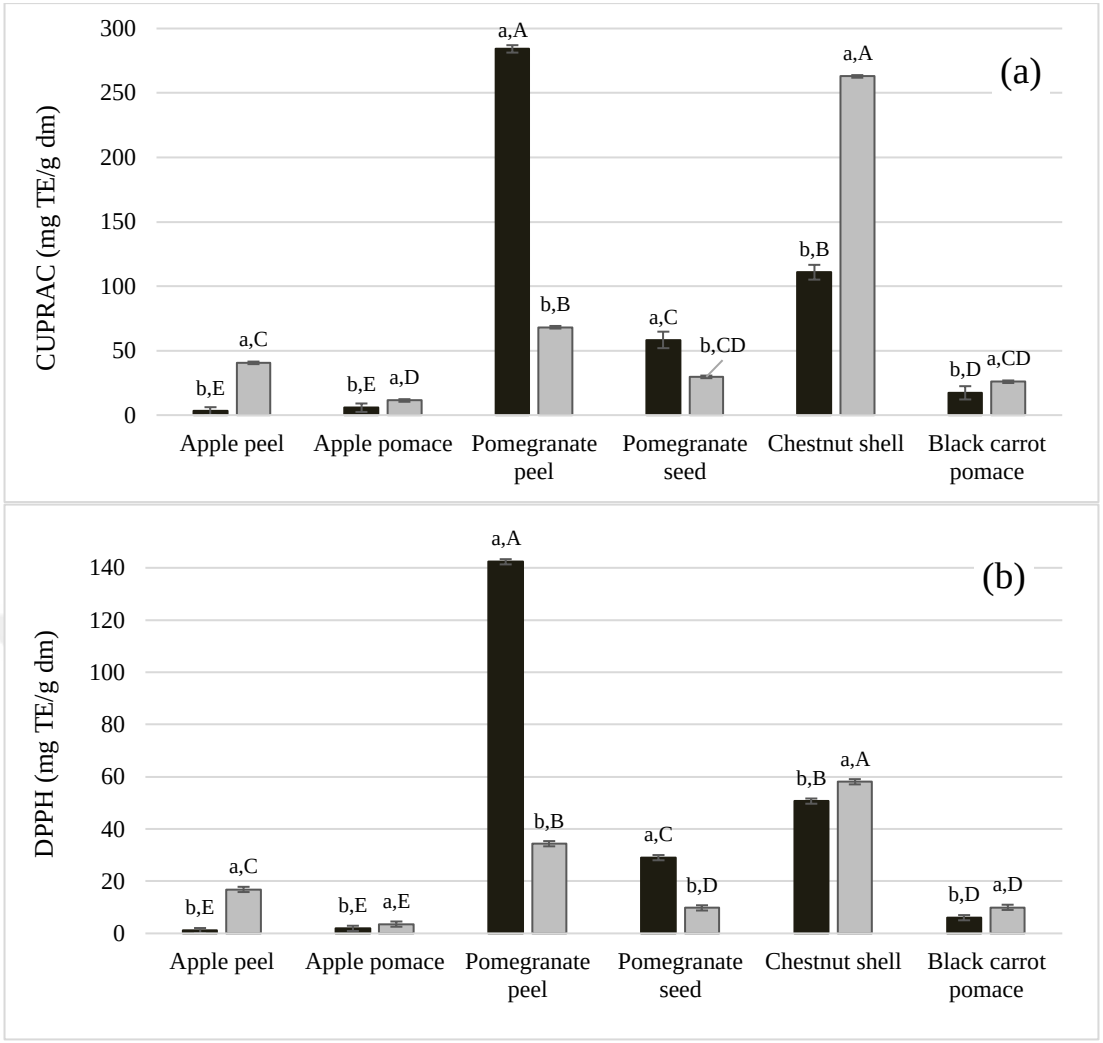


Figure 4.8 : Antioxidant activity in soluble (black) and bound (gray) extracts of wastes by DPPH (a) and CUPRAC (b) assays. Means of soluble and insoluble-bound phenolics for each waste marked with different lowercase letters are significantly different (P<0.05). Means of soluble or insoluble-bound phenolics of wastes marked with different uppercase letters are significantly different (P<0.05).

Many agricultural residues and byproducts from the food industry contain a variety of phenolics which are associated with the prevention of degenerative diseases and this may be ascribed to their action as antioxidants (Kim et al, 2005). Results of this study showed that plant wastes have antioxidant potential. Antioxidant activity measured by CUPRAC and DPPH methods of bound extract were found 17-20, 2.5, 1.5-2.9 and 2-folds higher than that of soluble extract for apple peel, pomegranate peel, chestnut shell and black carrot pomace, respectively. In addition to these, when we compared black carrot pomace with the fresh black carrot which studied by Koley et al. (2014), the antioxidant activity of black carrot pomace still had antioxidant activity of fresh black carrot around 10% and 24% in soluble and bound form, respectively.

4.3.4 Phenolic profile of wastes

Soluble-free and insoluble-bound phenolic compounds of wastes were identified and quantified using HPLC (Figure D.1 and D.2). Phenolic profile can vary depending on cultivar grown in different geographical conditions. The individual phenolic compounds present in plants can be influenced by soil quality, climate and stress conditions where plants are grown as well as different extraction conditions (Ambigaipalan et al, 2017).

Gallic acid, ellagic acid, cyanidin and punicalagin derivatives were identified in soluble phenolic extract of pomegranate peel (Table 4.7). Ellagic acid was the major peak in soluble phenolic extract of pomegranate peel followed by punicalagin derivatives. Ellagic acid and gallic acid concentration were higher in soluble phenolic extract of pomegranate peel than insoluble-bound extract. Abid et al. (2017) reported that the phenolic profile of pomegranate peel consists of ellagic acid and its derivatives, punicalagin derivatives and cyanidin derivatives. Punicalagin was reported as the major soluble phenolic of pomegranate husk which was also identified in pomegranate juice as a result of processing (Aloqbi et al, 2016; Gil et al, 2000). Gallic acid, catechin, epicatechin, ellagic acid and other hydrolyzable tannins were also identified in soluble form in pomegranate peel in these studies. Middha et al. (2013) reported that pomegranate peel of Indian cultivar contains gallic acid, ellagic acid, punicalagin, quercetin and rutin derivatives. Elfalleh et al. (2011) found that gallic acid, ellagic acid, caffeic acid and *p*-coumaric acid were the major phenolic acids identified in peel extract of Tunisia pomegranate cultivar. The phenolic profile of pomegranate peel of cultivar from Italy contains hydrolysable ellagitannins such as α- and β-punicalagin, pedunculagin, ellagic acid derivatives (Pagliarulo et al, 2016). Phenolic profiles and their concentration can vary due to the pomegranate cultivars grown in different geographical conditions (Sing et al, 2018).

Ellagic acid, gallic acids and punicalagin derivatives were found in the soluble phenolics extract of pomegranate seed, however, at lower concentrations than those in pomegranate peel. Antioxidant activity of punicalagin was reported to be 6-fold and 2.5-fold higher than those of ellagic and gallic acids (Gil et al, 2000) which explains high antioxidant activity of soluble phenolics extract of pomegranate peel. In addition, high amount of ellagic acid derivatives possibly gave rise to antioxidant activity of the peel (Gil et al, 2000). Bound extract of pomegranate seed contained only ellagic acid

in significant amount. Ambigaipalan et al. (2017) found that ellagic acid was the main hydrolysable tannin in bound form in pomegranate seed. They also identified phenolic acids and flavonoids in bound form in pomegranate seed. He et al. (2011) reported that caffeic acid, pedunculagin, procyanidin dimer and trimer, catechin, *p*-coumaric acid, quercitrin, kaempferol and ferulic acid were found in pomegranate seed.

Gallic acid, ellagic acid and catechin were identified in soluble extract and gallic acid, protocatechuic acid and ellagic acid were detected in bound extract of chestnut shell. Ellagic acid was the most abundant compound followed by gallic acid in both soluble and insoluble-bound phenolics extracts of chestnut shell. Squillaci et al. (2018) reported that gallic acid was the most abundant phenolic compound identified in aqueous soluble extract of chestnut shell followed by protocatechuic acid, ellagic acid, epicatechin, catechin, *p*-coumaric acid and scopoletin. Insoluble-bound phenolics extract of chestnut shell was not rich in phenolic compounds, however, it showed higher antioxidant activity than its soluble phenolics extract. The TFC content of bound phenolics extract of chestnut shell was higher than those of other wastes. There could be other flavonoid compounds in chestnut shell contributing to the antioxidant activity that were not identified by the HPLC analysis.

The three major anthocyanins detected in soluble extract of black carrot pomace were cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, malvinidin-3-*O*-glucoside. The major phenolic acid was identified as chlorogenic acid. In bound extract, the major phenolic acid was found as ferulic acid, followed by sinapic acid, chlorogenic acid, protocatechuic acid, *p*-coumaric acid and 4-hydroxybenzoic acid in insoluble-bound phenolics extract of black carrot pomace. Kamiloglu et al. (2016) also found that chlorogenic acid as the most abundant soluble phenolic in black carrot pomace accounting for approximately 90% of the total phenolic acids. They also reported cyanidin derivatives, caffeic acid, ferulic acid, cryotochlorogenic acid and neochlorogenic acid were found in methanolic extracts of black carrot pomace. According to phenolic profiles, soluble phenolics extract of black carrot pomace was expected to have higher antioxidant activity than that of its bound phenolics extract but this was not the situation. Bound phenolics extract might have other components that could contribute to its antioxidant activity.

Table 4.7 : Concentration of soluble free and insoluble-bound phenolic compounds in wastes.

Compound	Concentration (mg/100 g dm)†											
	Apple peel		Apple pomace		Pomegranate peel		Pomegranate seed		Chestnut shell		Black carrot pomace	
	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound
Hydroxybenzoic acids												
Gallic acid	0.09±0.02	-‡	0.50±0.02	-	19.0±3.9	7.71±1.25	2.81±0.22	0.44±0.12	7.82±0.38	18.96±0.27	-	-
4-hydroxybenzoic acid	0.39±0.01	-	-	-	-	-	-	-	-	-	0.21±0.03	0.62±0.14
Protocatechuic acid	-	0.56±0.05	-	0.28±0.02	-	-	-	-	-	3.98±0.02	-	0.62±0.13
Ellagic acid	-	-	-	-	3697±99	199±7	1426±118	14.62±0.14	52.1±1.0	52.1±1.9	-	-
Hydroxycinnamic acids												
Chlorogenic acid	-	-	17.69±0.02	-	-	-	-	-	-	-	79.1±3.6	2.01±0.12
p-coumaric acid	-	0.22±0.01	1.38±0.03	0.51±0.01	-	-	-	-	-	-	-	0.76±0.06
Ferulic acid	-	-	-	-	-	-	-	-	-	-	-	4.55±0.42
Sinapic acid	-	-	-	-	-	-	-	-	-	-	-	2.12±0.21
Anthocyanins												
Cyanidin-3,5-*O*-diglucoside	-	-	-	-	4.68±0.31	-	-	-	-	-	-	-
Cyanidin-3-*O*-galactoside	-	-	-	-	-	-	-	-	-	-	18.0±1.6	-
Cyanidin-3-*O*-glucoside	-	-	-	-	4.51±0.26	-	14.73±0.45	-	-	-	70±6	-
Peonidin-3-*O*-glucoside	-	-	-	-	-	-	0.44±0.03	-	-	-		-
Malvidin-3-*O*-glucoside	-	-	-	-	-	-	-	-	-	-	35.1±2.7	-
Isoflavonoids												
Daidzein	3.17±0.38	-	-	-	-	-	-	-	-	-	-	-
Flavanols												
Catechin	-	2.05±0.18	6.67±0.01	-	-	-	-	-	17.1±1.7	-	-	-
(-)-Epicatechin	-	-	7.05±0.01	-	-	-	-	-	-	-	-	-
Flavanones												
Naringenin	-	-	9.62±0.21	-	-	-	-	-	-	-	-	-
Flavonols												
Quercetin-3-*O*-galactoside	10.48±0.11	-	-	-	-	-	-	-	-	-	-	-
Quercetin-3-*O*-glucoside	3.96±0.04	-	-	-	-	-	-	-	-	-	-	-
Quercetin-3-*O*-glucuronide	5.57±0.05	-	-	-	-	-	-	-	-	-	-	-
Quercitrin	7.73±0.01	-	-	-	-	-	-	-	-	-	-	-
Chalcones												
Phlorizin	2.91±0.10	-	2.81±0.07	-	-	-	-	-	-	-	-	-
Hydrolyzable tannins												
Punicalagin derivative 1	-	-	-	-	1076±92	-	194±13	-	-	-	-	-
Punicalagin derivative 2	-	-	-	-	698±83	-	136±28	-	-	-	-	-

†Means ± standard deviation (n=3).

‡ -: Not detected

While soluble phenolics extract of apple peel was rich in daidzein, quercetin, quercitrin and phlorizin, that of apple pomace contained high amounts of chlorogenic acid, *p*-coumaric acid, catechin, epicatechin, naringenin and phlorizin. Suarez et al. (2010) reported that quercetin glycosides, dihydrochalcones, epicatechin and procyanidin B2 were determined as major phenolics in apple pomace. Dineiro-Garcia et al. (2009) also identified six quercetin glucosides as major phenolics in the apple pomace, hyperin, isoquercitrin, rutin, reynoutrin, avicularin and quercitrin. The presence of quercetin derivatives in soluble phenolics extract of apple peel which was not found in bound form, could be a result of degradation of these phenolics by alkali treatment.

Protocatechuic and *p*-coumaric acids were identified as major phenolics in insoluble-bound form in apple peel and apple pomace (Table 4.7). Catechin was also found in insoluble bound phenolic extract of apple peel. Catechin in bound phenolics extract of apple peel could originate from residual pomace present. Bound phenolics extract of apple peel had higher antioxidant activity than that of its soluble one. Presence of catechin in bound phenolics extract of apple peel possibly affected its antioxidant activity positively compared to that of apple pomace.

There was a positive correlation between phenolic content and antioxidant activity. Correlation coefficients were over 0.95 between soluble TPC or TFC and DPPH or CUPRAC values. Correlation coefficients for bound phenolics and antioxidant activity were relatively lower (0.88 for bound TPC-DPPH, 0.72 for bound TPC-CUPRAC, 0.87 for bound TFC-DPPH, 0.95 for bound TFC-CUPRAC). Correlation coefficient between TPC and antioxidant activity was also reported to be higher than that between TFC and antioxidant activity by DPPH or ORAC tests done for different fruit samples (Kalita and Jayanty, 2014).

Even though, bound phenolics extracts of wastes except those of pomegranate had higher antioxidant activity, concentrations and number of phenolics in them were generally lesser compared to those of corresponding soluble phenolics extract. This could be due to the presence of unidentified constituents with reducing activity other than phenolics in bound extracts that are released by alkali hydrolysis. In addition, synergistic action of phenolics in a particular profile can enhance antioxidant activity.

