ISOLATION OF ANTIMICROBIAL MOLECULES FROM AGRICULTURAL BIOMASS AND UTILIZATION IN XYLAN-BASED BIODEGRADABLE FILMS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

UMUT ÇEKMEZ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

JANUARY 2010

Approval of the thesis:

ISOLATION OF ANTIMICROBIAL MOLECULES FROM AGRICULTURAL BIOMASS AND UTILIZATION IN XYLAN-BASED BIODEGRADABLE FILMS

submitted by **UMUT ÇEKMEZ** in partial fulfillment of the requirements for the degree of **Master of Science in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen ________________
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. İnci Eroğlu ________________
Head of Department, **Biotechnology, METU**

Prof. Dr. Ufuk Bakır ________________
Supervisor, **Chemical Engineering Dept., METU**

Prof. Dr. Zümrüt Begüm Ögel ________________
Co-Supervisor, **Food Engineering Dept., METU**

Examining Committee Members:

Prof. Dr. Necati Özkan ________________
Polymer Science and Technology, METU

Prof. Dr. Ufuk Bakır ________________
Chemical Engineering Dept., METU

Prof. Dr. Zümrüt Begüm Ögel ________________
Food Engineering Dept., METU

Assoc. Prof. Dr. Candan Gürakan Gültekin ________________
Food Engineering Dept., METU

Assoc. Prof. Dr. Göknur Bayram ________________
Chemical Engineering Dept., METU

Date: 18.01.2010

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Umut ÇEKMEZ

Signature:

ABSTRACT

ISOLATION OF ANTIMICROBIAL MOLECULES FROM AGRICULTURAL BIOMASS AND UTILIZATION IN XYLAN-BASED BIODEGRADABLE FILMS

Çekmez, Umut
M.Sc., Department of Biotechnology
Supervisor: Prof. Dr. Ufuk Bakır
Co-Supervisor: Prof. Dr. Zümrüt Begüm Ögel

January 2010, 94 pages

Cotton stalk lignin extractions were performed via alkaline methods at different conditions. Crude and post treated cotton stalk lignins, olive mill wastewater and garlic stalk juice were examined in terms of antimicrobial activity. Antimicrobial lignin was isolated depending on alkaline extraction conditions. Lignin extracted at 60°C exhibited significant antimicrobial effect towards both *Escherichia coli* and *Bacillus pumilus*. However different post treatments such as ultrasonication and TiO_2-assisted photocatalytic oxidation did not result in antimicrobial compounds. Olive mill wastewater and garlic stalk juice exerted substantial antimicrobial effects towards tested microorganisms.

Xylan-based biodegradable films containing lignin, garlic stalk juice, tannic acid and olive mill wastewater were characterized against both *B. pumilus* and *E. coli* by means of their antimicrobial activities. *E. coli* exhibited lesser sensitivity to all tested antimicrobial xylan films except tannic acid-integrated xylan film than *B. pumilus*. Antimicrobial lignin integrated-xylan film exhibited stronger effect towards tested microorganisms than tannic acid-integrated film. In the case of both antimicrobial lignin and tannic acid integrated xylan films,

4% was found to be the maximum antimicrobial compound percentage in film forming solutions to observe continuous film formation.

Lignin samples with/without antimicrobial activity were characterized by means of their chemical structure via FTIR and LC-MS. FTIR results revealed that cotton stalk lignins were significantly broken down via alkaline treatment and this breakdown resulted in the formation of new fractions and also ester & ether bonds between antimicrobial hydroxycinnamic acids and lignin were cleaved during the alkaline treatments of cotton stalk lignins. By FTIR results, C=C bonds were found to be characteristic for antimicrobial lignin sample and it was suggested that these bonds might be the reason of the antimicrobial activity. By LC-MS qualitative mass analysis, antibacterial lignin fractions were found to be quite different from non-antibacterial lignin fractions. LC-MS results indicated that the antimicrobial lignin fractions might be lignin-derived oligomers and/or might be flavonoids. Cotton stalk lignin fractions demonstrated different antimicrobial activities depending on the method of isolation and chemical treatment.

Keywords: cotton stalk lignin, antimicrobial activity, photocatalytic oxidation, ultrasonication, xylan-based films, biodegradable, garlic stalk juice, olive mill wastewater

ÖZ

TARIMSAL BİYOKÜTLEDEN ANTİMİKROBİYEL MOLEKÜL ELDE EDİLMESİ VE KSİLAN TEMELLİ BİYOBOZUNUR FİLMLERDE KULLANIMI

Çekmez, Umut
M.Sc., Department of Biotechnology
Supervisor: Prof. Dr. Ufuk Bakır
Co-Supervisor: Prof. Dr. Zümrüt Begüm Ögel

Ocak 2010, 94 sayfa

Pamuk sapı lignini özütlemeleri alkali yöntemler ile farklı koşullarda yapılmıştır. Ham ve elde edilme sonrası muamele görmüş pamuk sapı ligninleri, zeytin karasuyu ve sarımsak sapı suyu antimikrobiyel aktivite açısından incelenmişlerdir. Antimikrobiyel lignin alkali özütleme koşullarına bağlı olarak elde edilmiştir. 60°C' de özütlenen lignin, *E. coli* ve *B. pumilus'* a karşı dikkate değer antimikrobiyel etki göstermiştir. Fakat ultrasonikasyon ve TiO_2-destekli fotokatalitik oksidasyon gibi değişik elde edilme sonrası muameleleri antimikrobiyel bileşik ile sonuçlanmamıştır. Zeytin karasuyu ve sarımsak sapı suyu denenen mikroorganizmalara karşı kayda değer antimikrobiyel etkiler göstermişlerdir.

Lignin, sarımsak sapı suyu, tannik asit ve zeytin karasuyu içeren ksilan temelli biyobozunur filmler antimikrobiyel aktiviteleri bağlamında *B. pumilus* ve *E. coli'* ye karşı karakterize edilmişlerdir. *E. coli*, tannik asit ilave edilen ksilan filmler hariç tüm denenen antimikrobiyel ksilan filmlere *B. pumilus*' tan daha az duyarlılık göstermiştir. Antimikrobiyel lignin katılmış ksilan filmi, denenen mikroorganizmalara karşı tannik asit katılmış ksilan filminden daha kuvvetli

etki göstermiştir. Ksilan filmlerin antimikrobiyel lignin ve tannik asit içerdiği durumlarda bütün film oluşumu gözlemlemek için film oluşma çözeltilerindeki maksimum antimikrobiyel bileşik yüzdesi yüzde 4 olarak bulunmuştur.

Antimikrobiyel aktiviteli ve aktivitesiz lignin örnekleri kimyasal yapıları bağlamında FTIR ve LC-MS ile karakterize edilmiştir. FTIR sonuçları pamuk sapı ligninlerinin alkali muamele yolu ile önemli ölçüde parçalandığını, bu parçalanmanın yeni parçaların oluşumuyla sonuçlandığını ve ayrıca pamuk sapı ligninlerinin alkali muameleleri sırasında lignin ve antimikrobiyel hidroksisinnamik asitler arasındaki ester ve eter bağlarının açıldığını ortaya çıkartmıştır. FTIR sonuçlarıyla C=C bağlarının antimikrobiyel lignin örneği için karakteristik olduğu bulunmuştur ve antimikrobiyel aktivitenin sebebinin bu bağlar olabileceği öne sürülmüştür. LC-MS niteleyici kütle analizi yardımıyla, antibakteriyel lignin parçalarının antibakteriyel olmayan lignin parçalarından oldukça farklı olduğu bulunmuştur. LC-MS sonuçları antimikrobiyel lignin parçalarının lignin türevli oligomerler ve/veya flavonidler olabileceğini göstermiştir. Pamuk sapı lignini parçaları kimyasal muamele ve elde edilme metoduna bağlı olarak farklı antimikrobiyel aktiviteler göstermişlerdir.

Anahtar sözcükler: Pamuk sapı lignini, antimikrobiyel aktivite, fotokatalitik oksidasyon, ultrasonikasyon, ksilan-temelli filmler, biyobozunur, sarımsak sapı suyu, zeytin karasuyu

TO MY FAMILY

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my supervisor Prof. Dr. Ufuk Bakır for her guidance, patience and unforgettable encouragement throughout not only this study but also my hopeless times.

I would like to thank Turkish National Scientific and Technological Foundation Research Fund (TUBITAK-BAP) and ARÇELİK Dishwasher Plant for their financial supports provided.

I would also thank the members of thesis examining committee; Prof. Dr. Zümrüt Begüm Ögel, Prof. Dr. Necati Özkan, Assoc. Prof. Dr. Candan Gültekin Gürakan, Assoc. Prof. Dr. Göknur Bayram for their suggestions and comments.

I would like to express my gratitude to all my lab colleagues and friends; Didem Sutay, Beril Korkmaz Erdural, Serpil Apaydın, Aytaç Kocabaş, Özlem İkinci, Erdem Boy, Merve Çınar, Zeynep Eker, Esra Uçkun and Barış Boylu for their friendship and support.

Special thanks are due to Gizem Seber, Erinç Bahçegül, Tarkan Uslu, Tuğçe Ertiş, Ömer Göktürk Az and Murat Engin for their endless support, extraordinary help and encouragement.

Finally, I am grateful to my mother Kamile, my father Ümit and my sister Sıla for their love, patience, and endless support.