4.3.5 Conclusion

Industrial plant wastes were found to be good resources for proteins, minerals and phenolic antioxidants. Agro-industrial wastes analysed in this study were found to be rich in some macro- and micronutrients and phenolic antioxidants that can be utilized in the future. Production processes need to be developed accordingly to extract both soluble and bound phenolics with high yield and bioactivity. Obtained information from this part of thesis would be helpful in design and optimization of extraction process of phenolics in valorization of plant wastes. Owing to its richness in polyphenols in bound form compared to its soluble form, apple peel and chestnut shell had a potential to utilize for releasing of bound phenolic compounds.

4.4 Bioconversion of Phenolic Compounds of Apple Peel Using *Aspergillus* spp. and Optimization Studies of Fermentation Media

4.4.1 Total phenolic and flavonoid contents of fermented apple peel

SSF of apple peel with different *Aspergillus* spp. was monitored during 7 days in order to determine changes in TPC by fermentation (Figure 4.9). TPC of naturally fermented sample was similar to that of unfermented apple peel. TPC of apple peel fermented with natural flora was not changed significantly ($P>0.05$) during fermentation time. TPC of apple peel increased by fermentation with *Aspergillus* spp. upto 7 days except the sample fermented with *A. aculeatus* ZGM6 compared to that of the sample with natural flora. Among the samples, the sample fermented with *A. niger* ZDM2 had the highest TPC followed by the sample fermented with *A. tubingensis* ZDM1.

After 7 days of fermentation by *Aspergillus* spp., the TFC of apple peel exhibited approximately 4-fold increase, whereas apple peel fermented by natural flora did not show any significant change during fermentation (Figure 4.10). Higher TFC values were observed in the apple peel fermented with *A. tubingensis* ZDM1 compared to those of other fermented samples.

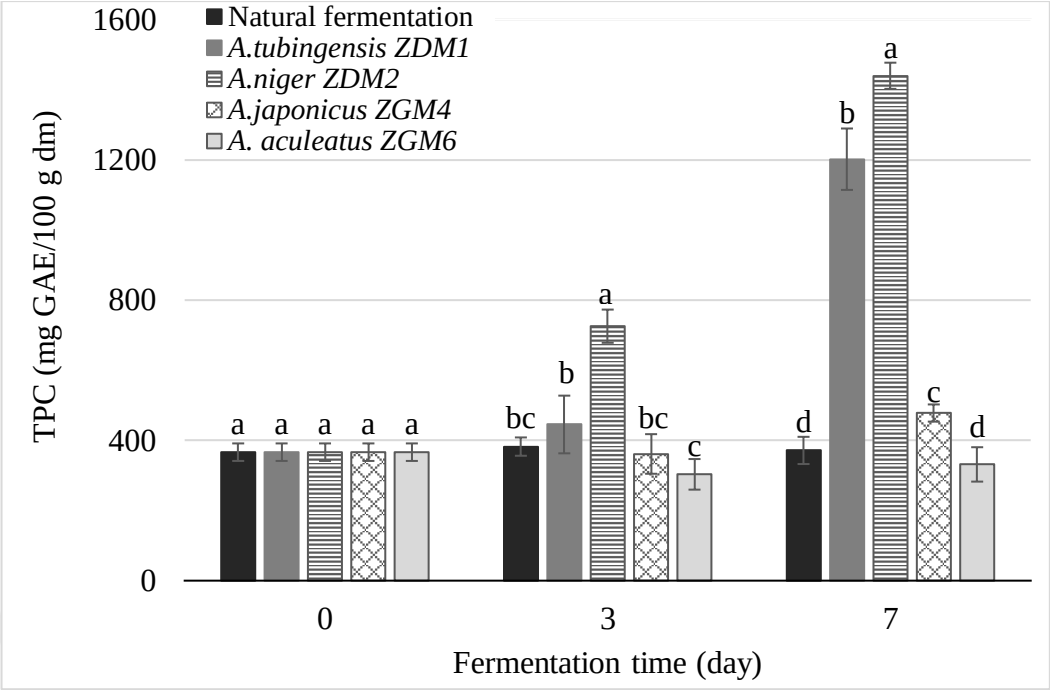


Figure 4.9 : Total phenolic content of apple peel fermented by natural flora and *Aspergillus* spp. Different letters above the bars indicate significant differences among the isolates at each day (P<0.05).

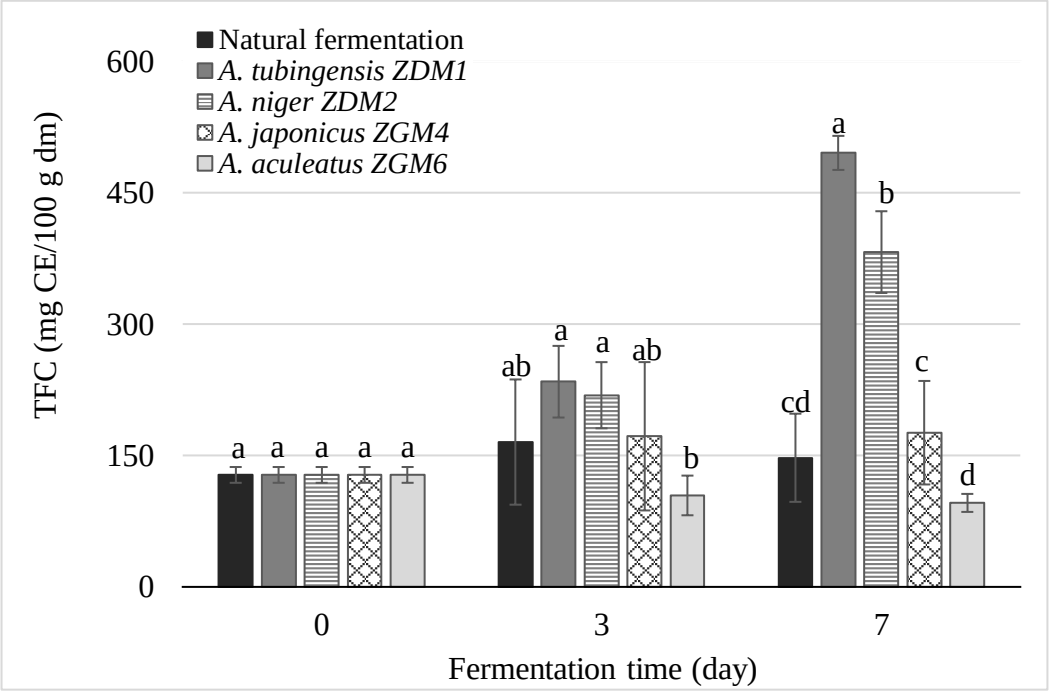


Figure 4.10 : Total flavonoid content of apple peel fermented by natural flora and *Aspergillus* spp. Means marked with different letters above the bars indicate significant differences among the isolates at each day (P<0.05).

Results from this study were in agreement with those reported by previous studies that mold fermentation could enhance phenolic and flavonoid contents. This situation can be due to either the formation of aglycones from glycosides or release of phenolics

from cell wall structure by microbial enzymes. Huynh et al. (2016) reported that the TPC in outer leaves of cauliflower increased by 3-fold after fermentation by *A. sojae*. Sadh et al. (2017) also reported that SSF using *Aspergillus* spp. enhanced TPC of *Oryza sativa* seed and flour by 3 to 6-fold, respectively.

4.4.2 Determination of phenolic profile of unfermented and fermented apple peels

The profiles of phenolic compounds extracted from the samples were quantified and identified by HPLC-PDA and LC-MS/MS (Figure 4.11, Table 4.8). The major compounds present in unfermented apple peel were quercetin and its derivatives (compounds 1, 2, 3, 4, 5 and 6). These compounds were initially identified by comparing their retention times and UV-VIS spectra with those of the standards. The identities of compounds were also confirmed by matching the mass spectra of them obtained from LC-MS/MS with those of the standards. Compounds 1, 2, 5 and 6 were assigned as quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin-3-*O*-rhamnoside and quercetin, respectively. Our laboratory did not have standards for compounds 3 and 4. Therefore, MS spectrum of these compounds was characterized by ions and both were assigned the molecular formula $C_{21}H_{20}O_{12}$ on the basis of molecular ion at *m/z* 433.0770. The corresponding loss of a pentose unit gave fragment ions at *m/z* 301.0345 and 301.0346, respectively (Figure E.1). Higher collision energies showed that characteristic fragment ions of quercetin were observed for these compounds and therefore, they are both likely quercetin derivatives containing one pentose attached to the aglycone. As a result, compound 3 and 4 were identified as quercetin-pentosides because the exact glycoside or its location on quercetin cannot be determined from the mass spectrometry. Sanchez-Rabaneda et al. (2004) also found the same fragment and molecular ions of the quercetin-pentoside in apple pomace.

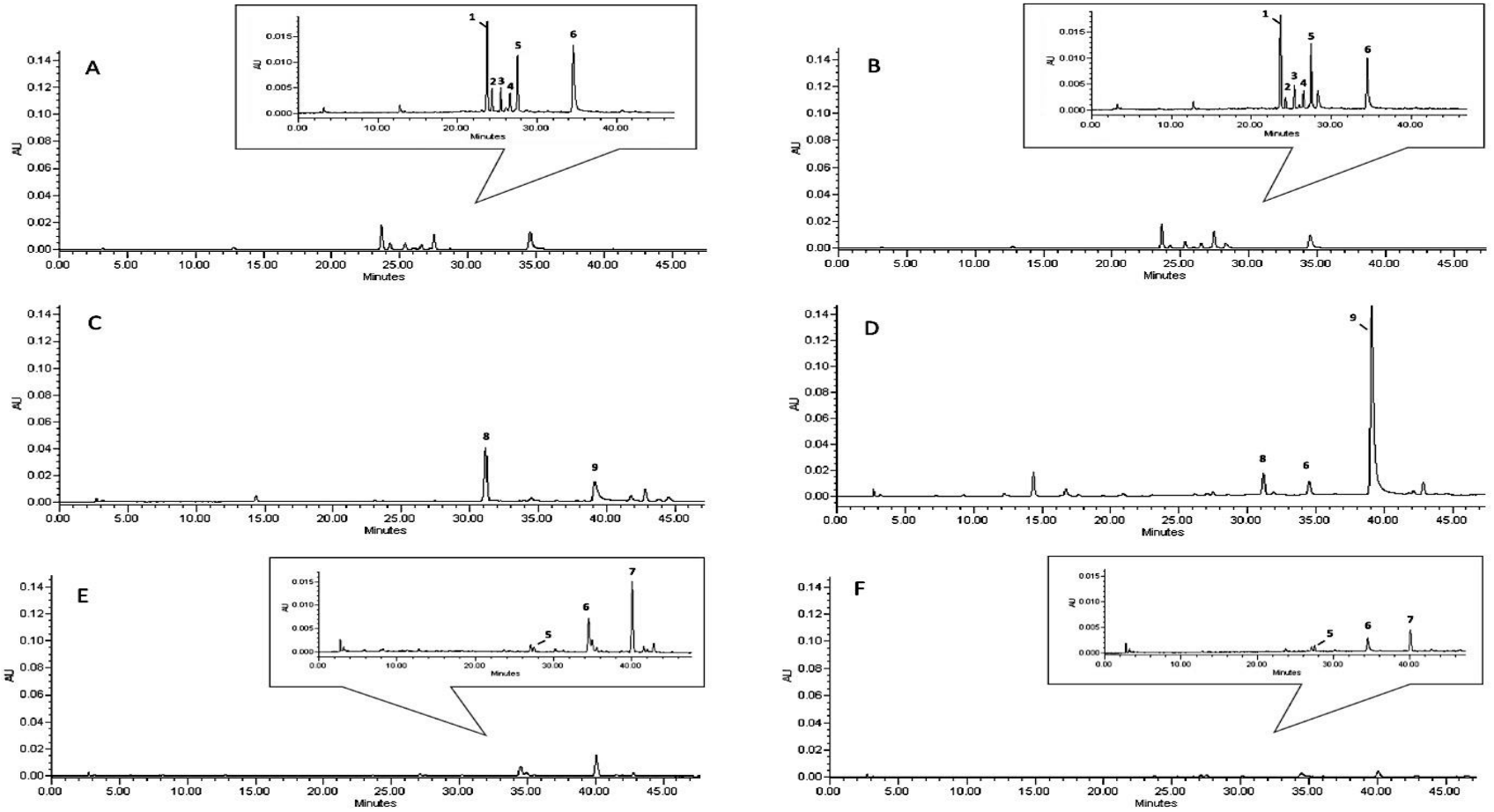


Figure 4.11 : HPLC chromatogram of phenolic compounds from unfermented (A), and fermented apple peel for 7 days by natural flora (B), *Aspergillus tubingensis* ZDM1 (C), *A. niger* ZDM2 (D), *A. japonicus* ZGM4 (E), *A. aculeatus* ZGM6 (F) recorded at 360 nm.

Table 4.8 : Phenolic compounds identified by HPLC-MS-MS in unfermented and fermented apple peel extracts.

Compound	RT (min)	*m/z* values		Proposed Identity
		Parent Exact Mass	Major Fragments*	
1	23.68	463.0879	300, 271, 255	Quercetin 3-*O*-galactoside+
2	24.30	463.0880	300, 271, 255	Quercetin 3-*O*-glucoside+
3	25.40	433.0773	300, 271, 255	Quercetin-pentoside
4	26.58	433,0773	300, 271, 255	Quercetin-pentoside
5	27.52	447.0927	300, 271, 255	Quercetin-3-*O*-rhamnoside+
6	34.56	301.0351	151, 121, 107	Quercetin+
7	40.06	303.0510	243, 215	Taxifolin isomer
8	31.15	289.0717	205, 190	Catechin isomer
9	39.09	287.0563	272, 257	Eriodictyol or isomer

* Major fragments with NCE=55 eV.
+ Confirmed with standard.

Based on the identification by LC-MS/MS, there was only one phenolic aglycone detected in the unfermented apple peel as compound 6 (quercetin), indicating that the phenolic compounds present in apple peel occur mainly in glycosidic forms. These findings are in agreement with the previous study of Marks et al. (2007) who also reported that predominant phenolic compounds in apple peel were mainly quercetin glycosides and (–)-epicatechin.

Naturally fermented apple peel did not show significant difference during fermentation (Figure D.3), while apple peel was fermented by *Aspergillus* spp., profile of phenolic compounds was changed (Table 4.9). Concentrations of compounds 2 (quercetin 3-*O*-glucoside), 3 and 4 (quercetin-pentoside) were reduced after 3 days and they were not detectable after 7-day fermentation. The concentration of compound 1 (quercetin 3-*O*-galactoside) decreased by upto 98% after 7 days of fermentation compared to the initial concentration. The concentration of compound 5 (quercetin-3-*O*-rhamnoside) was also reduced by 90% after 7-day fermentation. While the concentration of compound 6 (quercetin) did not change significantly after 7-day fermentation of apple peel fermented with *A. niger* ZDM2, it was reduced in other samples (Figure D.4).

Table 4.9 : The concentration of phenolic compounds in unfermented and fermented apple peel by natural flora and *Aspergillus* spp.

Compound	Initial	Concentration (µg/g dm)†									
		Natural flora		*A. niger* ZDM2		*A. tubingensis* ZDM1		*A. aculeatus* ZGM6		*A. japonicus* ZGM4	
		Day 3	Day 7	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7
Quercetin 3-*O*-galactoside	178±6	179±13	162±26	25±9	6±2	14±3	3±1	19±5	4±1	8±2	4±1
Quercetin 3-*O*-glucoside	38±6	39±5	16±4	6±1	-‡	-	-	-	-	-	-
Quercetin-pentoside	52±6	48±7	41±5	13±3	4±1	8±1	-	13±4	-	-	-
Quercetin-pentoside	63±8	35±5	38±13	7±3	-	5±1	-	12±5	-	-	-
Quercetin-3-*O*-rhamnoside	114±8	117±18	128±27	69±4	33±9	29±2	-	44±13	10±1	24±8	10±1
Quercetin	199±9	179±24	142±36	225±7	181±39	142±21	32±1	70±14	36±3	112±23	117±6
Taxifolin isomer	-	-	-	-	-	-	-	23±3	40±5	61±6	142±1
Catechin isomer	-	-	-	330±1	580±22	586±167	1434±96	-	-	-	-
Eriodictyol isomer	-	-	-	2641±50	6392±145	627±188	806±23	-	-	-	-

†Means ± standard deviation (n=3).
‡ - : not detected.

Three major phenolic aglycones (compounds 7, 8, 9) were observed in fermented apple peel, while these compounds were absent in unfermented apple peel. Production and concentration of these new phenolics were dependent on the *Aspergillus* spp. used. Their concentration increased continuously during fermentation for 7 days.

Compound 7 was produced by *A. japonicus* ZGM4 and *A. aculeatus* ZGM6 in apple peel. The MS spectrum of compound 7 was assigned the molecular formula $C_{15}H_{12}O_7$ on the basis of a molecular ion at *m/z* 303.0510 and fragment ions at *m/z* 243 and 215. The UV absorption spectra of compound 7 was observed at 285 nm. Although the compound was tentatively assigned as taxifolin based on a previous study reported by Kocabova et al. (2016) the fragmentation was not consistent with taxifolin so we suspect the compound is actually an isomer and has been referred to as such throughout the text.

Compound 8 was formed by *A. niger* ZDM2 and *A. tubingensis* ZDM1 in apple peel. Chemical formula of compound 8 was determined as $C_{15}H_{14}O_6$ from the molecular ion $[M\text{-}H]^-$ at *m/z* 289.0717 and a fragment ion at *m/z* 205 (Figure E.2). Although compound 8 has the same chemical formula as (+)-catechin and (-)-epicatechin and even shared some similar fragments, the retention time was different. Therefore, it could not be identified neither with the standards nor by the literature. In conclusion, compound 8 appears to be a structural isomer of catechin and we have referred to it as such throughout the text.

Compound 9 was observed in apple peel fermented with *A. niger* ZDM2 and *A. tubingensis* ZDM1. This compound was tentatively identified on the basis of its *m/z* value and UV spectrum (λ_{max}= 398 nm) based on the previous reports (Gonzales et al, 2016; Sanchez-Rabaneda et al, 2004). MS analysis of the compound gave a molecular ion at *m/z* 287.0563 and a fragment ion at *m/z* 272 in negative mode, which is consistent with eriodictyol (Figure E.3). However, we did not see a characteristic fragment peak at m/z 151 at any collision energy and this peak is reported as a dominant fragment ion in the literature. Therefore, we assigned compound 9 as eriodictyol, but this is a low confidence assignment and we recognize that this peak is more likely an isomer of eriodictyol.

4.4.3 Antioxidant activity of fermented apple peel

Antioxidant activity of apple peel with natural flora was not changed during fermentation of 7 days which was also supported by its phenolic profile (Table 4.10). On the other hand, the samples fermented with *Aspergillus* spp. had higher antioxidant activity compared to those of the unfermented sample and the sample fermented with natural flora. While the sample with *A. aculeatus* ZGM6 did not exhibit a significant change in antioxidant activity during fermentation, other samples showed an increasing trend in antioxidant activity. Especially, fermentation with *A. tubingensis* ZDM1 and *A. niger* ZDM2 resulted in approximately 3-fold and 5-fold enhancement of antioxidant activity after 7-day fermentation by CURPAC and DPPH methods, respectively. There was a strong correlation between antioxidant activity and phenolic content of fermented apple peel with correlation coefficients of 0.98 and 0.97 for CUPRAC and DPPH methods, respectively. Similar results were also obtained by Lin et al. (2014) where SSF by *A. awamori* significantly increased the phenolic content and enhanced the antioxidant activity of the litchi pericarp.

Table 4.10 : Antioxidant activity of apple peel fermented by natural flora and *Aspergillus* spp.

Isolates	CUPRAC* (mg TE/100 g dm)			DPPH* (mg TE/100 g dm)		
	Initial	Day 3	Day 7	Initial	Day 3	Day 7
Natural Fermentation	$868\pm45_{a,A}$	$892\pm63_{a,C}$	$878\pm123_{a,C}$	$772\pm40_{a,A}$	$738\pm30_{a,C}$	$788\pm82_{a,C}$
A. tubingensis ZDM1	$868\pm45_{c,A}$	$1506\pm265_{b,B}$	$2780\pm161_{a,A}$	$772\pm40_{c,A}$	$1569\pm178_{b,A}$	$4297\pm261_{a,A}$
A. niger ZDM2	$868\pm45_{c,A}$	$1831\pm60_{b,A}$	$3092\pm245_{a,A}$	$772\pm40_{c,A}$	$1597\pm15_{b,A}$	$4081\pm85_{a,A}$
A. japonicus ZGM4	$868\pm45_{c,A}$	$981\pm17_{b,C}$	$1389\pm111_{a,B}$	$772\pm40_{c,A}$	$895\pm72_{b,B}$	$1213\pm47_{a,B}$
A. aculeatus ZGM6	$868\pm45_{b,A}$	$1030\pm144_{ab,C}$	$1065\pm123_{a,C}$	$772\pm40_{b,A}$	$841\pm20_{ab,BC}$	$935\pm157_{a,C}$

*Each value was expressed as mean±SD (n=3). Means marked with different lower case letters within a row are significantly different. Means marked with different upper case letters within a column are significantly different ($P<0.05$).

Increase in antioxidant activity of apple peel by fermentation can be explained by different antioxidant activity of individual phenolics and concentration of these phenolics in extract. In addition, there could be interactive effects among phenolic antioxidants in a medium that could result in varying antioxidant activity (Wang et al, 2012). Antioxidant activity of phenolic compounds enhances with increasing number of OH groups in the structure. When the phenolic compound bind a sugar molecule

like glucose, rhamnose or rutinose, its antioxidant activity is reduced due to reduction in scavenging of radicals by the sugar moiety. The trolox equivalent antioxidant capacity (TEAC) of quercetin (4.7 mM) was reported to be approximately 2-fold higher compared to that of quercetin-3-*O*-rutinoside (2.4 mM) (Heim et al, 2002). Quercetin has stronger antioxidant activity when compared with those of other phenolic compounds which are saturated at the double bonds at 2' and 3' position. Taxifolin differs from quercetin by the double bound in the C-ring, while eriodictyol differs from quercetin by the double bound between 2´ and 3´ and one OH group at the position 3´ of the C ring (Braune et al. 2001). The TEAC of quercetin (4.7 mM) is almost two times higher than those of the TEAC of catechin (2.4 mM), taxifolin (1.9 mM) and eriodictyol (1.8 mM) (Heim et al. 2002).

Fermentation with *A. japonicus* ZGM4 yielded more taxifolin isomer compared to that with *A. aculeatus* ZGM6. Apple peel fermented with *A. japonicus* ZGM4 had also significantly higher TPC and TFC than the sample with *A. aculeatus* ZGM6. In addition, the sample fermented by *A. japonicus* ZGM4 still contain 60% of the initial amount of quercetin which is the strongest antioxidant among the ones identified in this study.

The catechin isomer was not found in the samples fermented with *A. aculeatus* ZGM6 and *A. japonicus* ZGM4, while the samples fermented with *A. niger* ZDM2 and *A. tubingensis* ZDM1 contained high concentration of this compound. Eriodictyol isomer was also present in the samples fermented with *A. niger* ZDM2 and *A. tubingensis* ZDM1. Quercetin was also present at higher concentration in these samples compared to those in the other mold-fermented samples. Presence of quercetin, catechin and eriodictyol isomers in these samples resulted in higher antioxidant activity compared to those of the others.

The control sample did not have any catechin, however the concentration of catechin increased after 7-day fermentation by *A. niger* ZDM2 and *A. tubingensis* ZDM1. In a previous study, we found that the cell wall hydrolyzing enzymes can be produced by *Aspergillus* spp. (Gulsunoglu et al, 2019a). The increase in the concentration of catechin by these species can be explained by enzymatic hydrolysis of cell wall-bound catechins. Martins et al. (2016) also found catechin can be released by hydrolytic enzymes in white and red grape pomace including peel.

Depending on the type of strain, phenolic compounds can be metabolized differently by molds which use different degradation pathways (Sridevi et al, 2012). Decrease in the concentration of quercetin and its glycosides could be associated with the bioconversion of these compounds into other metabolites. During fermentation, molds can produce enzymes which hydrolyze bound or conjugated phenolics into biologically more active free phenolics (Huynh et al, 2016; Lin et al, 2014).

This is the first report about the production of eriodictyol and taxifolin isomers in apple peel using *Aspergillus* spp. under SSF. When quercetin derivatives decreased, eriodictyol and taxifolin isomers increased during fermentation time in two fermented samples. This bioconversion can be explained by either hydrogenation and dehydroxylation of quercetin and its derivatives or the production of these phenolic compounds by the secondary metabolism of microorganisms during fermentation (Lin et al, 2014; Liu et al, 2010).

Increase in antioxidant activity by fermentation possibly caused by release of bound phenolics and formation of new reductants during fermentation process. Reductants react with free radicals and stabilize them by reducing power. In addition, the intracellular antioxidants and peptides of the microorganisms and their hydrogen donating ability might also contribute to the increase in reducing ability of fermented wastes (Xiao et al, 2015).

4.4.4 Optimization studies of fermentation conditions by PBD and RSM

Nine variables (5 nitrogen sources, 3 carbon sources and fermentation time) were evaluated with regard to their effects on phenolic compounds of apple peel under SSF of *A. niger* ZDM2 using a PBD (Table B.1). The Pareto chart of effects is a useful plot for identifying the factors that are important. In these charts, bar lengths are proportional to the absolute value of the estimated effects, helping to compare their relative importance (Table B.2). Working at a 95% confidence level determined by a vertical line (T-value limit: 2.78) as critical limit, the Pareto chart shows that the significant effects were fermentation time, concentrations of lactose and urea (Figure 4.12).

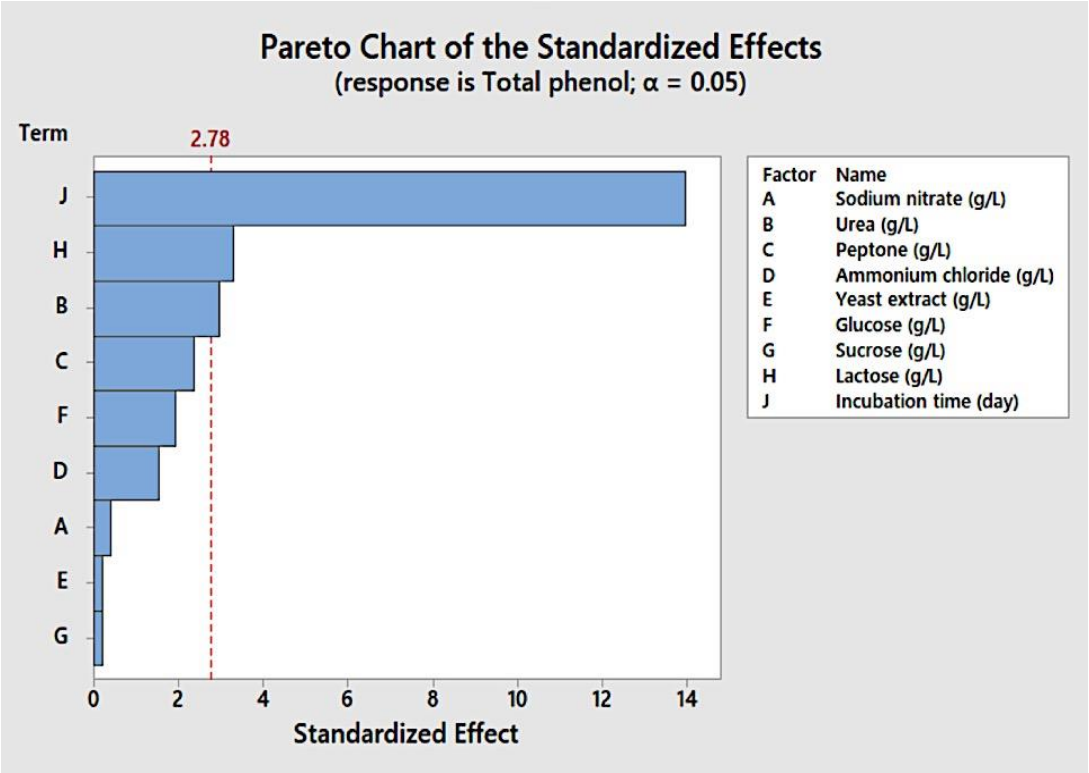


Figure 4.12 : Standardized Pareto chart for various factors influencing the total phenolic compounds of apple peel.

Lactose concentration and fermentation time were found to have positive significant effects with T-value of 3.29 and 13.96, respectively. On the other hand, concentration of urea exhibited significant negative effect with a T-value of –2.98 (Table B.3). When the effect has positive sign, the influence of the factor on the phenolic content of fermented apple peel is greater at a high concentration, and when the effect has negative sign, opposite is valid. Therefore, ranges of lactose concentration and fermentation time were chosen to cover their higher levels and range of the concentration of urea was selected to cover its lower level in further optimization study by RSM.

PBD is well established and widely used statistical tool for evaluation of different variables. Following the initial screening, the next step is optimizing the variables selected through PBD. Several researchers in biotechnological processes have applied these statistical techniques for optimization of different parameters. Zarate-Chaves et al. (2013) optimized fermentation conditions using PBD for production of phenolic compounds by *Ganoderma lucidum*. According to the PBD results, sucrose, yeast extract and olive oil was found as effective factors for production of phenolics within tested variables (glucose, sucrose, lactose, yeast extract, peptone, ammonium chloride,

olive oil and thiamine). Liu et al. (2008) studied optimization of fermentation conditions for xylanase production by *A. niger* SL-05 using PBD. Due to optimization results, urea and KH_2PO_4 was found as significant factors. Yeast extract, moisture content, KH_2PO_4 and Na_2HPO_4 were found as effective parameters for fermentation of pineapple waste for production of citric acid (Imandi et al, 2008). The nutrient requirements can be changed due to the microorganisms. However, all these studies were shown that statistical methodologies are useful for optimizing fermentation conditions.