TABLE OF CONTENTS

ABSTRACT......iv

ÖZ......vi

ACKNOWLEDGEMENT......ix

TABLE OF CONTENTS......x

LIST OF TABLES......xiv

LIST OF FIGURES......xvi

LIST OF ABBREVIATIONS......xviii

CHAPTERS

1. INTRODUCTION 1

1.1 Application of Biological Polymers as Packaging Materials...... 1

1.2 Lignocellulosic Biomass...... 3

1.3 Xylan-based Biodegradable Films 8

1.4 TiO_2 and Its Photocatalytic activity...... 9

1.5 Lignin Fragmentation Processes 10

1.6 The Antimicrobial Effects of Both Different Lignin Types and Different Lignin Fragments 13

1.7 The Antimicrobial Activity of Some Natural Plant Based Compounds .. 17

1.8 Cotton Stalk Waste 19

1.9 Aim of the Study...... 20

2. MATERIALS AND METHODS...... 22

2.1. Materials 22

2.2. Microorganisms and Growth Conditions 22

2.3. Xylan and Lignin Extraction Methods.. 23

2.3.1. Zilliox and Debeire (1998) & Xu *et al.* (2005) Method.................. 23

2.3.2. Xu *et al.* (2005) Method and Modified Version of Zilliox and Debeire (1998) Method... 24

2.4. Garlic Stalk Juice Extraction 24

2.5. Olive Mill Wastewater (OMW)... 25

2.6. Lignin Breakdown Processes.. 25

2.6.1. Ultrasonication of Cotton Stalk Lignin 25

2.6.2. TiO_2-assisted Photocatalytic Oxidation of Cotton Stalk Lignin...... 25

2.7. Antimicrobial Property Control Tests against *E. coli* & *B. pumilus* Cells 26

2.7.1. Liquid Medium Tests.. 26

2.7.2. Solid Medium Tests 27

2.8. Antimicrobial Xylan-based Biodegradable Film Preparation................ 27

2.9. The Antimicrobial Activity Test For Xylan-based Biodegradable Films against *E. coli* & *B. pumilus* Cells 28

2.10. Dynamic Light Scattering Method for Molecular Size Characterization of Photocatalytically Oxidized Cotton Stalk Lignin 29

2.11. Chemical Structure Characterization Methods for Lignin Molecule ... 29

2.11.1. Fourier Transform Infrared Spectroscopy (FTIR)....................... 29

2.11.2. Liquid Chromatography-Mass Spectroscopy (LC-MS) 30

3. RESULTS AND DISCUSSION 31

3.1. Antimicrobial Property Control Tests 32

3.1.1. Liquid Medium Tests 32

3.1.1.1. Thymol 32

3.1.1.2. Carvacrol 34

3.1.2. Solid Medium Tests 36

3.1.2.1. Thymol, Carvacrol and Tannic acid 37

3.2. Extraction of Low-cost Antimicrobial Compounds from Plant-Based Waste Materials 38

3.2.1. The Assessment of Antimicrobial Properties of Cotton Stalk Lignins 38

3.2.1.1. Application without any post treatments 39

3.2.1.2. Application of cotton stalk lignin after exposed to ultrasonication 42

3.2.1.3. Application of cotton stalk lignin after exposed to TiO_2-assisted photocatalytic oxidation 44

3.2.2. The Assessment of Antimicrobial Properties of Green Garlic Stalk Juice 48

3.2.3. The Evaluation of Antibacterial Activities of Olive Mill Wastewater 52

3.3. Natural Antimicrobial Compounds and Their Utilization in Xylan-Based Biodegradable Films 54

3.3.1. The Assessment of the Antibacterial Activity of Tannic acid-integrated Birchwood Xylan Biodegradable Films 55

3.3.2. The Evaluation of Antimicrobial Effect of Birchwood Xylan-based Biodegradable Films Containing Green Garlic Stalk Juice 58

3.3.3. The Assessment of Antimicrobial Activity of Birchwood Xylan-based Biodegradable Films Containing Olive Mill Wastewater............... 60

3.3.4. The Characterization of Cotton Stalk Lignin-Integrated Birchwood Xylan-based Biodegradable Films by Means of Antibacterial Activity 62

3.4. Molecular Characterization of Cotton Stalk Lignins via Different Methods 65

3.4.1. FTIR Spectra of Different Types of Lignins.................................. 65

3.4.2. Molecular Characterization of the Fragmentation Products of Cotton Stalk Lignins by LC-MS 72

4. CONCLUSION AND RECOMMENDATIONS 77

REFERENCES 79

APPENDICES

A. MEDIUM AND AGAR BASES 91

B. BACTERIAL CELL CALIBRATION CURVES 93

LIST OF TABLES

TABLES

Table 3.1 Antimicrobial property of thymol in liquid medium against both *E. coli* & *B. pumilus*.. 33

Table 3.2 Antimicrobial property of carvacrol in liquid medium against both *E. coli* & *B. pumilus* 35

Table 3.3 The antimicrobial activity of thymol, carvacrol and tannic acid against both *E. coli* & *B. pumilus* in solid medium 37

Table 3.4 The antibacterial effects of cotton stalk lignins and their extraction chemicals against both *E. coli* & *B. pumilus* in liquid medium 40

Table 3.5 The antimicrobial activity of Lignin A and Lignin B against both *E. coli* & *B. pumilus* in solid medium 41

Table 3.6 Antibacterial activity of ultrasonicated lignin A in liquid medium against both *E. coli* & *B. pumilus* 43

Table 3.7 The antimicrobial effect of lignin A oxidized by TiO_2 based photocatalysis against both *B. pumilus* and *E. coli* in liquid medium 45

Table 3.8 Molecular size distributions in both oxidized and non-oxidized lignin A obtained from DLS via cumulant fit method 46

Table 3.9 Molecular size distributions in both oxidized and non-oxidized lignin A obtained from DLS via distribution function fit method 47

Table 3.10 Antibacterial effect of garlic stalk juice in liquid medium against both *E. coli* & *B. pumilus*.. 49

Table 3.11 The antibacterial activity of OMW against both *E. coli* & *B. pumilus* in liquid medium... 53

Table 3.12 The antimicrobial effect of green OMW against both *E. coli* & *B. pumilus* in solid medium 54

Table 3.13 The antimicrobial activity of tannic acid-integrated birchwood xylan biodegradable films against both *E. coli* & *B. pumilus* 56

Table 3.14 The antibacterial activity of birchwood xylan biodegradable films containing GGSJ against both *E. coli* & *B. pumilus* 59

Table 3.15 The antibacterial effect of birchwood xylan biodegradable films containing OMW against both *E. coli* & *B. pumilus*... 61

Table 3.16 The antibacterial effect of cotton stalk lignin B-integrated birchwood xylan-based biodegradable films against both *E. coli* & *B. pumilus*................ 63

Table 3.17 LC/MS results of fragmentation products isolated from both lignin A & lignin B... 75

LIST OF FIGURES

FIGURES

Figure 1.1 Cellulose Structure (Brown, 2004)... 3

Figure 1.2 Structure of O-acetyl-4-O-methylglucuronoxylan from hardwood (Puls and Schuseil, 1993)... 4

Figure 1.3 Lignin Structure (Lee, 1997).. 5

Figure 1.4 Secondary Cell Wall Structures of Plants (Bidlack *et al.*, 1992)...... 6

Figure 1.5 The Value-added Products Isolated from Lignocellulosic Biomass (Kamm *et al.*, 2006)... 7

Figure 1.6 A sketch of band gap energy between valence band and conduction band in semiconductor particle (Benedix *et al.*, 2000) 9

Figure 1.7 Three Monomeric Alcohols of Lignin Structure (Zemek *et al.*, 1979) 13

Figure 1.8 Phenolic Monomeric Fragments of Lignin (Zemek *et al.*, 1979). ... 14

Figure 3.1 The antimicrobial activity of garlic stalk juice against *B. pumilus* in solid medium.. 50

Figure 3.2 The antimicrobial activity of garlic stalk juice against *E. coli* in solid medium... 51

Figure 3.3 Continuous xylan-based biodegradable film containing 4% (w/w) tannic acid.. 57

Figure 3.4 Continuous xylan-based biodegradable film containing 4% (w/w) antibacterial lignin B... 62

Figure 3.5 FTIR spectra of three different lignins in the 400-4000 cm^{-1} range 66

Figure 3.6 FTIR spectrum of commercial lignin in the 1800-900 cm^{-1} region ……………… 67

Figure 3.7 FTIR spectrum of non-antibacterial lignin A in the 1800-900 cm^{-1} region 68

Figure 3.8 FTIR spectrum of antibacterial lignin B in the 1800-900 cm^{-1} region 69

Figure 3.9 FTIR spectrum obtained by the subtraction of antibacterial lignin spectrum from non-antibacterial lignin in the 1800-900 cm^{-1} region 71

Figure 3.10 Mass spectrum of fragmentation products isolated from lignin A 73

Figure 3.11 Mass spectrum of fragmentation products isolated from lignin B 73

Figure B.1 Calibration curve for *Bacillus pumilus*. 93

Figure B.2 Calibration curve for *Escherichia coli*. 94

LIST OF ABBREVIATIONS

B. pumilus: *Bacillus pumilus*

CFU: Colony-Forming unit

CSL: Cotton stalk lignin

DLS: Dynamic light Scattering

E. coli: *Escherichia coli*

FTIR: Fourier Transform Infrared Spectroscopy

Gr(+): Gram positive

Gr(-): Gram negative

GGSJ: Green garlic stalk juice

LB medium: Luria-Bertani medium

LC-MS: Liquid Chromatography-Mass Spectroscopy

ml: Milliliter

OMW: Olive mill wastewater

w/v: weight/volume

w/w: weight/weight

CHAPTER 1

INTRODUCTION

1.1 Application of Biological Polymers as Packaging Materials

Food packaging, an important discipline in the area of food technology, is concerned with the preservation and protection of all types of foods and their raw materials (Tharanathan, 2003). The main purposes of the studies on food packaging materials are preventing the spoilage of food, prolonging the shelf life of packed foods and guaranteeing consumer safety (Cha and Chinnan, 2004).

There are various materials used in packaging. The most commonly used ones are petrochemical based plastics such as polyethylene, polyamides, and polyesters. Their satisfactory mechanical properties and low prices render them preferential in packaging applications. On the other hand, most of these widely used plastics are hydrophobic, demonstrating very low water vapor permeation rates and especially, they are fully non-biodegradable leading to environmental problems (Davis and Song, 2006).

The fragmentation of polymers may originate from biodegradation, photo-degradation or chemical degradation. Biodegradation is a natural way of recycling wastes, or breakdown of organic matters into smaller compounds, mineralized and redistributed through elemental cycles such as the carbon and sulphur cycles. Biodegradation is carried out only within the biosphere by a huge assortment of bacteria, insects, fungi and yeast (Chandra and Rustgi, 1998).