CCD in RSM was applied to determine optimum levels of fermentation time, urea and lactose concentrations on the phenolic content and antioxidant activity of fermented apple peel. Effects of all studied factors on responses were found statistically significant (Table B.4) (P<0.05). The F-values of the models were found to be 263.11 and 123.39 for TPC and DPPH, respectively, implying that the models were significant (Table B.5 and B.6). The lack of fit was not found significant showing that the quadratic model was valid for the data. The models for TPC and DPPH activity responses in terms of uncoded significant factors were shown in equations 4.1 and 4.2. Regression coefficients for the model for TPC were found as: R_2=99.51%, R_2(adjusted)=99.14%. Regression coefficients for the model for DPPH activity were found as: R_2=98.97%, R_2(adjusted)=98.17%.

$$TPC = 753 + 17.76X_1 - 189.8X_2 - 257.2X_3 - 0.07823X_1^2 + 1.074X_1X_2 + 1.184X_1X_3 + 27.88X_2X_3 \quad (4.1)$$

$$DPPH = 4548 + 44.8X_1 - 1174X_2 - 688X_3 - 0.2622X_1^2 + 8.15X_1X_2 + 3.209X_1X_3 + 73.0X_2X_3 \quad (4.2)$$

The effects of interaction between factors were also found significant on the responses. In order to elaborate the effects of factors on the TPC, three-dimensional response surface plots were presented in Figure 4.13. Three-dimensional plots for TPC were obtained by keeping one variable constant at the center point and changing the other two variables within the experimental range. The model equations with the help of surface plots are used to describe the individual and cumulative effects on the responses in these plots. Figure 4.13a indicated that an increase in the concentration of urea with extending fermentation time had a positive effect on the TPC. Increasing

concentration of lactose in fermentation medium and longer fermentation time led to an increase in TPC (Figure 4.13b). Maximum TPC was obtained at the highest lactose and urea concentrations when fermentation time was held at 120 h (Figure 4.13c).

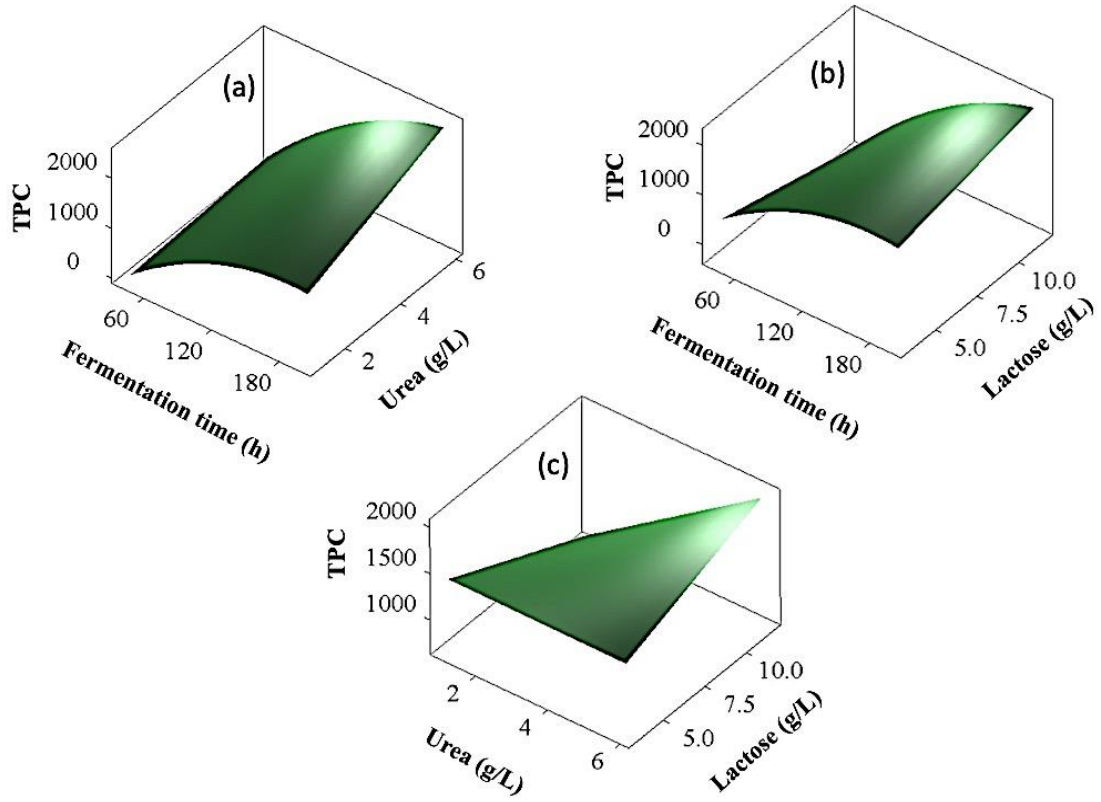


Figure 4.13 : Surface plot illustrating the effect of fermentation time, lactose and urea concentrations on TPC of apple peel fermented by *Aspergillus niger* ZDM2. (a) Effects of fermentation time and urea at 7.5 g/L lactose concentration. (b) Effects of fermentation time and lactose at 3.5 g/L urea concentration. (c) Effects of lactose and urea concentration after fermentation for 120 h.

Fermentation time and lactose concentration affected DPPH activity significantly (P<0.05), while urea concentration had no influence. Interactive effects between factors on DPPH activity were shown in Figure 4.14. The interactions between factors also affected DPPH activity significantly (Figure 4.14a, b, c). An increase in the concentration of urea with longer fermentation time had a positive effect on the DPPH activity. A similar interactive effect between lactose and fermentation time was observed. Maximum DPPH was obtained at the highest lactose and urea concentrations when fermentation time was held at 120 h.

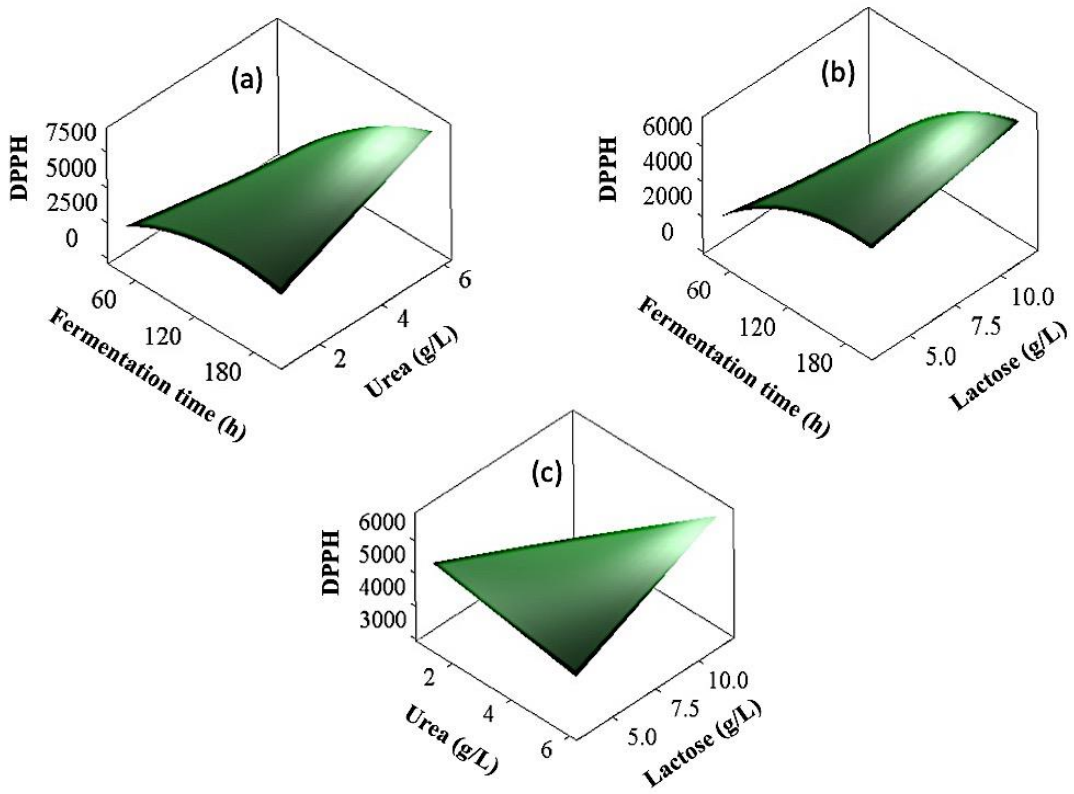


Figure 4.14 : Surface plot illustrating the effect of fermentation time, lactose and urea concentration on DPPH of apple peel fermented by *Aspergillus niger* ZDM2. (a) Effects of fermentation time and urea at 7.5 g/L lactose concentration. (b) Effects of fermentation time and lactose at 3.5 g/L urea concentration. (c) Effects of lactose and urea concentration after fermentation for 120 h.

At the final step, to validate of the optimum values, three replicated trials were conducted at optimum conditions predicted by the model. Optimum conditions for TPC and DPPH activity was determined as 11.5 g/L lactose, 5.9 g/L urea, and 198 h fermentation time. Trials yielded TPC at a concentration of 2864±105 mg GAE/100 g dm which was close to the predicted concentration of 2998±95 mg GAE/100 g dm by the model. The experimental and predicted values of DPPH activity were also found to be close which were 9088±64 mg TE/100 g dm and 10187±452 mg TE/100 g dm, respectively. These results indicated that developed models can be successfully applied for predicting the production level of TPC and DPPH activity under the optimum conditions.

4.4.5 Conclusion

The fermentation of apple peel with *Aspergillus* spp. was found to be a suitable way of increasing phenolic content and producing significant levels of different phenols depending on the isolate. SSF of apple peel by *Aspergillus niger* ZDM2 for 7 days caused a marked increase in phenolic and flavonoid contents and enhanced antioxidant activity. By optimization studies, TPC and DPPH activity of fermented apple peel was increased approximately 6-fold and 10-fold, respectively, by fermentation of *A. niger* ZDM2 compared to those of unfermented apple peel. Based on the results, fermentation of fruit waste is recommended as a possible way of valorization to produce phenolics with high antioxidant activity which could be used in manufacture of new functional foods and nutraceuticals.

4.5 Bioconversion of Phenolic Compounds of Chestnut Shell Using *Aspergillus* spp. and Optimization Studies for Fermentation Media

4.5.1 Screening of *Aspergillus* spp. for production of ellagic acid

Four different *Aspergillus* isolates were investigated for the production of ellagic acid with chestnut shell under SSF for 7 days (Table 4.11). The initial concentration of ellagic acid in chestnut shell measured as 0.48 mg/g dm. Sorice et al. (2016) reported that the ellagic acid concentration in chestnut shell was 1.05 mg/g dm, whereas Abe et al. (2010) found free ellagic acid in chestnut shell was 0.0074 mg/g dm and after acid hydrolysis (released from ellagitannins and/or glycosides) total ellagic acid was reported as 2.98 mg/g dm. These results showed that fungal biotransformation of chestnut shell can be a good alternative to chemical hydrolysis methods for production of ellagic acid. The difference between the initial ellagic acid concentrations can be due to the variety, cultivar, maturity, harvesting year as well as soil type, soil composition, and climatic factors (Neri et al. 2010). Another possible reason could be extraction method and solvents used.

Table 4.11 : Ellagic acid production by *Aspergillus* spp. on chestnut shell.

Isolates	Ellagic acid (mg/g)		
	Day 0	**Day 3**	**Day 7**
Control	$0.48\pm0.02^{b,A}$	$1.20\pm0.24^{a,B}$	$0.52\pm0.06^{b,B}$
A. tubingensis ZDM1	$0.48\pm0.02^{b,A}$	$1.40\pm0.03^{a,AB}$	$1.67\pm0.23^{a,A}$
A. niger ZDM2	$0.48\pm0.02^{b,A}$	$1.63\pm0.17^{a,AB}$	$1.69\pm0.24^{a,A}$
A. japonicus ZGM4	$0.48\pm0.02^{b,A}$	$1.77\pm0.23^{a,A}$	$2.04\pm0.31^{a,A}$
A. aculeatus ZGM6	$0.48\pm0.02^{b,A}$	$1.61\pm0.14^{a,AB}$	$1.42\pm0.35^{a,A}$

*Each value was expressed as mean±SD (n=3). Means marked with different lower case letters within a row are significantly different. Means marked with different upper case letters within a column are significantly different ($P<0.05$).

Fermentation by *A. japonicus* ZGM4 exhibited approximately 4-fold (2.04 mg/g dm) increase in concentration of ellagic acid (Figure D.5), whereas the control sample did not show any significant difference at 7-day fermentation compared with the initial ellagic acid concentration (Figure D.6). Shi et al. (2005) reported that ellagic acid concentration of valonea tannins increased 1.47-fold and 1.78-fold after fermentation by *A. niger* and *Candida utilis*, respectively. Vattem and Shetty (2002) reported increase in ellagic acid concentration from 0.26 mg/g dm to 0.35 mg/g dm under SSF of cranberry pomace by *Lentinus edodes*. Madeira et al. (2014) showed that the orange waste fermented by *Paecilomyces variotti* for 120 h yielded ellagic acid at a concentration of 10 mg/g dm from an initial concentration of 0.7 mg/g dm. Sepuldeva et al. (2016) reported that partially purified polyphenols from pomegranate peel, cranberry and creosote bush yielded higher amount of ellagic acid after fermentation by *A. niger*. This was explained by the structure of purified polyphenols that have a glucose core making them more susceptible to attack by microbial enzymes. Sepulveda et al. (2018) achieved a yield of 21 mg/g ellagic acid from pomegranate husk polyphenols by using *A. niger* PSH under SSF which was performed with polyurethane foam as a support material. Fermentation of pomegranate peel seems to yield more ellagic acid compared to that from chestnut shell. This situation can be explained by more hydrolysable tannins of pomegranate peel corresponding to ellagitannins (Aguilar et al, 2008). However, chestnut shell tannins seem to be of the condensed type in view of their high Stiasny number (Vazquez et al, 2008).

A. japonicus ZGM4 was selected for further optimization studies. To explain the production of ellagic acid by *A. japonicus* ZGM4, phenolic profiles of unfermented and fermented chestnut shell were examined in detail. When the HPLC chromatograms of phenolic compounds extracted from the unfermented and fermented chestnut shell were compared, there were five major peaks showing a change after fermentation. Concentration of compound 1 (Peak 1) was increased and those of compounds 2, 3, 4 and 5 (Peaks 2, 3, 4 and 5) were decreased by fermentation (Figure 4.15). Compound 1 showed a peak at a retention time of 19.31 min. Compound 1 had the molecular formula of $C_{14}H_6O_8$ on the basis of molecular ion at *m/z* 300.9985 which was assigned as ellagic acid using standard (Figure E.4). Presence of ellagic acid in chestnut shell has been previously reported by Aires et al. (2016).

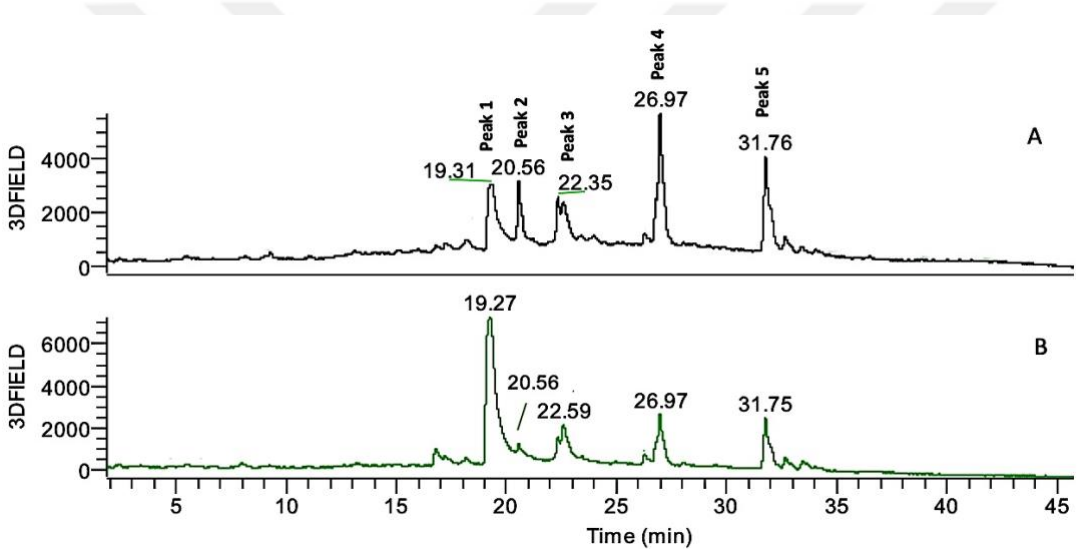


Figure 4.15 : The differences in phenolic profile of chestnut shell (A) and fermented chestnut shell by *A. japonicus* ZGM4 (B) as determined by LC-MS/MS.

Compound 2 found in fermented and unfermented chestnut shell produced an $[M–H]^-$ ion at *m/z* 507.1137 with fragment ions at *m/z* 344, 286, and 258 and the maximum absorbance was obtained at 272 nm (Figure E.5). This compound with a molecular formula of $C_{23}H_{24}O_{13}$ was tentatively identified as syringetin-hexoside (Fischer et al, 2011). Compound 3 with an $[M–H]^-$ ion at *m/z* 575.1195 was annotated as procyanidin A2 with the molecular formula of $C_{30}H_{24}O_{12}$ (Taeye et al, 2017; Yuzuak et al, 2018). Compound 4 and 5 were determined as isomers because they showed the same MS/MS fragment ions with different retention times at 26.97 and 31.76 min, respectively. They presented a molecular ion at m/z 343.0455 ($C_{17}H_{12}O_8$) with fragment ions at *m/z* 328, 312 and 297 corresponding to the losses of three methyl groups which allowed its tentative identification as trimethyl-*O*-ellagic acid (Figure E.6) (Taeye et al, 2017).

Similarly, Carocho et al. (2014) identified this compound in chestnut flowers with a molecular ion $[M–H]^-$ at *m/z* 343 releasing three fragments at m/z 328, 313 and 298.

Ellagic acid was the most abundant phenolic compound found in fermented chestnut shell, syrigetin-hexoside, procyanidin A2 and trimethyl-*O*-ellagic acid were also present at lesser amounts compared to those in unfermented chestnut shell. The reduction in concentrations of syringetin-hexoside, procyanidin A2 and trimethyl-*O*-ellagic acids after fermentation can be explained by degradation and/or biotransformation by molds (Krastanov et al, 2013). The increase in concentration of ellagic acid by fungal fermentation can be explained either by demethylation of trimethyl-*O*-ellagic acids (Ibrahim et al, 2003) or releasing of ellagic acid from cell wall structure by the action of fungal enzymes (Ascacio-Valdes et al, 2014; Shi et al, 2005). Concentration of ellagic acid in bound form was found as 0.52 mg/g dm in chestnut shell previously by our group (Gulsunoglu et al, 2019b) which can be released by fungal enzymes. Ascacio-Valdes et al. (2014) also mentioned that β-glucosidase enzyme was involved in degradation of ellagitannins, however it was not found to be directly associated with the ellagic acid accumulation. In the same study, they linked ellagitannase enzyme and ellagic acid accumulation because the ester bonds between HHDP group and glycosides could be degraded by the enzyme. In addition, increasing of polyphenol oxidase activity in the medium might cause the conversion of the intermediate HHDP groups to ellagic acid through coupled oxidation.

4.5.2 Optimization studies of fermentation conditions by PBD and RSM

Effects of various carbon and nitrogen sources and fermentation time on production of ellagic acid by *A. japonicus* ZGM4 were determined by PBD (Table C.1). The Pareto chart obtained is given in Figure 4.16 where absolute value of effect of each factor is shown with a bar (Table C.2). In the Pareto chart, the effects of factors were presented in decreasing order. The T-value limit (2.78) marked with a vertical line was used to determine significant effects which were fermentation time, lactose, glucose and yeast extract. For further optimization studies, one carbon source (lactose), one nitrogen source (yeast extract) and fermentation time were selected as independent factors. Glucose with lesser significance than lactose was added to each fermentation medium at a constant level.

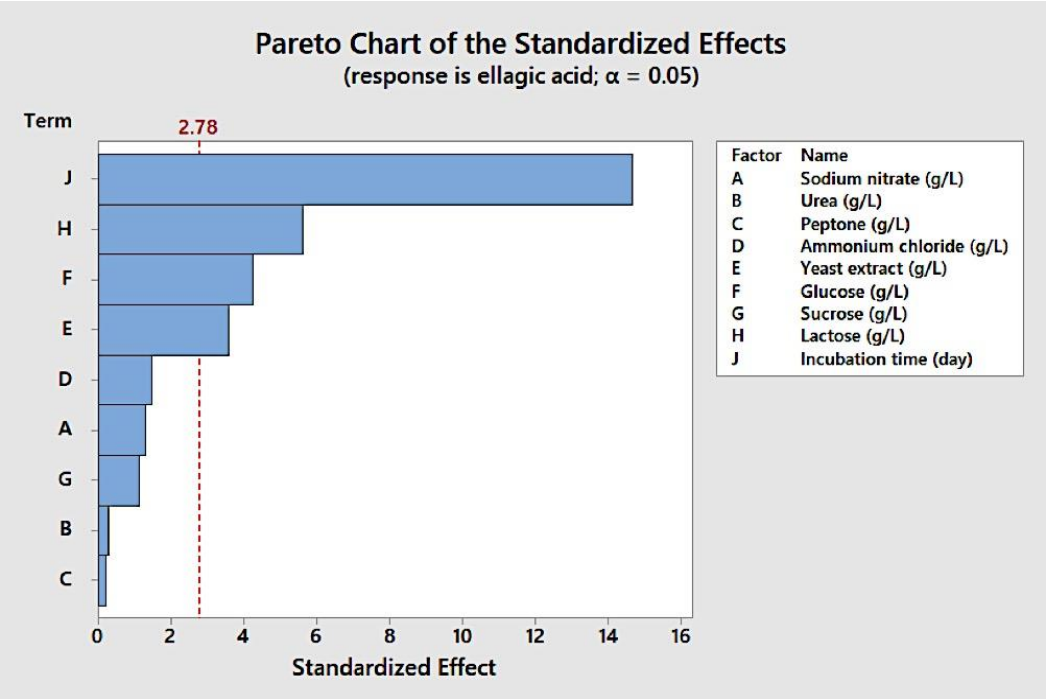


Figure 4.16 : Standardized Pareto chart for various factors influencing the ellagic acid production by *A. japonicus* ZGM4.

Lactose concentration was found to have a positive significant effect (T-value: 5.07), while concentration of glucose (T-value: –3.81), yeast extract (T-value: –3.20) and fermentation time (T-value: –13.52) exhibited significant negative effects on ellagic acid production by *A. japonicus* ZGM4 on chestnut shell (Table C.3). When the effect is positive, the influence of the factor on the ellagic acid production is greater at a high concentration, and when the effect is negative an opposite trend is observed. The level of factors was determined accordingly.

Sepulveda et al. (2012) reported that fermentation temperature, amount of inoculum, initial moisture content and concentration of salts as major factors for production of ellagic acid by fermentation with *A. niger* GH1 from pomegranate peel. Madeira et al. (2014) found that optimum conditions for accumulation of ellagic acid from citrus residues were 2:1 water:residue, fermentation temperature of 32 °C and 1.20 mm particle size of the residue. In this study, final fermentation conditions were determined by PBD. A nutrient solution containing various salts was used for sufficient growth of molds.

CCD in RSM was applied to determine optimum levels of fermentation time, concentrations of yeast extract and lactose on the production of ellagic acid. The multiple regression analysis was used to fit a model to data in terms of significant

factors which resulted in the following equation (4.3). The model was shown to be adequate by lack of fit test. In addition, a close agreement was found between experimental data and those predicted by the model with values of R^2 and R^2 (adjusted) as 95.02%, and 94.68%, respectively (Table C.4).

$$[Ellagic\ acid] = 2.683 + 0.01009X_1 - 0.2094X_2 - 0.1240X_3 - 0.000095X_1^2 + 0.001597X_1X_2 + 0.01967X_2X_3 \quad (4.3)$$

The ellagic acid production on chestnut shell ranged from 1.66 mg/g dm to 2.68 mg/g dm. The interactions between fermentation time-lactose and yeast extract-lactose had significant effects on ellagic acid production (Table C.5). In order to elaborate the effects of factors on the ellagic acid production, three-dimensional response surface plots were presented in Figure 4.17. There was a maximum level of ellagic acid produced at a certain fermentation time depending on the yeast and lactose concentrations. The effect of fermentation time was slight at low concentration of lactose, while there was a maximum production of ellagic acid after 142 h of fermentation at the highest concentration of lactose (Figure 4.17a). A similar trend was observed between fermentation time and concentration of yeast extract (Figure 4.17b). The decline after maxima could be due to degradation of ellagic acid by *A. japonicus* ZGM4 with extended fermentation. Maximum amount of ellagic acid was obtained at the highest lactose or yeast extract concentrations (Figure 4.17c). Minimum amount of ellagic acid was recorded at maximum lactose and minimum yeast extract concentrations.

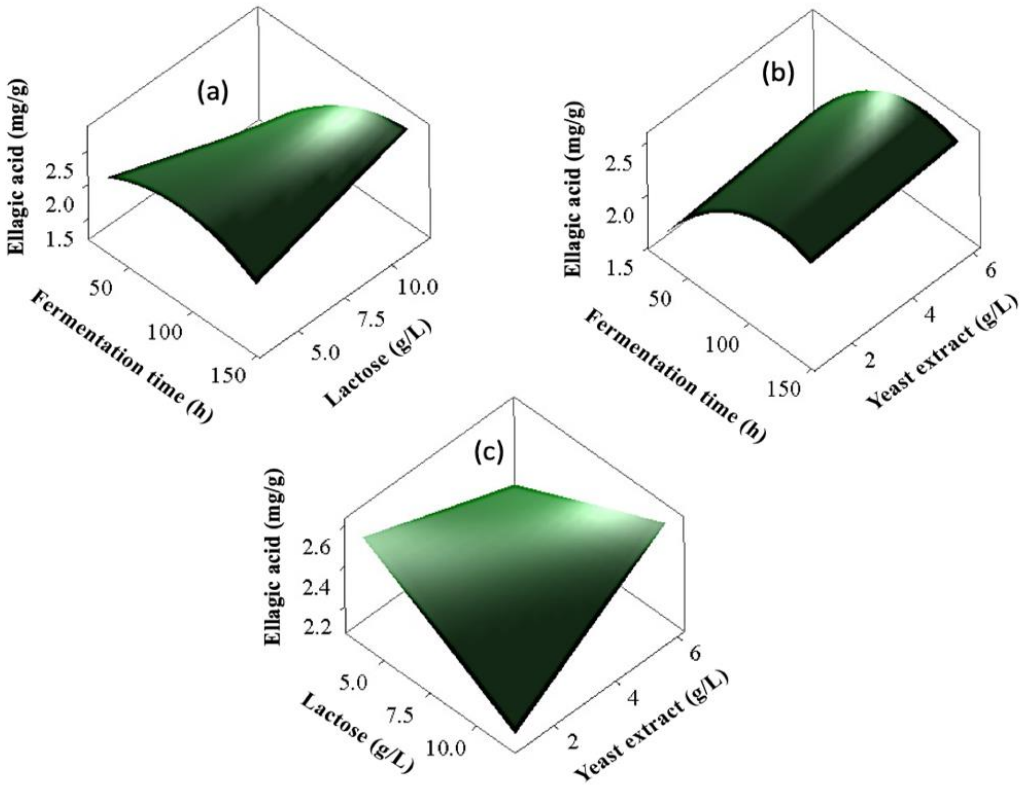


Figure 4.17 : Surface plots illustrating the effect of fermentation time, lactose and yeast extract concentration on ellagic acid production by *Aspergillus japonicus* ZGM4 on chestnut shell. (a) Effect of fermentation time and lactose at 3.5 g/L yeast extract concentration. (b) Effect of fermentation time and yeast extract at 7.5 g/L lactose concentration. (c) Effect of lactose and yeast extract concentration after fermentation for 84 h.

4.5.3 Conclusion

PBD and CCD were applied as statistical tools to optimize the fermentation conditions for improving ellagic acid production from chestnut shell by SSF with four *Aspergillus* isolates. *A. japonicus* ZGM4 showed the highest ellagic acid production among *Aspergillus* spp. tested. The optimized fermentation process resulted in the enrichment of the chestnut shell with ellagic acid resulting in approximately 6 times higher concentration compared to that in the unfermented chestnut shell. Results of this study contribute to efforts for establishment of new bioprocesses for production of ellagic acid and similar bioactive compounds. The development of bioprocesses for production of bioactive compounds would offer economical and environmental advantages over traditional chemical methods.

4.6 Phenolic and Antioxidant Fortification of Yoghurt with Fermented and Unfermented Chestnut Shell and Apple Peel

Yoghurt is one of the most common dairy product typically produced by fermentation with *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. Yoghurt is a highly-consumed product all over the world, at the same time, dairy industry is in constant research for innovative products. Therefore, yoghurts fortified with natural antioxidants could be a good choice to satisfy consumer demands for healthy products. Even though yoghurt contains various health-promoting nutrients, plain yoghurt is considered a poor source of phenolic compounds. Therefore, plant-based additives have been used to enhance the phenolic content of yoghurt. There is an increasing interest in agro-industrial wastes as a source of functional ingredients. The incorporation of phenolic extracts from wastes to yoghurt could be a way to increase the phenolic content and antioxidant activity of the product.