Biopolymers are polymers that are biodegradable. The input materials for the production of these polymers may be either renewable (based on agricultural plant or animal products) or synthetic. The synthesis of biopolymers always involves enzyme-catalyzed, chain growth polymerization reactions of activated monomers, which are generally formed within cells by complex metabolic processes (Gross *et al.*, 2001).

Organic material can be degraded aerobically, with oxygen, or anaerobically, without oxygen. The final products of the degradation process are carbon dioxide, water and biomass under aerobic conditions, and hydrocarbons, methane and biomass under anaerobic conditions (Gross and Kalra, 2002).

Current and future developments in biodegradable polymers and renewable input materials focus relate mainly to the scaling-up of production and improvement of product properties. Larger scale production will increase availability and reduce prices (Tharanathan, 2003).

The term antimicrobial packaging includes any packaging techniques used to control bacterial growth in food. In recent years, antimicrobial packaging has gained huge importance in food industry owing to the rise in client demand for minimally processed, preservative-free products (Cha and Chinnan, 2004).

Antimicrobial packaging can take several forms; integration of volatile and non-volatile antimicrobial agents directly into polymers; coating of antimicrobials onto polymer surfaces; immobilization of antimicrobials to polymers by covalent linkages; and use of polymers that are natively antimicrobial (Appendini and Hotchkiss, 2002). There are so many studies in literature about both biopolymer-based and petrochemical-based antimicrobial packaging. Low density polyethylene (LPDE) and polyamides films containing natural antimicrobial compounds such as thymol, carvacrol and trans-cinnamaldehyde exhibited significant antimicrobial effect towards both *Escherichia coli* & *Listeria innocua* and furthermore addition of these active compounds improved the properties of films such as film flexibility and moisture barrier capability (Han *et al*., 2007).

The cellulose packaging produced via laccase initiated grafting of antibacterial compounds such as caffeic acid and isoeugenol etc. demonstrated significant antimicrobial effect towards *E. coli* & *Staphylococcus aureus* (Elegir *et al.*, 2007). Several studies on the antimicrobial characteristics of films made from chitosan (inherently antimicrobial biopolymer) have been carried out earlier (Chen *et al.*, 1996; Coma *et al.*, 2002).

1.2 Lignocellulosic Biomass

Lignocellulosic biomass makes up about 50% of all biomass. It is composed of three major polymeric constituents, namely; cellulose, hemicellulose and lignin (Biely, 1993). Cellulose is famous for being the most abundant of Earth's organic polymers. It is composed of monomers of glucose, a hexose (6-carbon) sugar, linked by ten thousand β(1,4)-glycosidic bonds. The structure of cellulose is shown in Figure 1.1.

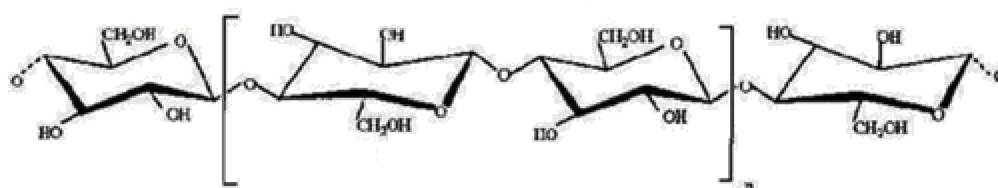

Figure 1.1 Cellulose Structure (Brown, 2004)

Hemicellulose is a highly branched low molecular weight polymer composed of both hexose and pentose (5-carbon) sugars and includes glucan, mannan, arabinan and xylan (Zheng *et al.*, 2009). The main hemicelluloses of softwood are galactoglucomannans and arabinoglucuronoxylan while in hardwood is glucuronoxylan. Hemicelluloses consist of D-glucose, D-mannose, D-xylose, D-glucuronic acid, L-arabinose-4-*O*-methyl-D-glucuronic acid and D-galacturonic acid in various amounts according to the natural source. The sugar units are mainly β-D-xylopyranose (β-D-Xyl*P*); β-D-xglucopyranose (β-D-Glc*p*); β-D-mannopyranose (β-D-Man*p*) α-Larobinofuranose (Ara*f*). They

constitute the backbone structure of glucomannans and xylans (Gabrielli and Gatenholm, 1998).

Xylan, which is the major hemicellulose and the second most abundant polymer in nature, accounts for one-third of renewable biomass available on earth (Gabrielii and Gatenholm, 1998). The main sugar of xylan is D-xylose. Depending on the xylan source, the structure of xylan differs from linear β-1-4-polyxylose main chains to highly branched heteropolysaccharides substituted with mainly acetyl, arabinosyl and glucuronosyl residues. The principle xylan of hardwood, *O*-acetyl-4-*O*-methylglucuronoxylan, consists of a backbone of 1-4-glycosidic bond linked β-xylopyranose residues. Moreover, being found in the cell wall of monocots like grasses and cereals, xylan consists of linear chains of β-D-1-4-linked D-xylopyranosyl residues. Those residues can be also substituted with α-L-arabinofuranosyl at the 2-*O* and/or 3-*O* position(s) and α-D-glucuronopyranosyl or its 4-*O*-methyl derivative at the 2-*O* position (Biely, 1993; Carpita, 1996). In contrast to softwood xylan, hardwood xylan is highly acetylated 4-*O*-methyl-α-D-glucuronoxylan, essentially without arabinosyl substitution. Approximately, seven out of ten xylosyl residue carries an α-*O*-methylglucuronyl residue at *O*-2 (Evtuguin *et al.*, 2003). The hardwood xylan structure is represented in Figure 1.2.

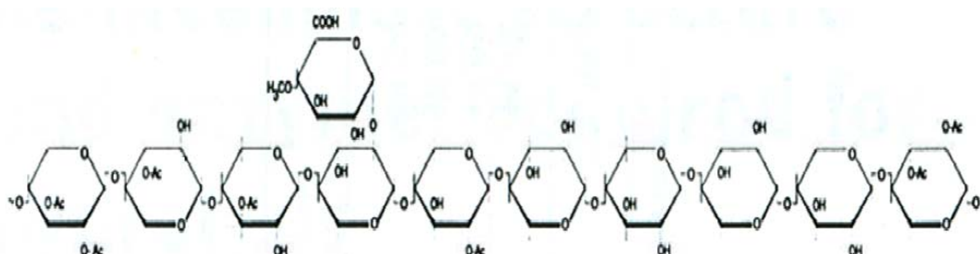

Figure 1.2 Structure of *O*-acetyl-4-*O*-methylglucuronoxylan from hardwood (Puls and Schuseil, 1993)

They are associated with the lignin via ester, ether and glycosidic bonds in plant cell walls (Scalbert *et.al*, 1985; Thompson, 2000). Xylan can be extracted from many different agricultural wastes including wheat straws, cotton stalks etc. via alkaline extraction (Zilliox and Debeire, 1998).

Lignin, the second most abundant natural compound after cellulose (Boudet and Grima-Pettenati, 1996), is a natural high molecular weight biopolymer consisting of phenylpropane units. The phenylpropane units are attached to one another by a series of characteristic linkages (β-O-4, β-5, β- β, etc.). Cellulose, lignin, and hemicelluloses are the main components of plant cell walls. Partly cellulose gives plant its strength and stiffness. However, without the matrix of lignin, and hemicelluloses, the plant cell walls would not stay intact (Sarkanen and Ludwig, 1971). A typical lignin structure is given in Figure 1.3.

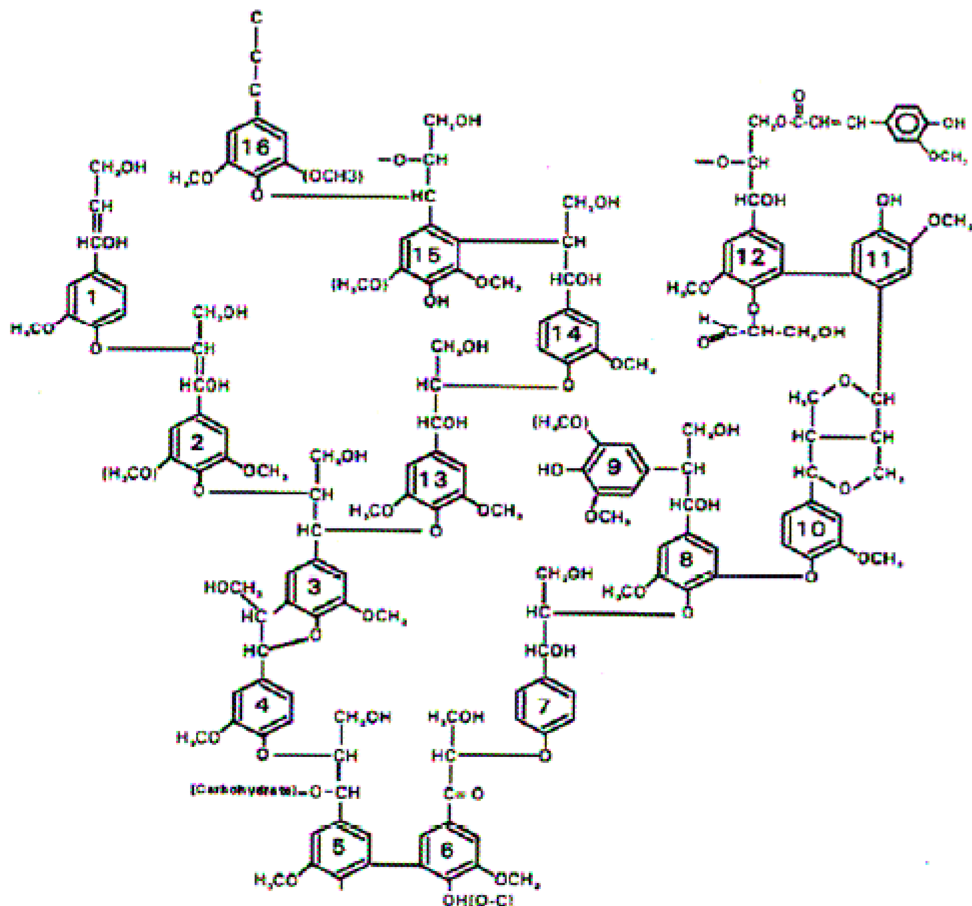

Figure 1.3 Lignin Structure (Lee, 1997)

As shown in Figure 1.4. the hemicellulose and cellulose are imbedded in the lignin.