Apple peel and chestnut shell were found to be good sources of phenolic compounds. Apple peel was reported as one of the most potent fruit waste containing phenolic compounds including catechin, quercetin, phlorizin, hydroxybenzoic acid, and gallic acid. Chestnut shell also contained phenolic compounds like catechin, gallic, and ellagic acids (Gulsunoglu et al, 2019b). The results given in Section 4.4 and 4.5 showed that TPC and antioxidant activity of these wastes increased after fermentation with *Aspergillus* spp. Therefore, supplementation of yoghurt samples with fermented and unfermented apple peel and chestnut shell would offer a new way for delivering biologically active phytochemicals to the human diet.

In this part of the study, yoghurts fortified with the extracts obtained from fermented and unfermented apple peel and chestnut shell was analyzed for TPC and antioxidant activity initially and after 3 days of storage period. As presented in Table 4.12, the TPC and antioxidant activity of yoghurt fortified with fermented and unfermented apple peel and chestnut shell extracts were higher than those of the plain yoghurt. Among the assayed samples, the highest phenolic content was observed in fermented chestnut shell extracts. Yoghurts fortified with unfermented and fermented chestnut shell extract increased the TPC by 3.1 and 6.3-fold compared to that of the plain yoghurt, respectively. On the other hand, the yoghurts containing unfermented and fermented apple peel extract increased the TPC as 2.2 and 5.8-fold, respectively.

Storage time significantly decreased the TPC of yoghurt samples fortified with fermented and unfermented chestnut shell on day 3, while other samples did not significant change during storage time. This decrease can be explained with the degradation of phenolic compounds during storage. Karaaslan et al. (2011) also detected phenolic degradation during storage of yoghurt samples fortified with grape and callus extracts. Protein-polyphenol complexes might also reduce the antioxidant activity by lowering the number of free hydroxyl groups. Kharchoufi et al. (2017) reported that the antioxidant activity and phenolic content of yoghurt samples fortified with pomegranate juice and aril extracts decreased during storage time.

Table 4.12 : Total phenolic content and antioxidant activity of fermented and unfermented extracts.

	Total phenolic content (mg GAE/100g dm)		**Antioxidant activity (mg TE/100g dm)**	
	Day 0	**Day 3**	**Day 0**	**Day 3**
Control	98±2$_{a*}$	96±1$_{a}$	84±11$_{a}$	79±11$_{a}$
Chestnut shell unfermented	309±9$_{a}$	231±7$_{b}$	1333±5$_{a}$	1002±53$_{b}$
Chestnut shell fermented	615±12$_{a}$	503±2$_{b}$	2620±90$_{a}$	2223±125$_{b}$
Apple peel unfermented	214±3$_{a}$	214±2$_{a}$	347±23$_{a}$	343±4$_{a}$
Apple peel fermented	569±14$_{a}$	532±8$_{a}$	1273±8$_{a}$	1194±68$_{a}$

*Different small letters in the same row indicate significant difference (P<0.05).

The free radical scavenging activity of the samples were measured by DPPH assay. The antioxidant activity of yoghurt samples carried out on the first day of storage showed the yoghurt containing the fermented chestnut shell displayed the highest antioxidant activity and the lowest activity was found in the control samples which had the least phenolic content. Fermented chestnut shell extract yielded higher TPC and antioxidant activity in fortified yoghurt samples compared to those of unfermented chestnut shell extract. This could be linked to increased ellagic acid concentration after fermentation. Antioxidant activity of yoghurt samples supplemented with fermented and unfermented chestnut shell decreased significantly, while other samples did not show any significant change after three days of storage at 4 °C. Antioxidant activity values are correlated with TPC of the samples. In a previous study, phenolic content of plants is associated with their antioxidant activities, probably due to their redox

properties which allow them to act as reducing agents, hydrogen donors and metal chelating potential (El-Said et al, 2014).

TPC and DPPH activity of unfermented apple peel extract were approximately 20 mg GAE/g and 44 mg TE/g, respectively. Those of fermented extract were 160 mg GAE/g and 514 mg TE/g, respectively. When these values were considered, there seems to be a synergistic interaction between yoghurt components and phenolic compounds that increase antioxidant activity.

TPC and DPPH activity of unfermented chestnut shell extract were approximately 438 mg GAE/g and 3011 mg TE/g, respectively. Those of fermented extract were 225 mg GAE/g and 1318 mg TE/g, respectively. When these values were considered, there seems to be a synergistic interaction between yoghurt components and phenolic compounds in the case of yoghurt fortified with fermented extract for antioxidant activity. On the other hand, there is an antagonistic interaction in the case of yoghurt fortified with unfermented extract.

In this chapter, TPC and antioxidant activity of yoghurt samples fortified with fermented and unfermented chestnut shell and apple peel were investigated and yogurts supplemented with fermented and unfermented wastes showed higher TPC and antioxidant activity than control samples. Additionally, fermented wastes revealed more TPC and antioxidant activity than unfermented wastes. In conclusion, fermented wastes may be utilized to provide functional properties to food products.

5. CONCLUSIONS AND RECOMMENDATIONS

This thesis aimed to assess the potential of mold fermentation for production of phenolic compounds from agro-industrial food wastes. For this purpose, *Aspergillus* spp. were isolated from local sources. Grape and date were found as a potential sources of black *Aspergillus* section *Nigri* members among the screened vegetables and fruits. According to the molecular and morphological results, these molds identified and named as *A. tubingensis* ZDM1 and ZGM5, *A. niger* ZDM2 and ZDM3, *A. japonicus* ZGM4 and *A. aculeatus* ZGM6. Variability is low in ITS region between closely related fungal species, while β-tubulin and calmodulin are better genes for identification of closely related species. In future studies, these genes can be used for confirmation of identification results. No ochratoxin A production was detected in all isolates grown on the CYA and YES media. Some *Aspergillus* spp. may be fumonisin producers, therefore isolates can be analyzed for fumonisin production in future studies. These isolates can be utilized in biotechnological applications in industry in future.

Isolated *Aspergillus* spp. were screened for hydrolytic enzyme production ability on solid and also in liquid standard media. Isolates were found to produce cellulase, pectinase and tannase enzymes. With this ability, isolates were shown to have potential for industrial enzyme production. Enzyme production and activity can be enhanced by some modifications in media using bioreactor and application of molecular biological techniques.

Agro-industrial food wastes including apple peel, apple pomace, pomegranate peel, pomegranate seed, black carrot pomace and chestnut shell were selected as substrate for production of phenolics by fermentation with *Aspergillus* spp. The gross and phenolic compositions of these wastes were determined to evaluate their potential as a fermentation medium component. While, pomegranate seed was found to be rich in protein, apple pomace was found to be rich in glucose. Chestnut shell contained higher amount of condensed tannin compared to others. Additionally, the wastes can be used as a source of macro- and micro-minerals such as Na, K, Ca, Mg, Fe, Cu and Zn. The

wastes contained various phenolics both in soluble-free and insoluble-bound forms. Obtained results showed that agro-industrial wastes can be used as a source of nutrients and bioactive compounds. These wastes can be valorized in terms of these components. Best of our knowledge, there were limited studies on the detailed analyses of phenolics in different forms present in plant wastes. The findings from this part contribute to studies on the utilization of agro-industrial wastes. Natural antioxidants produced in this way can be formulated to functional food ingredients or nutraceuticals beneficial for human health.

Apple peel and chestnut shell were found to be suitable for fermentation by *Aspergillus* spp. due to the higher insoluble-bound phenolics than soluble-free ones. *A. niger* ZDM1 and *A. japonicus* ZGM4 were selected for fermentation of apple peel and chestnut shell by preliminary experiments, respectively. Fermentation conditions including fermentation time, carbon (glucose, sucrose, lactose) and nitrogen (sodium nitrate, urea, peptone, ammonium chloride, yeast extract) sources were screened for their effects on total phenolic content by PBD. Fermentation of apple peel was optimized by RSM for maximizing TPC and antioxidant activity in terms of selected significant factors from PBD, including concentrations of urea and lactose and fermentation time. Phenolic content and antioxidant activity of apple peel was increased by 6-fold and 10-fold, respectively, by fermentation with *A. niger* ZDM2 under optimized conditions. In addition, eriodictyol and taxifolin were tentatively identified in fermented apple peel by *Aspergillus* spp. for the first time in the literature. This shows that phenolics can be manufactured industrially by development of new bioprocess.

Fermentation conditions including fermentation time, carbon (glucose, sucrose, lactose) and nitrogen (sodium nitrate, urea, peptone, ammonium chloride, yeast extract) sources were screened for their effects on production of ellagic acid by PBD. Fermentation of chestnut shell was optimized by RSM for maximizing ellagic acid concentration in terms of selected significant factors from PBD, including concentrations of yeast extract and lactose and fermentation time. *A. japonicus* ZGM4 showed 6-fold increase in ellagic acid content from chestnut shell. Chestnut shell was used for the first time to produce ellagic acid by fermentation with *Aspergillus* spp. The isolate might also be used for bioconversion of other phenolic compounds from different agro-industrial wastes. Bioprocesses can be developed for conversion of

wastes into valuable phenolics instead of chemical synthesis in view of enviromental and economical benefits.

Phenolics were extracted from fermented and unfermented wastes and they were added into yoghurt to provide functional properties. Fermented extracts of chestnut shell and apple peel increased phenolic content and antioxidant activity of yoghurt samples compared to those of unfermented samples. This study showed that wastes can be added as functional components to impart bioactivity to food products. However, stability and interactions of bioactive components with other food components during storage need to be evaluated in future studies. The bioactivity and bioavailability of phenolics in wastes and food products fortified with them need to be assessed by *in vitro* and *in vivo* studies. Sensory analysis should be conducted to determine the consumer acceptability of yoghurt samples fortified with food grade and more pure extracts.

Food processing industry should valorize wastes to minimize the amounts of generated wastes. Wastes include many functional and nutritional components that can be recovered and utilized in food and pharmaceutical industry. The findings and newly isolated *Aspergillus* spp. from this thesis contribute to the efforts for valorization of agro-industrial wastes in future.

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APPENDICES

APPENDIX A: Supplementary Materials for Section 2.2
APPENDIX B: Supplementary Materials for Section 4.4
APPENDIX C: Supplementary Materials for Section 4.5
APPENDIX D: HPLC-PDA chromatograms of phenolics obtained from wastes
APPENDIX E: LC-MS/MS spectra of phenolics obtained from fermented wastes

APPENDIX A

Table A.1 : Cellulase enzyme activity during 7 days incubation.

Isolates	Enzyme activity (U/g dry biomass)*				
	24 h	**48 h**	**72 h**	**96 h**	**168 h**
A. tubingensis ZDM1	-†	$12\pm10_{a,BC}$	$16\pm11_{a,AB}$	$22\pm17_{a,AB}$	$20\pm16_{a,AB}$
A. niger ZDM2	-	$21\pm3_{a,AB}$	$20\pm8_{a,AB}$	$31\pm3_{a,A}$	$28\pm5_{a,AB}$
A. niger ZDM3	-	$15\pm5_{a,B}$	$20\pm8_{a,A}$	$26\pm7_{a,AB}$	$29\pm4_{a,AB}$
A. japonicus ZGM4	-	$33\pm6_{a,A}$	$31\pm9_{a,A}$	$40\pm4_{a,A}$	$34\pm4_{a,A}$
A. tubingensis ZGM5	-	-	$4\pm7_{a,B}$	$5\pm6_{a,B}$	$7\pm7_{a,B}$
A. aculeatus ZGM6	$3\pm1_{b,A}$	$25\pm5_{a,AB}$	$26\pm6_{a,AB}$	$35\pm15_{a,A}$	$32\pm10_{a,A}$

*Each value was expressed as mean±SD (n=3). Means marked with different lower case letters within a row are significantly different. Means marked with different upper case letters within a column are significantly different (P<0.05). †No activity found.

Table A.2 : Tannase enzyme activity during 7 days incubation.

Isolates	Enzyme activity (U/g dry biomass)*				
	24 h	**48 h**	**72 h**	**96 h**	**168 h**
A. tubingensis ZDM1	$234\pm99_{a,A}$	$138\pm9_{a,A}$	-†	-	-
A. niger ZDM2	$325\pm57_{a,A}$	$191\pm92_{a,A}$	$21\pm78_{b,A}$	-	-
A. niger ZDM3	$168\pm36_{a,B}$	$184\pm46_{a,A}$	$75\pm74_{ab,A}$	-	-
A. japonicus ZGM4	$343\pm108_{a,A}$	$254\pm55_{ab,A}$	$145\pm94_{ab,A}$	-	-
A. tubingensis ZGM5	$152\pm45_{a,B}$	$180\pm54_{a,A}$	$34\pm29_{b,A}$	-	-
A. aculeatus ZGM6	$262\pm109_{a,A}$	$243\pm66_{a,A}$	$127\pm75_{a,A}$	-	-

*Each value was expressed as mean±SD (n=3). Means marked with different lower case letters within a row are significantly different. Means marked with different upper case letters within a column are significantly different (P<0.05). †No activity found.

Table A.3 : Pectinase enzyme activity during 7 days incubation.

Isolates	Enzyme activity (U/g dry biomass)*				
	24 h	**48 h**	**72 h**	**96 h**	**168 h**
A. tubingensis ZDM1	$86\pm30_{a,A}$	$106\pm17_{a,A}$	$86\pm63_{a,A}$	$98\pm22_{a,A}$	$18\pm44_{a,B}$
A. niger ZDM2	$59\pm40_{a,A}$	$82\pm16_{a,A}$	$69\pm25_{a,B}$	$90\pm31_{a,A}$	$33\pm21_{a,B}$
A. niger ZDM3	$87\pm25_{a,A}$	$95\pm40_{a,A}$	$91\pm29_{a,A}$	$67\pm39_{a,A}$	$20\pm10_{a,B}$
A. japonicus ZGM4	$55\pm1_{a,A}$	$68\pm39_{a,A}$	$68\pm16_{a,B}$	$53\pm21_{a,A}$	$73\pm13_{a,AB}$
A. tubingensis ZGM5	$110\pm33_{a,A}$	$104\pm30_{a,A}$	$130\pm66_{a,A}$	$116\pm65_{a,A}$	$63\pm14_{a,AB}$
A. aculeatus ZGM6	$117\pm29_{a,A}$	$87\pm42_{a,A}$	$70\pm18_{a,B}$	$96\pm17_{a,A}$	$113\pm39_{a,A}$

*Each value was expressed as mean±SD (n=3). Means marked with different lower case letters within a row are significantly different. Means marked with different upper case letters within a column are significantly different (P<0.05).

APPENDIX B

Table B.1 : Experimental design of PBD with observed and predicted TPC results of each run for fermented apple peel.

Run	Variables									TPC	
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**J**	**Observed**	**Predicted**
1	+1	-1	+1	-1	-1	-1	+1	+1	+1	1048±59	1006±65
2	+1	+1	-1	+1	+1	-1	+1	-1	-1	386±10	344±65
3	+1	+1	+1	-1	+1	+1	-1	+1	-1	421±32	390±65
4	-1	+1	+1	-1	+1	-1	-1	-1	+1	700±71	732±65
5	0	0	0	0	0	0	0	0	0	1162±114	1207±41
6	0	0	0	0	0	0	0	0	0	1214±59	1207±41
7	0	0	0	0	0	0	0	0	0	1246±141	1207±41
8	+1	-1	-1	-1	+1	+1	+1	-1	+1	1016±88	1059±65
9	+1	-1	+1	+1	-1	+1	-1	-1	-1	399±38	431±65
10	-1	+1	-1	-1	-1	+1	+1	+1	-1	437±36	471±65
11	-1	+1	+1	+1	-1	+1	+1	-1	+1	907±120	875±65
12	+1	+1	-1	+1	-1	-1	-1	+1	+1	995±68	1037±65
13	-1	-1	-1	-1	-1	-1	-1	-1	-1	400±39	369±65
14	-1	-1	+1	+1	+1	-1	+1	+1	-1	442±41	486±65
15	-1	-1	-1	+1	+1	+1	-1	+1	+1	1274±85	1232±65

Table B.2 : ANOVA table of TPC analysis in PBD.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	1764452	176445	34.66	0.002
Linear	9	1153452	128161	25.17	0.004
Sodium nitrate (g/L)	1	878	878	0.17	0.699
Urea (g/L)	1	45125	45125	8.86	0.041
Peptone (g/L)	1	29021	29021	5.70	0.075
Ammonium chloride (g/L)	1	11973	11973	2.35	0.200
Yeast extract (g/L)	1	227	227	0.04	0.843
Glucose (g/L)	1	19413	19413	3.81	0.123
Sucrose (g/L)	1	206	206	0.04	0.850
Lactose (g/L)	1	55050	55050	10.81	0.030
Fermentation time (h)	1	991559	991559	194.77	0.000
Curvature	1	611000	611000	120.02	0.000
Error	4	20364	5091		
Lack-of-Fit	2	16745	8372	4.63	0.178
Pure Error	2	3619	1809		
Total	14				

Table B.3 : Coded Coefficients table of TPC analysis in PBD.

Term	Effect	Coef	SE Coef	95% CI	T-Value	P-Value
Constant		702.5	20.6	(645.4; 759.7)	34.11	0.000
Sodium nitrate (g/L)	17.1	8.6	20.6	(-48.6; 65.7)	0.42	0.699
Urea (g/L)	-122.6	-61.3	20.6	(-118.5; -4.1)	-2.98	0.041
Peptone (g/L)	-98.4	-49.2	20.6	(-106.4; 8.0)	-2.39	0.075
Ammonium chloride (g/L)	63.2	31.6	20.6	(-25.6; 88.8)	1.53	0.200
Yeast extract (g/L)	8.7	4.3	20.6	(-52.8; 61.5)	0.21	0.843
Glucose (g/L)	80.4	40.2	20.6	(-17.0; 97.4)	1.95	0.123
Sucrose (g/L)	8.3	4.1	20.6	(-53.0; 61.3)	0.20	0.850
Lactose (g/L)	135.5	67.7	20.6	(10.5; 124.9)	3.29	0.030
Fermentation time (h)	574.9	287.5	20.6	(230.3; 344.6)	13.96	0.000
Central Point		504.6	46.1	(376.7; 632.4)	10.96	0.000

Table B.4 : Observed and predicted results of TPC and DPPH assays of each run for fermented apple peel in RSM design.

Run	TPC		DPPH	
	Observed	**Predicted**	**Observed**	**Predicted**
1	1612.9±68.5	1584.6±55.0	5510.5±158.3	5393.0±286.3
2	1448.8±71.3	1412.6±24.6	5129.5±329.5	4998.9±128.1
3	1755.3±54.3	1803.6±28.8	5892.5±230.5	6189.7±150.2
4	1010.5±62.8	1022.8±48.5	4288.6±368.6	4444.6±252.7
5	2258.5±121.3	2226.2±54.8	8135.7±355.6	7869.4±285.4
6	730.7±50.6	725.9±32.0	2711.9±265.1	2921.4±166.6
7	1935.1±114.4	1943.2±53.5	6474.7±327.6	6481.7±278.8
8	979.3±27.7	1033.3±36.9	3941.3±148.6	2921.4±166.6
9	801.0±44.1	856.6±50.8	3566.0±39.3	3786.8±264.7
10	702.8±19.16	761.8±50.8	2644.1±138.7	2749.1±264.4
11	1410.6±66.9	1412.6±24.6	5181.0±177.7	4998.9±128.1
12	1888.2±8.4	1898.3±49.6	6401.2±100.3	6485.2±258.0
13	1427.0±14.8	1412.6±24.6	5202.9±152.4	4998.9±128.1
14	1361.2±56.3	1315.6±39.9	5220.0±149.0	4951.2±207.7
15	1718.7±36.9	1676.3±37.7	5649.5±91.5	5739.8±196.5
16	276.9±71.9	242.5±56.0	864.5±209.9	669.0±291.4
17	1107.0±17.0	1033.3±36.9	4480.4±40.57	4136.0±192.3

Table B.5 : ANOVA table of TPC analysis in RSM design.

Source	**DF**	**Adj SS**	**Adj MS**	***F*-value**	***P*-value**
Model	7	4375547	625078	263.11	0.000
Linear	3	2740593	913531	384.53	0.000
Fermentation time (h)	1	2210857	2210857	930.61	0.000
Lactose (g/L)	1	422189	422189	177.71	0.000
Urea (g/L)	1	15804	15804	6.65	0.030
Square	1	185105	185105	77.92	0.000
Ferm. time * Ferm. time	1	185105	185105	77.92	0.000
2-Way Interaction	3	275039	91680	38.59	0.000
Ferm. time * Lactose	1	25424	25424	10.70	0.010
Ferm. time * Urea	1	129526	129526	54.52	0.000
Lactose * Urea	1	63198	63198	26.60	0.001
Error	9	21381	2376		
Lack-of-Fit	6	12495	2082	0.70	0.674
Pure Error	3	8887	2962		
Total	16				

Table B.6 : ANOVA table of DPPH analysis in RSM design.

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	7	46141709	6591673	123.39	0.000
Linear	3	22959050	7653017	143.26	0.000
Ferm. time (h)	1	19478489	19478489	364.63	0.000
Lactose (g/L)	1	2367297	2367297	44.31	0.000
Urea (g/L)	1	117807	117807	2.21	0.172
Square	1	2079828	2079828	38.93	0.000
Ferm. time * Ferm. time	1	2079828	2079828	38.93	0.000
2-Way Interaction	3	4158077	1386026	25.95	0.000
Ferm. time * Lactose	1	1465559	1465559	27.43	0.001
Ferm. time* Urea	1	951073	951073	17.80	0.002
Lactose * Urea	1	432660	432660	8.10	0.019
Error	9	480782	53420		
Lack-of-Fit	6	332628	55438	1.12	0.502
Pure Error	3	148154	49385		
Total	16				

APPENDIX C

Table C.1 : Experimental design of PBD with observed and predicted ellagic acid results of each run for fermented chestnut shell.

Run	Variables									Ellagic acid	
	A	B	C	D	E	F	G	H	J	Observed	Predicted
1	+1	-1	+1	-1	-1	-1	+1	+1	+1	1.15±0.18	1.21±0.12
2	+1	+1	-1	+1	+1	-1	+1	-1	-1	1.40±0.01	1.46±0.12
3	+1	+1	+1	-1	+1	+1	-1	+1	-1	1.72±0.15	1.76±0.12
4	-1	+1	+1	-1	+1	-1	-1	-1	+1	0.53±0.05	0.49±0.12
5	0	0	0	0	0	0	0	0	0	1.35±0.07	1.20±0.08
6	0	0	0	0	0	0	0	0	0	1.19±0.22	1.20±0.08
7	0	0	0	0	0	0	0	0	0	1.07±0.20	1.20±0.08
8	+1	-1	-1	-1	+1	+1	+1	-1	+1	0.33±0.06	0.27±0.12
9	+1	-1	+1	+1	-1	+1	-1	-1	-1	1.57±0.10	1.53±0.12
10	-1	+1	-1	-1	-1	+1	+1	+1	-1	1.87±0.13	1.83±0.12
11	-1	+1	+1	+1	-1	+1	+1	-1	+1	0.21±0.02	0.25±0.12
12	+1	+1	-1	+1	-1	-1	-1	+1	+1	1.19±0.01	1.13±0.12
13	-1	-1	-1	-1	-1	-1	-1	-1	-1	1.80±0.04	1.84±0.12
14	-1	-1	+1	+1	+1	-1	+1	+1	-1	1.86±0.03	1.80±0.12
15	-1	-1	-1	+1	+1	+1	-1	+1	+1	0.44±0.16	0.50±0.12

Table C.2 : ANOVA table of ellagic acid analysis in PBD.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	4.41769	0.44177	23.87	0.004
Linear	9	4.41544	0.49060	26.51	0.003
Sodium nitrate (g/L)	1	0.03554	0.03554	1.92	0.238
Urea (g/L)	1	0.00433	0.00433	0.23	0.654
Peptone (g/L)	1	0.00001	0.00001	0.00	0.987
Ammonium chloride (g/L)	1	0.04415	0.04415	2.39	0.197
Yeast extract (g/L)	1	0.18948	0.18948	10.24	0.033
Glucose (g/L)	1	0.26793	0.26793	14.48	0.019
Sucrose (g/L)	1	0.01519	0.01519	0.82	0.416
Lactose (g/L)	1	0.47517	0.47517	25.68	0.007
Fermentation time (h)	1	3.38364	3.38364	182.86	0.000
Curvature	1	0.00224	0.00224	0.12	0.745
Error	4	0.07402	0.01850		
Lack-of-Fit	2	0.03455	0.01727	0.88	0.533
Pure Error	2	0.03947	0.01973		
Total	14				

Table C.3 : Coded coefficients table of ellagic acid analysis in PBD.

Term	Effect	Coef	SE Coef	95% CI	T-Value	P-Value
Constant		1.173	0.039	(1.06; 1.28)	29.87	0.000
Sodium nitrate (g/L)	0.109	0.054	0.039	(-0.06; 0.16)	1.39	0.238
Urea (g/L)	-0.038	-0.019	0.039	(-0.13; 0.09)	-0.48	0.654
Peptone (g/L)	0.001	0.001	0.039	(-0.11; 0.11)	0.02	0.987
Ammonium chloride (g/L)	-0.121	-0.061	0.039	(-0.17; 0.05)	-1.54	0.197
Yeast extract (g/L)	-0.251	-0.126	0.039	(-0.24; -0.02)	-3.20	0.033
Glucose (g/L)	-0.299	-0.149	0.039	(-0.26; -0.04)	-3.81	0.019
Sucrose (g/L)	-0.071	-0.036	0.039	(-0.15; 0.07)	-0.91	0.416
Lactose (g/L)	0.398	0.199	0.039	(0.09; 0.31)	5.07	0.007
Fermentation time (h)	-1.062	-0.531	0.039	(-0.64; -0.42)	-13.52	0.000
Ct Pt		0.031	0.088	(-0.21; 0.27)	0.35	0.745

Table C.4 : Observed and predicted ellagic acid results of each run for fermented chestnut shell in RSM design.

Run	Ellagic acid (mg/g)		
	Observed	**Predicted**	**Residual**
1	1.66	1.70	-0.04
2	2.19	2.29	-0.10
3	2.46	2.41	0.05
4	2.41	2.42	-0.01
5	2.35	2.41	-0.06
6	2.36	2.52	-0.16
7	2.37	2.41	-0.04
8	2.51	2.52	-0.01
9	2.50	2.41	0.09
10	2.23	2.20	0.03
11	2.36	2.41	-0.05
12	2.22	2.18	0.03
13	2.23	2.27	-0.04
14	2.51	2.41	0.10
15	1.95	2.01	-0.06
16	2.68	2.67	0.01
17	1.82	1.73	0.09

Table C.5 : ANOVA table of ellagic acid analysis in RSM design.

Source	DF	Adj SS	Adj MS	*F*-value	*P*-value
Model	6	1.05307	0.175511	26.18	<0.0001
Linear	3	0.66601	0.222003	33.11	<0.0001
Ferm. time (h)	1	0.64595	0.645955	96.35	<0.0001
Lactose (g/L)	1	0.00342	0.003424	0.51	0.491
Yeast extract (g/L)	1	0.01663	0.016632	2.48	0.146
Square	1	0.17823	0.178230	26.59	<0.0001
Ferm. time * Ferm. time	1	0.17823	0.178230	26.59	<0.0001
2-Way Interaction	2	0.20882	0.104412	15.57	0.001
Ferm. time * Lactose	1	0.16531	0.165313	24.66	0.001
Lactose * Yeast extract	1	0.04351	0.043513	6.49	0.029
Error	10	0.06704	0.006704		
Lack-of-Fit	8	0.05297	0.006622	0.94	0.610
Pure Error	2	0.01407	0.007033		
Total	16				

APPENDIX D

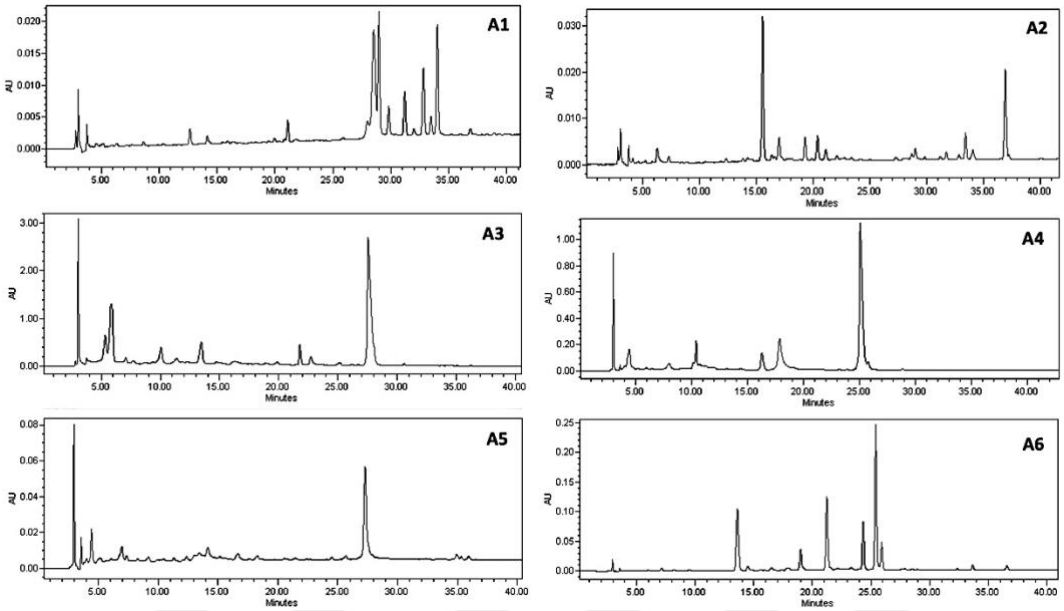

Figure D.1 : HPLC chromatograms (PDA, recorded at 280 nm) of phenolic compounds of soluble extract of apple peel (A1), apple pomace (A2), pomegranate peel (A3), pomegranate seed (A4), chestnut shell (A5) and black carrot pomace (A6).