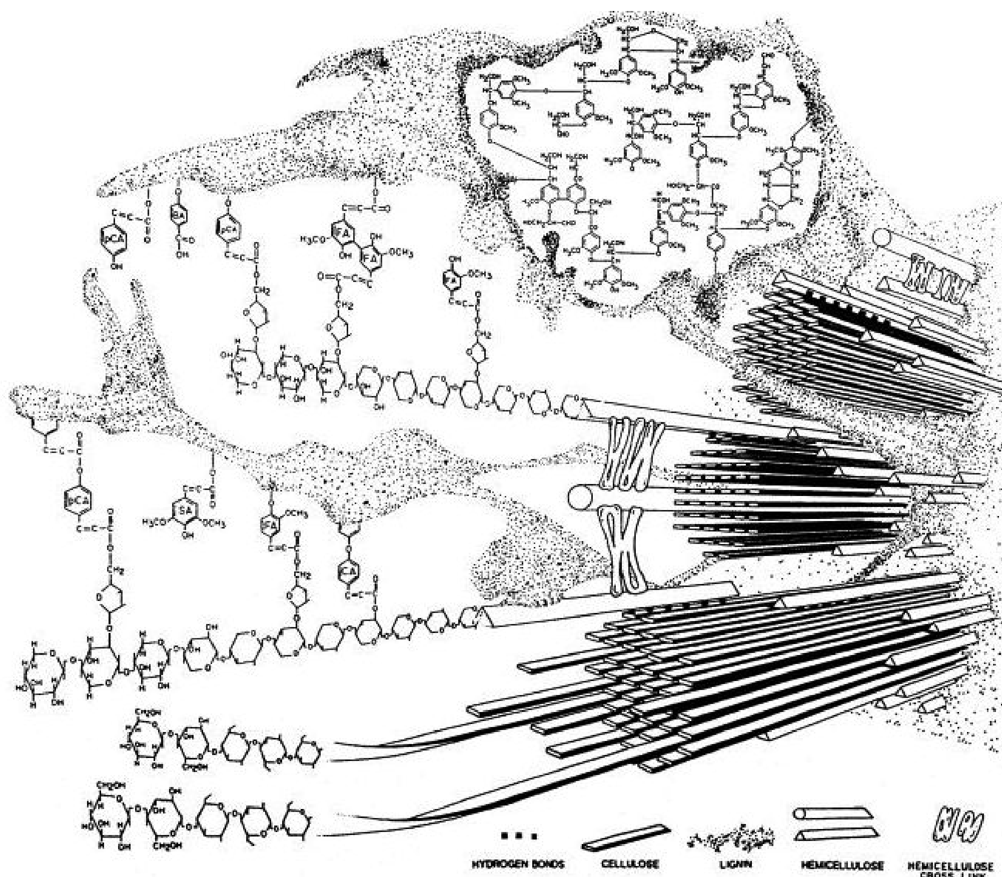

Figure 1.4 Secondary Cell Wall Structures of Plants (Bidlack *et al.*, 1992)

Alkali soluble and acid soluble lignins were isolated from agricultural waste, sugarcane bagasse via series of chemical & physical treatments ended up with pH precipitation (Xu *et al.*, 2005). Also lignin was isolated via acidolytic dioxane extraction from cotton stalks, jute stick and dhaincha (Jahan *et al.*, 2006).

The literature studies on the structure, hydrodynamic and physiochemical properties of lignin molecule have demonstrated that it is tough to get a general picture of the exact shape and size of an isolated lignin (Belgacem, 2000; Goring, 1989). The size and shape of an isolated lignin is highly dependent on the lignin concentration, type of solvent and type of extraction processes (Vainio *et al.*, 2004).

Gupta and Goring (1960) studied the range of lignin molecule size via using sedimentation and viscometry as analytical method and found that the radius of gyration of alkali lignin molecule is between 44-170 nm.

Lignocellulosic biomass can be converted to value-added products via different thermochemical, biochemical, chemical and physical methods as illustrated in Figure 1.5.

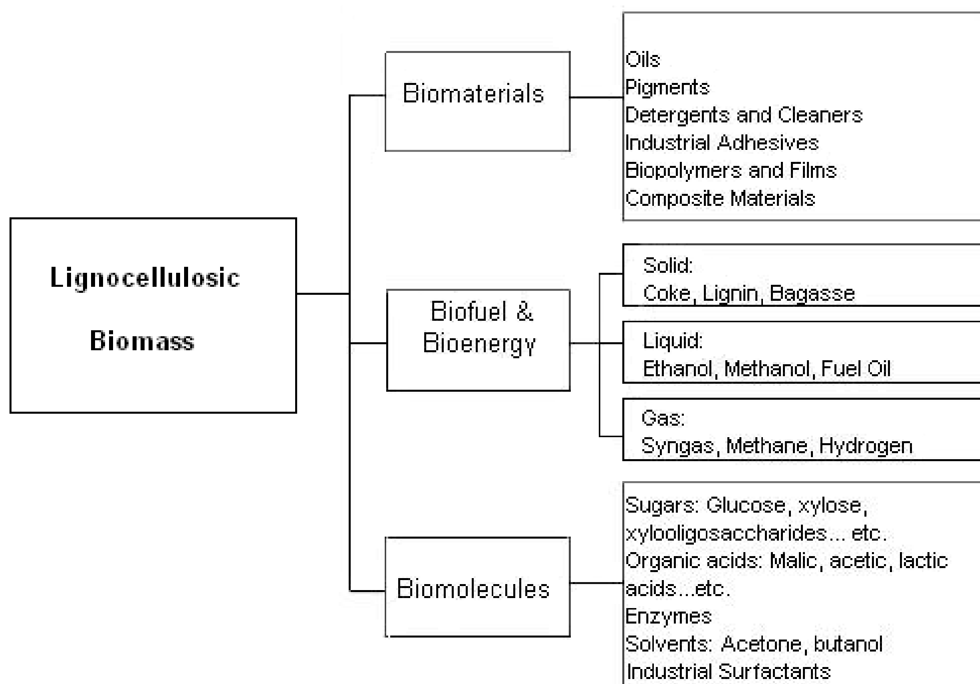

Figure 1.5 The Value-added Products Isolated from Lignocellulosic Biomass (Kamm *et al.*, 2006)

Lignocellulosic biomass can be chemically converted into value-added alcohols, acids, phenol derivatives, hydrocarbons etc. by the process of fermentation, hydrolysis, hydrogenation, crystallization, etc. For example, Ou *et al.* (2009) represented a technology for production of coumaric acids (phenolic acids) from sugarcane bagasse.

Lignin can be also chemically converted to value-added products including ink, paint, food and cement additives, adhesives, antioxidants and antimicrobial agents as well (Lignin.org).

1.3 Xylan-based Biodegradable Films

Xylan can be used as film forming biopolymer. But xylan in its pure form cannot be able to form continuous films. The films produced with xylan isolated from aspen (Gabrielli and Gatenholm, 1998) and birchwood (Gabrielli et al., 2000) were characterized by means of their film forming properties and it was concluded that continuous and self-supporting xylan films are able to be produced only with chitosan at its 10% and above concentrations in film forming solutions.

Composite film production of cotton stalk xylan was also studied. The complete film formation was observed via using xylan with partial removal of lignin during its extraction. As the xylan concentration increases in film forming solutions, thickness of the films increases which decreases the water vapor permeability of the film. To increase the water vapor permeability of xylan films, glycerol was added as an extra plasticizer to film forming solutions and it worked (Göksu, 2005).

Lignin was found to be necessary additive to produce continuous and self-supporting birchwood xylan films. The minimum percentage was determined as 1% lignin in xylan (w/w) for complete film formation (Karamanlıoğlu, 2008).

1.4 TiO_2 and Its Photocatalytic activity

Titanium dioxide, also known as titanium (IV) oxide or titania, is the naturally occurring oxide of titanium. Three polymorphs of TiO_2 are anatase, rutile and brookite phases. This semiconductor is inexpensive, chemically stable and harmless to human health and environment, and has no absorption in visible region. Semiconductors including TiO_2, SnO_2 and ZnO are highly active under UV light irradiation with their higher wavelength UV absorption (UV-A, 320-400 nm) (Thiruvenkatachari *et al.*, 2008).

The electronic structure of semiconductors is characterized by a filled valance band and an empty conduction band. The energy difference between the lowest energy level of the conduction band and the highest energy level of the valance band is called band gap energy (E_g) (shown in Figure 1.6); electrons are able to jump from one band to another (Benedix *et al.*, 2000).