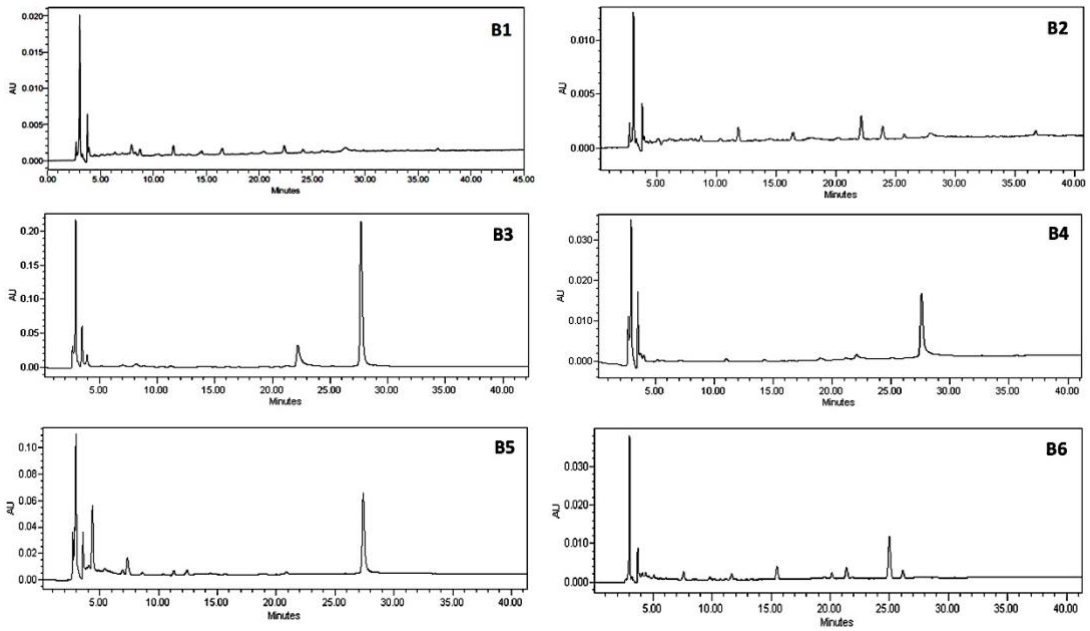

Figure D.2 : HPLC chromatograms (PDA, recorded at 280 nm) of phenolic compounds of insoluble-bound extract of apple peel (B1), apple pomace (B2), pomegranate peel (B3), pomegranate seed (B4), chestnut shell (B5) and black carrot pomace (B6).

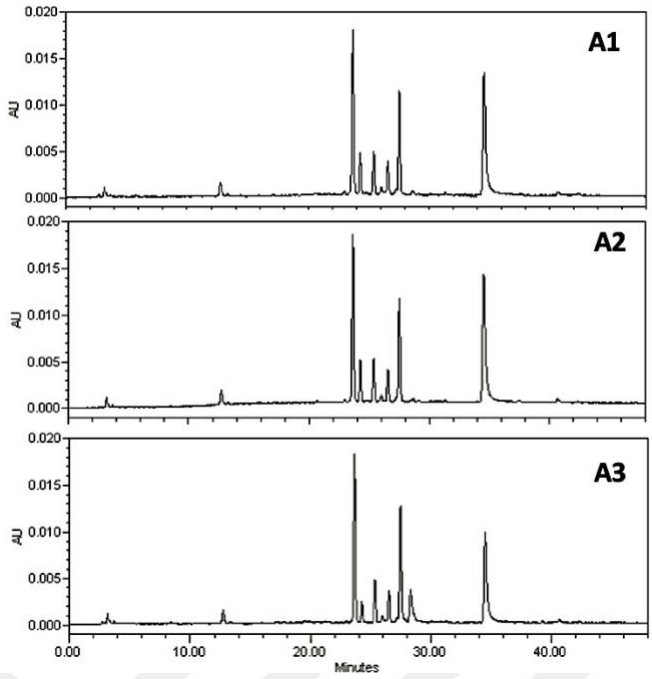

Figure D.3 : HPLC chromatogram (at 360 nm) of apple peel fermented by naturally for 7-days; A1: at 0 day; A2: at 3th day; A3: at 7th day.

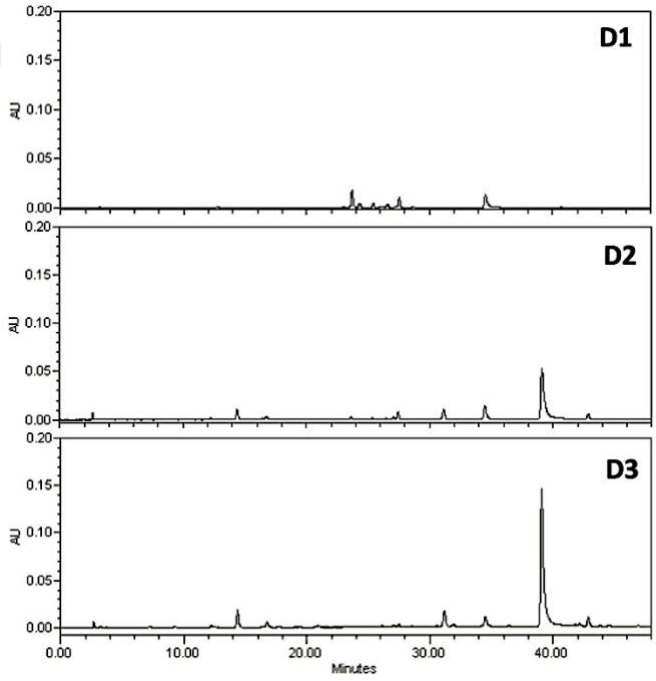

Figure D.4 : HPLC chromatogram (at 360 nm) of apple peel fermented by *A. niger* for 7-days; D1: at 0 day; D2: at 3th day; D3: at 7th day.

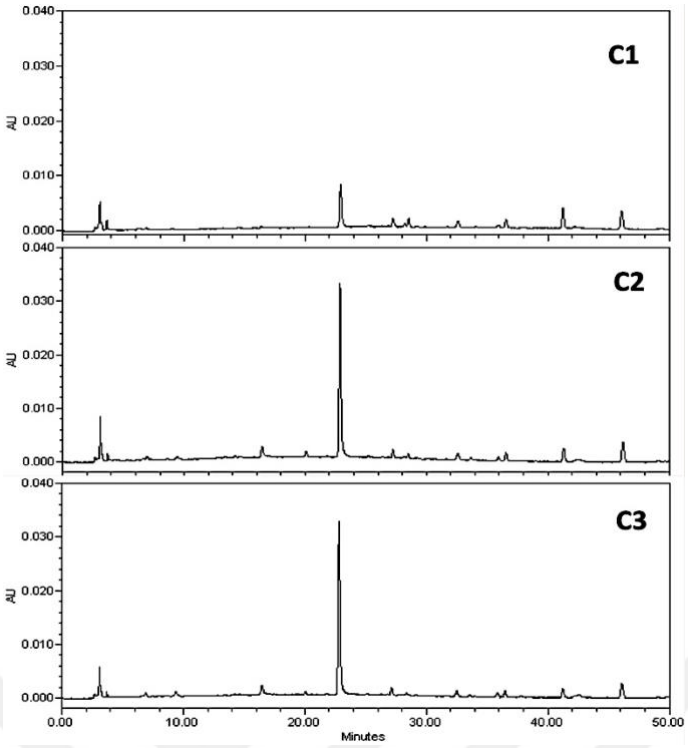

Figure D.5 : HPLC chromatogram (at 254 nm) of chestnut shell fermented by *A. japonicus* for 7-days; C1: at 0 day; C2: at 3th day; C3: at 7th day.

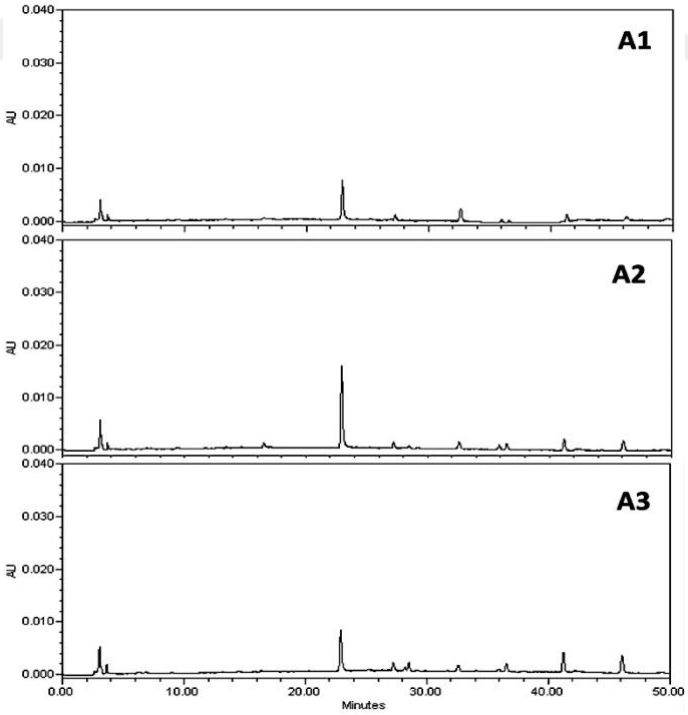

Figure D.6 : HPLC chromatogram (at 254 nm) of chestnut shell fermented by naturally for 7-days; A1: at 0 day; A2: at 3th day; A3: at 7th day.

APPENDIX E

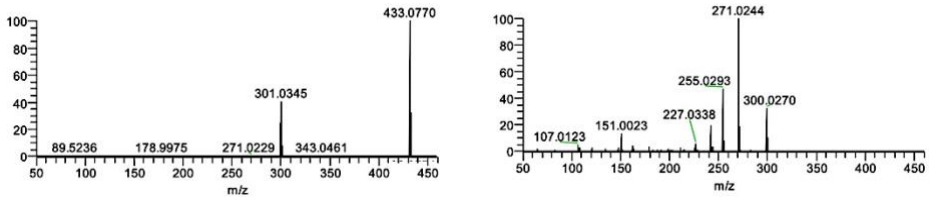

Figure E.1 : LC-MS/MS spectra of phenolic compound extracted from apple peel and identified as quercetin pentoside with low (15eV, left side) and high (55eV, right side) energy.

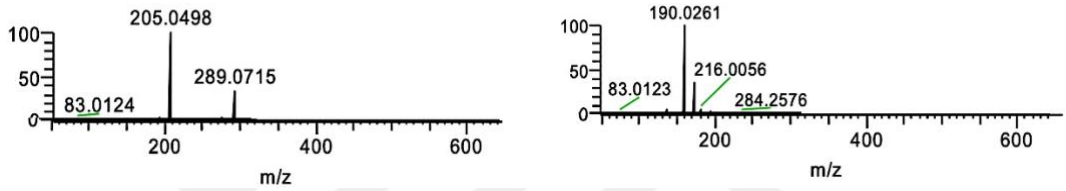

Figure E.2 : LC-MS/MS spectra of phenolic compound extracted from apple peel and identified as catechin with low (15eV, left side) and high (55eV, right side) energy.

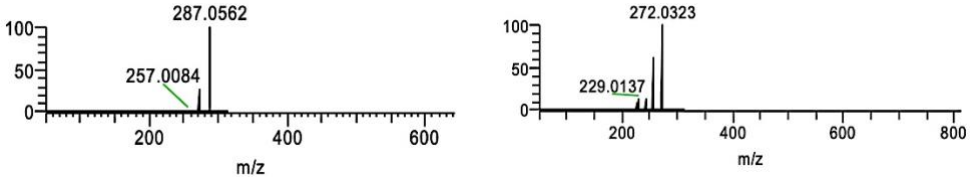

Figure E.3 : LC-MS/MS spectra of phenolic compound extracted from apple peel and identified as eriodictyol with low (15eV, left side) and high (55eV, right side) energy.

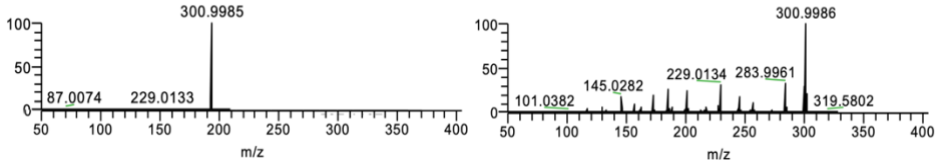

Figure E.4 : LC-MS/MS spectra of phenolic compound extracted from chestnut shell and identified as ellagic acid in with low (15eV, left side) and high (55eV, right side) energy.

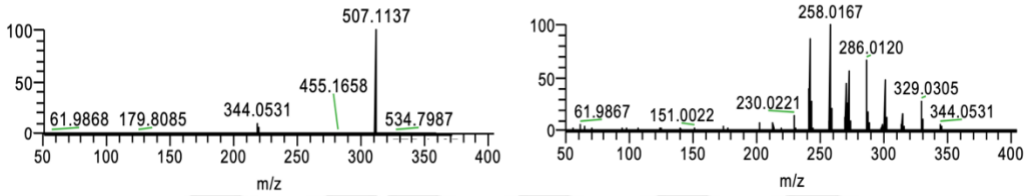

Figure E.5 : LC-MS/MS spectra of phenolic compound extracted from chestnut shell and identified as syringetin hexoside in with low (15eV, left side) and high (55eV, right side) energy.

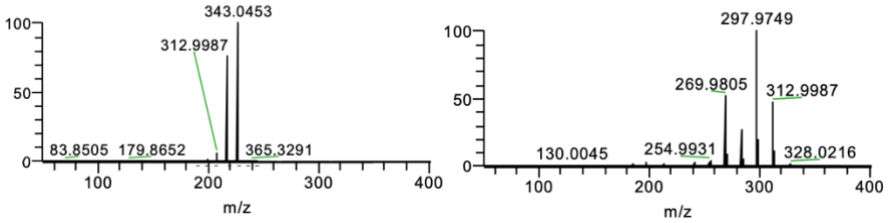

Figure E.6 : LC-MS/MS spectra of phenolic compound extracted from chestnut shell and identified as trimethyl-*O*-ellagic acid in with low (15eV, left side) and high (55eV, right side) energy.

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