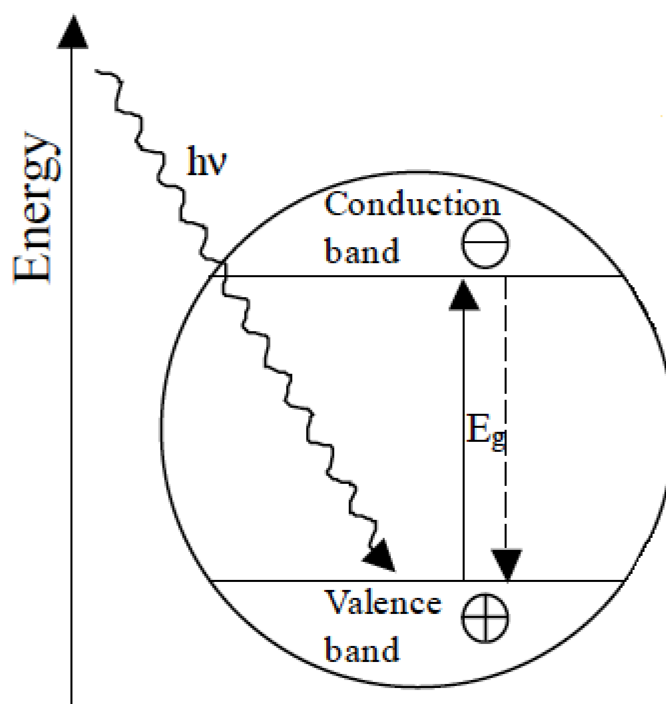

Figure 1.6 A sketch of band gap energy between valence band and conduction band in semiconductor particle (Benedix *et al.*, 2000)

The band gap energy of anatase type titanium oxide is 3.2 eV, which is equivalent to 388 nm wavelength. The absorption of ultraviolet rays shorter than this wavelength induces photocatalysis. When light is absorbed by titanium oxide, two carriers; electrons (e-) and positive holes (h+) are formed via jumping of an electron from the valence band to the conduction band. In ordinary substances, electrons and positive holes pair quickly; however, in titanium oxide photocatalyst the formation of the electron/hole (e-/h+) pairs more slowly. The carrier recombination percentage is major dependant on the photocatalytic efficiency. The surface of a photocatalyst contains water, which is referred to as "absorbed water." When this water molecule reacts with positive holes, water converts to hydroxyl radicals (• OH) which have strong oxidative breakdown power. If oxygen is present when this process takes place, the organic matter decomposes to carbon dioxide and water due to the reaction between (• OH) and H_2O. On the other hand, electron pairs cause the generation of superoxide radicals (• O_2^-) by the reduction of O_2 molecules. Superoxide anions are capable of many selective/non-selective oxidation reactions (Fox and Dulay, 1993; Hashimoto *et al.*, 2005).

1.5 Lignin Fragmentation Processes

In wood, lignin tends to form covalently bonded complexes with cellulose and hemicellulose it is not easy to separate from the plant cell walls (Perez *et al.*, 2002). The strong carbon-carbon and ether linkages in lignin molecule make it resistant to degradation (Besle *et al.*, 1994).

In paper industry, purified lignin is recovered as a by-product of cellulose production via different chemical treatments during wood-pulping processes. Chemical pulping involves the use of chemicals able to break down and dissolve lignin from the wood cell walls, are releasing cellulose fibers. Lignin is contained in pulp and paper mill effluents, which is a major chemical oxygen demand (COD) component and is responsible for color of the effluents (Franta *et al.*, 1994). Therefore, study of lignin degradation is of major environmental interest.

The oxidative breakdown of lignin molecule has been studied by using ozone, permanganate and UV-illuminated H_2O_2 (Tanaka *et al.*, 1999). These studies demonstrated that such breakdown treatments alone were not capable of reducing COD effectively. Further studies have been performed on the combination of oxidation with biological treatment (Nakamura *et al.*, 1997).

Photocatalytic oxidation method has been extensively studied on the breakdown of lignin molecule. The (UV/semiconductor) photocatalytic systems have been found to be effective on complete mineralization of lignin molecule at ambient temperatures and pressures without the production of any risky by-products such as phenols, aromatics etc. (Kobayakawa *et al.*, 1989; Khodja *et al.*, 2001; Lathasree *et al.*, 2004). Tanaka *et al.* (1999) have been studied the photocatalytic depolymerization of lignin by using TiO_2 as photocatalyst and found that it was successively depolymerized to oxygenated compounds such as aldehydes and carboxylates. Daneshvar *et al.* (2004) have been reported that ZnO can be appropriate alternative photocatalyst to TiO_2 due to their resemblance in photocatalysis mechanisms.

Kansal *et al.* (2008) studied the effect of different variables including catalyst type, catalyst dose, solution pH, oxidant concentration and initial concentration of substrate on photocatalytic breakdown of lignin and found that ZnO was more effective photocatalyst than TiO_2. The optimum values for studied variables were determined for an effective photocatalysis of lignin molecule and it was showed that COD removal from lignin can be accomplished via photocatalysis without any extra biological processes.

Lignin can also be fragmented to its monomeric phenols via several thermochemical depolymerization methods reviewed by Amen-Chen *et al.* (2001). In thermochemical fragmentation methods, high pressures and high temperatures are used and its breakdown products are so complex and separation cost is high. Due to these economic issues, there is limited information is available in literature.

Hammel *et al*. (1997) reviewed the breakdown of lignin via extracellular fungal enzymes including lignin peroxidases, manganese peroxidases and laccases. Both the characteristic properties and different breakdown mechanisms of these enzymes were explained and were discussed.

Ultrasonication is also a breakdown process which is used in the degradation of long chain macromolecules. It is well established that prolonged exposure of solutions of macromolecules to high-energy ultrasonic waves causes the degradation of macromolecules (Mason and Lorimer, 2002). Grönroos *et al*. (2008) studied the ultrasonic degradation of aqueous carboxymethylcellulose and found that the high molecular weight polymers degraded faster than low molecular weight ones and breakdown of polymers depended on the molecular mass and the concentration of polymers. Azhar and Hamdy (1979) studied the ultrasonication effect on potato starch and reported that the breakage of C-C bonds in starch molecule was accomplished via ultrasonication.

Also, Buranov and Mazza (2008) reported that monomeric phenolic fragments including ferulic acids and coumaric acids formed via the breakage of ester bonds present in lignin during its alkaline extraction.

1.6 The Antimicrobial Effects of Both Different Lignin Types and Different Lignin Fragments

Lignin is synthesized by peroxidase and/or laccase enzymes which initiate dehydrogenative polymerization of three monomeric aromatic phenylpropanoid precursors; coniferyl, p-coumaryl, and synapyl alcohols (Boudet and Grima-Pettenati, 1996). The structures of these monomeric alcohols are illustrated in Figure 1.7.

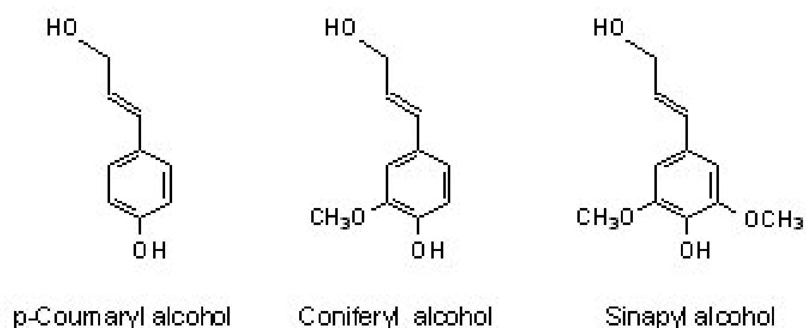

Figure 1.7 Three Monomeric Alcohols of Lignin Structure (Zemek *et al.*, 1979)

Variance in lignin content among plant species is caused by the differences in the proportions of these monomeric alcohols. Softwood lignins are approximately 80% coniferyl, 14% p-coumaryl and 6% synapyl alcohols. However, hardwood lignins are approximately 56% coniferyl, 4% p-coumaryl and 40% synapyl alcohols (Baurhoo et al., 2008).

Wood lignin contains 11 monomeric phenolic fragments, with p-coumaric acid and ferulic acids being the major phenolics in forages (Jung and Fahey, 1983) as seen in Figure 1.8.

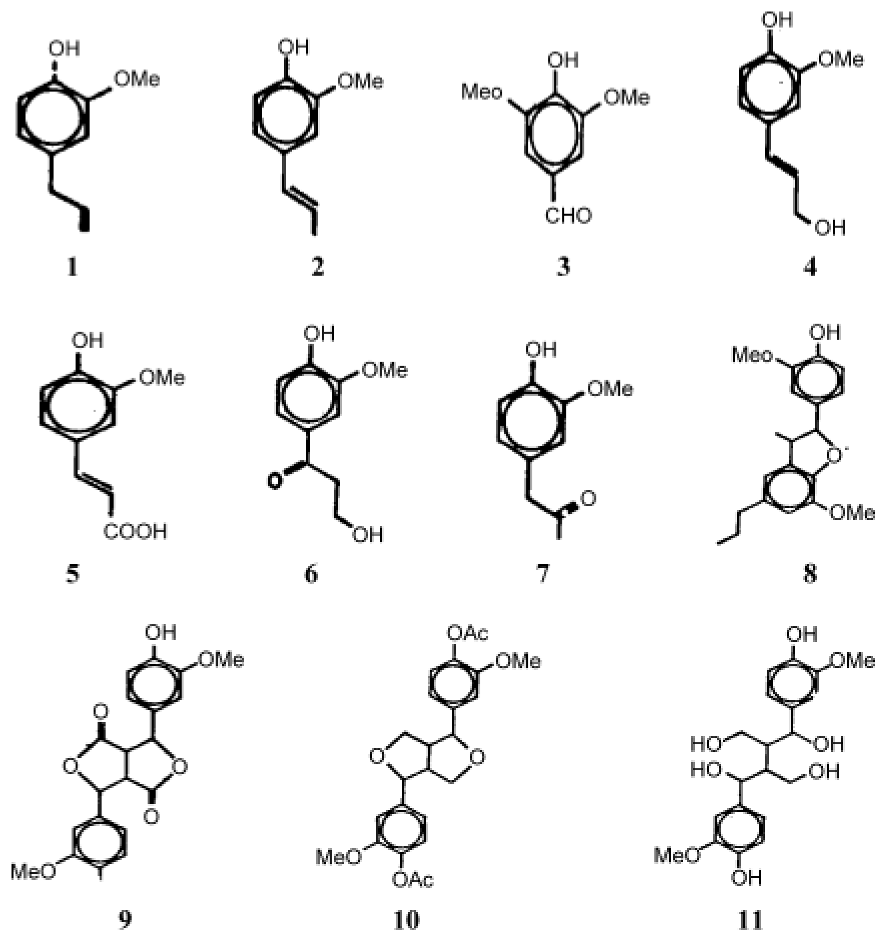

Figure 1.8 Phenolic Monomeric Fragments of Lignin (Zemek *et al.*, 1979).
1: Eugenol; 2: isoeugenol; 3: syringaldehyde; 4: coniferyl alcohol; 5: ferulic acid; 6: 4-hydroxy-3-methoxy-β- hydroxy-propiophenone; 7: 1-(4-hydroxy-3-methoxyphenyl)-2-propanone; 8: 2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-3-methyl-5-propyl-coumaran ("dehydro-diisoeugenol"); 9: pinoresinoldione ("dehydrodiferulic acid"); 10: Di-*O*-acetylpinoresinol; and 11: 2,3-bis(α-hydroxy-vanillyl)-1,4-butane-diol

The antimicrobial effect of 11 phenolic fragments of lignin was investigated against microorganisms including *E. coli*, *Saccharomyces cerevisiae*, *Bacillus licheniformis* and *Aspergillus niger* (Zemek *et al.*, 1979) and they found that the most inhibitory phenolic fragment was isoeugenol among all tested microorganisms and the order of antimicrobial effectiveness of isoeugenol

against tested microorganisms was *B. licheniformis* > *E. coli* = *S. cerevisiae* > *A. niger*. The other fragments of lignin were also found to be antimicrobial but they exhibited weaker antimicrobial effects among tested microorganisms than isoeugenol.

In general, the antimicrobiality of lignin is highly dependent on its side chain structure and the nature of the functional groups of its phenolic fragments (Baurhoo *et al.*, 2008). The reason for the antimicrobial effect of isoeugenol was explained via the presence of double bond in α, β position of the side chain and the presence of a methyl group in the γ position (Jung and Fahey, 1983).

Gosselink *et al.* (2004) reported that the major chemical functional groups in lignin include the hydroxyl, methoxyl, carbonyl, and carboxyl groups in various numbers and proportions, depending on origin and extraction processes. Depending on the method of isolation and chemical treatment, new functional groups not present in natural lignin may appear. Baurhoo *et al.* (2008) have reported that lignins may possess different biological activities depending on their extraction methods.

Alcell lignin (Alcell Technologies Incorporation, Montreal, Quebec, Canada), which is a trade name for compound generally known as organosolv lignin, is recovered as a by-product of cellulose production during wood pulping process in the paper industry. Rousu *et al.* (2002) demonstrated that the Alcell process broke down the lignin molecule and produced hydrophobic low molecular weight lignin fragments as a co-product. In Alcell process, woods are cooked in aqueous ethanol at 190°C.

Alcell lignin (50g/l) itself was found to be capable of inhibiting the growth of *E. coli*, *S. aureus* and *Pseudomonas* by the study of Nelson *et al*. (1994).

Kraft Lignin is also produced as a co-product of the paper-making industry, separated from cellulose in the wood fibers via kraft pulping process. Kraft process involves a mixture of sodium hydroxide and sodium sulphide (known as white liqour) that breaks the linkages between cellulose and lignin in wood structure. The phenolic fragments of Kraft lignin, which are isolated during pulping process, were found to be very effective antioxidants by *in vivo* studies (Catignani and Carter, 1982). Nada *et al*. (1989) studied the antimicrobial effect of lignin isolated from bagasse via Kraft process against *Bacillus subtilis*, *Bacillus mycoids*, *E. coli* and *A. niger* and found that bagasse lignin exhibited antimicrobial effect only towards gram positive bacteria namely *B. subtilis* and *B. mycoids*.

Also Nada *et al*. (1989) studied the antimicrobial activity of lignin isolated from cotton stalk via different extraction procedure against *B. subtilis*, *B. mycoids*, *E. coli* and *A. niger*. Only difference between new extraction process and Kraft process was the addition of anthraquinone in cooking liquor of cotton stalks. Cotton stalk lignin was found to be only effective against gram positive bacteria as in the case of bagasse lignin. No significant antimicrobial effect of lignins was observed against both gram negative bacterium, *E. coli* and fungus, *A. niger*.

1.7 The Antimicrobial Activity of Some Natural Plant Based Compounds

The natural products derived from medicinal plants have proven to be a rich source of biologically active compounds, many of which have been the beginning for the improvement of new chemicals for pharmaceuticals. The general antimicrobial activities of natural plant based products, such as essential oils, have been reviewed previously (Cowan, 1999; Kalemba and Kunicka, 2003).

Thymol and carvacrol, which are the major phenol derivatives found in the oil of oregano and thyme, are isomeric with each other. Thymol was found to be effective against several species of bacteria such as *Listeria monocytogenes*, *S. aureus*, *B. subtilis*, *Bacillus cereus*, *E. coli*, *Salmonella enterica*, *Pseudomonas aeruginosa* and *Campylobacter jejuni* (Sivropoulou *et al.*, 1996; Friedman *et al.*, 2002). Moreover, Gr(-) bacteria have been proven to be more sensitive to thymol than Gr(+) bacteria. In agar disc diffusion experiments of thymol, zone of inhibition values were determined as 7.2 mm and 8.5 mm against *B. cereus* and *E. coli*, respectively (Tippayatum and Chonhenchob, 2007). Hydrophobicity characteristic of thymol enable it to penetrate the lipopolysaccharide part of the Gr(-) cell membrane, and the leakage of cell content occurred by lesion of the membrane and quick discharge of potassium ions (Burt, 2004 and Pina-Vaz *et al.*, 2004).

In liquid broth experiments of carvacrol, minimal inhibition concentration was determined as 0.25 g/l against both *B. subtilis* and *E. coli* and carvacrol demonstrated same antimicrobial effect towards both Gr(+) and Gr(-) bacteria (Ben Arfa *et al.*, 2006 and Burt *et al.*, 2005). Michiels *et al.* (2007) have reported that carvacrol was more effective among tested Gr(+) and Gr(-) bacteria than thymol and studied the synergistic effect of thymol and carvacrol among tested bacteria species and no significant synergistic effect of thymol and carvacrol was observed.

The antimicrobial effect of carvacrol is associated with its lipophilic character, which affects both the lipid ordering and the bilayer stability of cell membranes, leads to the decrease of membrane integrity and depletion of intracellular ATP (Friedman, 2006 and Sikkema *et al.*, 1995). Plant phenolics, carvacrol and thymol possess similar mechanisms of antibacterial activity resulting from their similar chemical structures including systems of delocalized electrons and hydroxyl groups (Ultee *et al.*, 2002).

Tannic acid (commercial name for tannin), which is a polymer of gallic acid molecules and glucose, present in oak wood, tea, berries and in red wine grape skins, seeds and stems with high concentrations. The general antimicrobial activities of tannic acid have been review previously (Chung *et al.*, 1998). Tannic acid was capable of inhibiting the growth of all 15 types of test bacteria such as *E. coli*, *Proteus vulgaris*, *S. aureus*, etc. at a concentration of 5mg/mL and Gr(-) bacteria exhibited stronger sensitivity to tannins than Gr(+) bacteria (Chung *et al.*, 1993).

Biological and medical activities of garlic are mainly due to its high organo-sulphur compounds content. Major determinants for the antimicrobial effect of garlic were found to be allicin, dially sulphide and ajoene (Corzo-Martinez *et al.*, 2007). Bacteria against which garlic is effective include strains of *E. coli*, *Salmonella*, *Pseudomonas*, *Proteus*, *S. aureus*, *Klebsiella*, *B. subtilis* (Harris *et al.*, 2001).

Among various natural antimicrobial agents, nisin which is a polypeptide produced by *Lactococcus lactis* has been most widely studied for its activities. Because it is produced by lactic acid bacteria that often found in human's digestive tract and recognized as probiotic. Nisin was found to be effective against a wide range of spoilage and pathogenic gram-positive bacteria (Han, 2005).

1.8 Cotton Stalk Waste

Cotton, which is a natural fiber and hardwood, is botanically known as *Gossypium hirsutum* from Malvaceae family. The average cotton production in the world is approximately 20 million tons and Turkiye is one of the major producers of cotton along with China, India, The United States, Greece, Syria and Turkmenistan. The production of cotton in Turkiye is about 2.5 million tons per year giving rise to approximately 1.5 million tons of available residues (agrowaste-tr.org; www.dtm.gov.tr). Large amounts of cotton waste from the field and gins result in environmental problems due to disposal issues, cotton diseases and pests. Development of economical and efficient methods for utilization of cotton waste has been investigated for years. Some previous studies about the conversion of cotton stalk into value-added products were; 1-) bioethanol production from cotton waste (Demirbaş, 2005), 2-) char, hydrogen, and pyrolysis gases production from cotton waste (Balat, 2009), 3-) hemicellulose based biodegradable film production from cotton stalk xylan (Göksu, 2005), 4-) xylan based biodegradable antimicrobial film production by the use of cotton stalk as raw material (Karamanlıoğlu, 2008), 5-) Phenol formaldehyde type adhesives production from cotton stalk (Fidan, 2005). Cotton stalk can also be reliable, renewable and environmentally acceptable resource with high potential for conversion into value-added products especially natural based antimicrobial phenolics due to its high lignin content (28%) which has been determined by the study of Akpınar *et al.* (2007).

1.9 Aim of the Study

Food packaging is an important field in food industry and in recent years, antimicrobial biodegradable packaging has gained huge importance in food industry owing to the rise in client demand for minimally processed, environmental friendly and preservative-free products. Current trend for biodegradable film production is using biopolymers such as xylan instead of petrochemicals leading to environmental problems.

Cotton stalk, which is an important agricultural waste in Turkiye, contains considerable amount of lignin. Lignin is a superior source for the isolation of natural antimicrobial compounds because; this phenolic compound contains 11 monomeric phenolic fragments whose antimicrobial activities have been previously studied (Zemek *et al.*, 1979) and some studies demonstrated that lignins exhibited antimicrobial activities against several microorganisms by itself depending on their origin and extraction procedure (Baurhoo *et al.*, 2008; Nada *et al.*, 1989). Also lignin is broken down via fungal enzymes (Hammel *et al.*, 1997), thermochemical degradation (Amen-Chen *et al.*, 2000), and semiconductor assisted photocatalytic oxidation (Kansal *et al.*, 2008) that are considered to be time consuming and expensive processes.

The antimicrobial activities of garlic have been reviewed by Martinez *et al.*, (2007) in literature. Up to now, there are several studies about antimicrobial activities of garlic components isolated from its bulb and/or its clove. However, there is very limited information about the content and the antimicrobial activity of components of fresh green garlic stalk which can be considered as agricultural waste. Also, olive mill wastewater (OMW), which is an agricultural waste of olive oil industry, contains phenolic compounds. In literature, OMW exhibited antibacterial effect towards tested bacterium (Gonzalez *et al.*, 1990).

The major aim of the study was to develop easy, cheap and environmentally acceptable processes for the isolation of value-added antimicrobial compounds from plant based agricultural wastes which can be further applied in biorefineries and to produce 100% natural xylan-based antimicrobial biodegradable films by using these antimicrobial compounds.

For this purpose, lignin was extracted from cotton stalk via both classical and modified version of Zilliox & Debeire method (1998) and extracted lignins were characterized by means of their antimicrobial activity against *E. coli* and *Bacillus pumilus* in both liquid and solid medium. Additionally, garlic juice extracted from green garlic stalk and olive mill wastewater were also characterized in the same way. Fragmentation processes such as ultrasonication and TiO_2-assisted photocatalytic oxidation were utilized to isolate lignin fragments that demonstrate antimicrobial activity and the antimicrobial effects of post-treated lignin samples were evaluated against tested bacteria. Following these steps, xylan-based biodegradable films containing compounds that demonstrate antimicrobial activity were characterized against *B. pumilus* & *E. coli* by means of their antimicrobial properties. Moreover, lignin based compounds having antimicrobial activity were characterized by means of their chemical structure via FTIR and LC/MS analysis.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Cotton stalk was brought from a local producer in Adıyaman, Turkey. Fresh green garlic was purchased from greengrocer in Ankara, Turkey. Olive mill wastewater was obtained from Marmara Birlik, olive plant in Bursa, Turkey.

Carvacrol ($C_{10}H_{14}O$), Mueller Hinton agar, Luria-Bertani (LB) Agar, thymol ($C_{10}H_{14}O$), alkali lignin, birchwood xylan, xylitol were purchased from Sigma (Germany). Titanium dioxide (TiO_2) was bought from Aldrich (Germany). Luria-Bertani (LB) broth, nutrient broth (NB), boric acid (H_3BO_3), tannic acid, acetic acid (CH_3COOH), ethanol (C_2H_5OH), potassium bromide (KBr), potassium hydroxide (KOH), 35% hydrochloric acid ($HCl_{aq.}$), sodium hydroxide (NaOH), sodium borohydride ($NaBH_4$) were purchased from Merck (Germany). Sterile discs were purchased from Fluka (Switzerland). Nylon syringe filters, 0.20 µm pore size, were purchased from Sartorius (Göttingen, Germany).

2.2. Microorganisms and Growth Conditions

Esherichia coli (XL1-blue) was maintained on Luria-Bertani (LB) agar. Plates were incubated at 37°C overnight and then stored at 4°C until use. *E. coli* was incubated in Luria-Bertani (LB) broth at 37°C on a rotary shaker (Minitron) at 170 rpm overnight.

Bacillus pumilus (SB-M13; isolated in our laboratory) was maintained on Luria-Bertani (LB) agar. Plates were incubated at 30°C for 24 hours and then stored

at 4°C until use. *B.pumilus* was incubated in nutrient broth (NB) at 30°C on a rotary shaker (Minitron) at 180 rpm for 24 hours.

Medium and agar bases are given in Appendix A.

2.3. Xylan and Lignin Extraction Methods

2.3.1. Zilliox and Debeire (1998) & Xu *et al.* (2005) Method

The method used by Zilliox and Debeire (1998) was followed for the xylan extraction. Dried cotton waste was milled. 1000 ml of distilled water was added to 20 g of milled cotton stalk and the sample was swelled at 60°C for 16 hours in an incubator (Nüve, Turkey). The swollen sample was filtered by a filter paper (Whatman No. 41) and the pellet was mixed with solution containing 170 ml of 24% (w/v) KOH + 1 % (w/v) $NaBH_4$ solution and stirred for 3 hours at room temperature. The suspension was filtered and the supernatant was mixed with 500 ml cold ethanol solution containing 10% (v/v) acetic acid. This suspension was shaken for several minutes and a centrifugation step was performed at 10,000 rpm for 10 minutes. The precipitated part was collected and dried at 60°C for 24 hours to recover xylan.

The method used by Xu *et al.* (2005) was adapted to the end of xylan extraction for the lignin extraction. After xylan isolation, supernatant was boiled until its ethanol and acetic acid were evaporated. After evaporation of ethanol and acetic acid, the solubilized alkali soluble lignin was obtained by reprecipitation at pH 1.5, adjusted with 6M HCl. The isolated lignin preparation was washed with acidified water (pH 2.0) and filtered through Whatman No.41 filter paper via vacuum pump (Millipore). Finally, filtrate was dried at 105°C for 4 hours in incubator to recover lignin.

2.3.2. Xu *et al.* (2005) Method and Modified Version of Zilliox and Debeire (1998) Method

The method used by Zilliox and Debeire (1998) was modified. Dried cotton waste was milled. 1000 ml of distilled water was added to 20 g of milled cotton stalk and the sample was swelled at 60°C for 1 hour in an incubator (Nüve, Turkey). The swollen sample was filtered by a filter paper (Whatman No. 41) and the pellet was mixed with solution containing 170 ml of 10% (w/v) NaOH + 1 % (w/v) H_3BO_3 solution and stirred for 3 hours at 60°C.

The suspension was filtered and the supernatant was mixed with 500 ml cold ethanol solution containing 10% (v/v) acetic acid. This suspension was shaken for several minutes and a centrifugation step was performed at 10,000 rpm for 5 minutes. The precipitated part was collected and dried at 60°C for 24 hours to recover xylan.

The method used by Xu *et al.* (2005) was adapted to the end of xylan extraction for the lignin extraction. After xylan isolation, supernatant was boiled until its ethanol and acetic acid were evaporated. After evaporation of ethanol and acetic acid, the solubilized alkali soluble lignin was obtained by reprecipitation at pH 1.5, adjusted with 6M HCl. The isolated lignin preparation was washed with acidified water (pH 2.0) and filtered through Whatman No.41 filter paper via vacuum pump (Millipore). Finally, filtrate was dried at 105°C for 4 hours in incubator to recover lignin.

2.4. Garlic Stalk Juice Extraction

The green stalks of fresh garlic were cut off and original raw garlic juice was prepared by direct squeezing of garlic stalks in a commercial juicer and then stored at 4°C until use. It was sterilized through nylon syringe filters (Sartorius, 0.20 μm pore size) before using it in antimicrobial property control tests.

2.5. Olive Mill Wastewater (OMW)

Olive mill wastewater (Marmara Birlik) was stored at 4°C and kept away from direct light (to prevent decomposition of phenolics present in OMW) until use. Before evaluation of its antibacterial effect, it was 10-fold concentrated via evaporating its water in an oven at 70°C and its pH was adjusted to 7 with HCl. Then it was sterilized through nylon syringe filters (Sartorius, 0.20 µm pore size).

2.6. Lignin Breakdown Processes

2.6.1. Ultrasonication of Cotton Stalk Lignin

50 g/l and 100 g/l solutions of lignin extracted from cotton stalk via method that was described in 2.3.1 were prepared in 100 ml glass beaker. Cotton stalk lignin solutions were exposed to ultrasonic waves at a frequency of 20 ± 0.4 KHz via ultrasonic processor (Cole Parmer, CP70-T) for 10 and 30 minutes. To prevent the formation of glass cracks originating from overheat; beaker was covered with crushed ice during ultrasonication.

2.6.2. TiO_2-assisted Photocatalytic Oxidation of Cotton Stalk Lignin

Photocatalytic oxidation was carried out in 1000 ml glass beaker. Beakers were covered with aluminum folio to prevent scattering of light and to achieve complete interaction between light and sample. Total reaction mixture volumes were 200 ml. The reaction mixtures contained 100 mg/l lignin (extracted from cotton stalk via method described in 2.3.1) sample and 1 g/L TiO_2. The pH of reaction mixtures were adjusted to 11 with 1M NaOH. The mixtures were mixed with a magnetic stirrer at 250 rpm at ambient temperature while irradiated with artificial irradiation source (bulb) which was applied vertically. In order to carry out photocatalytic oxidation, Osram Ultra-Vitalux (Product Number: 003313) 300W bulb with similar spectral distribution to solar spectrum between 280 and 780 nm was used as artificial irradiation source.

The light intensity over the test bench was adjusted to achieve 100 W/m^2 by adjusting the distance between the bench and light source. Lignin samples were exposed to TiO_2 assisted photocatalytic oxidation for 1 and 3.5 hours. After photocatalysis was accomplished, TiO_2 was removed from oxidized reaction mixture via centrifugation (at 10,000 rpm for 5 minutes).

2.7. Antimicrobial Property Control Tests against *E. coli* & *B. pumilus* Cells

2.7.1. Liquid Medium Tests

Before bacterial inoculation, all liquid media (NB and LB broths) were sterilized in autoclave at 121°C for 15 minutes. All solutions of compounds and/or aqueous wastes, whose antimicrobial effects were under consideration, were sterilized through nylon syringe filters (Sartorius, 0.20 μm pore size) before test.

Test media were LB broth and nutrient broth (NB) for *E. coli* and *B. pumilus*, respectively. Test medium volumes were 20 ml. Overnight incubated *E. coli* (corresponding to $1x10^9$ CFU/ml) and 24 hours incubated *B. pumilus* (corresponding to $9x10^8$ CFU/ml) suspensions were used as inoculum in liquid medium tests. Firstly, 1% (v/v) inoculum and 5% (v/v) solution of antimicrobial test compounds were transferred aseptically to sterile liquid test media. For blank media, only 1% (v/v) inoculum was added aseptically to sterile test media. Then, the optical density values of test media were recorded via UV-vis spectrophotometer (Shimadsu, UV-1601) at 550 nm wavelength before and after overnight incubation with the presence/absence of 5% antimicrobial test compound. Then measured optical density values (OD_{550}) were converted to the number of bacterial cells via the characteristic correlation lines (Appendix B) for *E. coli* & *B. pumilus*. This conversion was done to calculate the bacterial inactivation percentage caused by antimicrobial test compounds.

2.7.2. Solid Medium Tests

Agar disc diffusion method was used to screen the antimicrobial activity. In vitro antimicrobial activity was screened by using Mueller Hinton Agar (MHA). The MHA plates were prepared by pouring 20 ml of molten autoclaved agar (121°C for 15 minutes) into sterile Petri plates. The plates were allowed to solidify for 5 minutes and were covered with aluminum foil to protect agars from direct light contact. It was stored at 4°C until use. Inocula were overnight-incubated *E. coli* (corresponding to $1x10^9$ CFU/ml) and 24 hours-incubated *B. pumilus* (corresponding to $9x10^8$ CFU/ml) suspensions.

Under aseptic conditions, 110 µl of inoculum suspensions were spread onto the MHAs and the inoculum was allowed to dry for 5 minutes. The sterile discs (Fluka, 10 mm in diameter) were hold by the edge with sterile forceps and dipped into the solutions of antimicrobial test compounds. The impregnated discs were placed onto the agar surface via sterile forceps and solutions were allowed to diffuse for 3 minutes and the plates were incubated at 37°C for 24 hours. At the end of the incubation, inhibition zones formed around each disc were measured with transparent ruler in millimeters (including the disc diameter). These studies were performed in duplicate.

2.8. Antimicrobial Xylan-based Biodegradable Film Preparation

For xylan-based film preparation, the solvent casting method used by Gröndahl *et al.* (2004) was modified. Blank xylan-based films were casted from birchwood xylan (Sigma) by dissolving 0.6 g (2.4% w/w) of birchwood xylan and 0.4 g (1.6% w/w) xylitol (Sigma) in 25 ml of ultra purified water and agitated by a magnetic stirrer at 450 rpm at 90°C until complete solubilization of the polymers has been accomplished. Differently from blank film preparation procedure, antimicrobial compounds and/or extracts with concentrations of 0.8%, 2% and 4% (w/w) were added to film forming solutions during antimicrobial xylan-based film preparation. At the stirring

stage, the pH of all films was adjusted to 7 with 6M NaOH except films containing alkali soluble cotton stalk lignin.

The pH of xylan films containing alkali soluble lignin (ASL) was adjusted to 9 in order to solubilize ASL. After all components of films were completely solubilized in water, the film forming solutions were poured into Petri dishes (diameter = 9 cm). Finally, film forming solutions were dried at room conditions for 4 days.

2.9. The Antimicrobial Activity Test For Xylan-based Biodegradable Films against *E. coli* & *B. pumilus* Cells

E. coli cells were cultivated in Luria-Bertani (LB) broth at 37°C on a rotary shaker (Minitron) at 170 rpm overnight. *B. pumilus* cells were cultivated in nutrient broth (NB) at 30°C on the rotary shaker at 180 rpm for 24 hours.

1.5 ml of *E. coli* and *B. pumilus* cell suspensions was transferred to Eppendorf tubes and tubes were centrifuged at 10,000 rpm for 4 min. Supernatants were removed and the pellets were mixed with 1 ml of peptone water and 0.1 ml of these concentrated cultures were put on the quarter of the both blank and antimicrobial xylan-based film samples. After 8-hour interaction time between films and tested bacteria, serial dilutions in peptone water (0.1%) were performed. The film samples were well mixed with 9.9 ml of peptone water until complete solubilization of xylan-based films has been achieved. Then, 0.1 ml of this mixture was taken and mixed with 9.9 ml of peptone water. After vortex mixing, this mixture was diluted with peptone water for counting. 0.1 ml of appropriately diluted (10^6 dilution) microbial suspensions was inoculated on Luria-Bertani (LB) agar. In the case of evaluation of antimicrobial activity against *E. coli* cells, agar plates were incubated at 37°C overnight. On the other hand, agar plates were incubated at 30°C for 24 hours in the case of antimicrobial activity test against *B. pumilus* cells. Next day, the number of bacterial cells survived was counted by viable colony count method.

Additionally, to determine the initial number of tested bacterial cells that were put on xylan-based films, 0.1 ml of liquid cell suspensions (serially diluted to 10^6) was spread onto agar plates and cultivated.

2.10. Dynamic Light Scattering Method for Molecular Size Characterization of Photocatalytically Oxidized Cotton Stalk Lignin

Both photocatalytically oxidized and non-oxidized cotton stalk lignin solutions at 100 mg/l concentration were filtered with 0.5 µm filters before dynamic light scattering (DLS) analysis. The sample volumes used for analysis were 2 ml. DLS measurements were performed using ALV (Germany)/Malvern CGS-3 (UK) compact goniometer system with a constant 90°C scattering angle, at 21°C ± 0.2°C. The wavelength of He-Ne laser in compact goniometer system was 632.8 nm. The scattering intensity data were processed via Malvern's software using both cumulant fit & distribution function fit methods to obtain molecular size distribution of scatterers in each sample. Scattered light intensity vs. size classes (nm) distribution plot was obtained. All samples were analyzed in duplicate.

2.11. Chemical Structure Characterization Methods for Lignin Molecule

2.11.1. Fourier Transform Infrared Spectroscopy (FTIR)

To elucidate the structural differences between different alkaline soluble cotton stalk lignins and commercial lignin (alkali lignin with low sulfonate content, Sigma), FTIR spectrometer (Bruker, Equinox 55) was used. All lignin samples used for FTIR were in powder form. The dried samples were embedded in KBr pellets in the concentrations of about 1mg/100mg KBr. The FTIR spectra were recorded in the absorption band mode in the range of 4000-400 cm^{-1}.

2.11.2. Liquid Chromatography-Mass Spectroscopy (LC-MS)

To investigate the differences in the structures of fragmentation products formed during the alkaline extractions of different cotton stalk lignins, qualitative mass analysis was performed via LC-MS (Agilent 1100 MSD). Different types of lignin were dissolved in tetrahydrofuran (THF) and were filtered through filters (0.45 μm pore size). After filtered lignin solutions were injected to LC-MS, mass spectroscopy spectra were recorded for fragmentation products of different cotton stalk lignins. The detailed MS conditions: atmospheric pressure interface electro spray (API-ES) was adopted with both negative and positive mode polarity, the fragmentor voltage was 55 eV and the scanning range was at m/z (mass to charge ratio) 55-1500 with 1.10 s/scan.

CHAPTER 3

RESULTS AND DISCUSSION

In this study, the major aim was to isolate renewable, natural, low-cost and non-toxic molecules having antimicrobial properties from agricultural biomass and to produce environmental friendly, biodegradable and antimicrobial films that could be further used in food packaging industry. For this purpose, different antimicrobial compounds were evaluated for their antimicrobial properties and later on integrated into xylan based biodegradable films to render them antimicrobial.

First of all, plant phenolics including thymol, tannic acid and carvacrol were characterized by means of their antimicrobial activity in solid and liquid media and used as positive controls for the rest of the experiments. Additionally, olive mill wastewater, which also contains plant phenolics, was also characterized in the same way. Both the chemicals and conditions of classical alkaline extraction of lignin were altered to isolate structurally different lignins from cotton stalk. Afterwards different types of lignins and garlic stalk extract were isolated with different extraction methods from biomass including cotton stalk and green garlic stalk, respectively and these isolates were also evaluated for their antimicrobial properties. In order to modify the structures of lignins by fragmentation, different methods such as ultrasonication and TiO_2 assisted photocatalytic oxidation were utilized. Following these steps, xylan based biodegradable films containing compounds that demonstrate antimicrobial activity were characterized against two microorganisms by means of their antimicrobial properties. *Escherichia coli* and *Bacillus pumilus* were used as Gr(-) and Gr(+) bacteria, respectively. Finally, lignin based compounds having antimicrobial activity were characterized by means of their chemical structure via FTIR and LC/MS analysis.

3.1. Antimicrobial Property Control Tests

3.1.1. Liquid Medium Tests

In liquid medium tests, different concentrations of compounds and definite percentage of bacterial cells in exponential growth phase were added to sterile liquid cultivation medium in test tubes and incubated for overnight. After incubation, optical densities of the cultures were measured and percentage reductions in the number of bacterial cells were calculated. The major aim is to observe complete inhibition against tested bacterial cells because; tested antimicrobial compounds will be used as positive control for further experiments. In this part, only the results of thymol and carvacrol are given and discussed. The reasons for not assessing the antimicrobial property of tannic acid in liquid medium are the solubility problem of tannic acid and precipitation of proteins in liquid cultivation medium incubated with tannic acid.

3.1.1.1. Thymol

For the evaluation of antimicrobial effect of thymol, different concentrations were tested on the growth of both *E. coli* and *B. pumilus* in liquid cultivation media. Well-known plant phenolics, thymol and carvacrol, were used as positive control in this study while investigating the antimicrobial property of isolated compounds from agricultural waste. So, the key point was to observe complete bacterial inactivation, not to determine the exact IC_{100} (100% inhibitory concentration) of thymol and carvacrol against tested microorganisms. Concentrations of thymol & percentage of bacterial inactivation are given in Table 3.1.

Table 3.1 Antimicrobial property of thymol in liquid medium against both *E. coli* & *B. pumilus*

Thymol concentration range (mg/ml)	The number of *E. coli* cells (CFU/ml) after overnight incubation at 37°C	Microbial log reduction in the number of *E. coli* cells	The number of *B. pumilus* cells (CFU/ml) after overnight incubation at 30°C	Microbial log reduction in the number of *B. pumilus* cells
0.00	$(1.80 \pm 0.01) \times 10^9$	0.000 ± 0.000	$(9.00 \pm 0.10) \times 10^8$	0.000 ± 0.000
0.05	$(1.10 \pm 0.02) \times 10^9$	0.214 ± 0.008	$(6.00 \pm 0.20) \times 10^8$	0.176 ± 0.014
5.00	$(9.00 \pm 0.10) \times 10^8$	0.301 ± 0.005	$(5.20 \pm 0.10) \times 10^8$	0.238 ± 0.008
10.00	$(2.00 \pm 0.30) \times 10^8$	0.954 ± 0.065	$(2.00 \pm 0.25) \times 10^8$	0.653 ± 0.050
50.00	0.00 ± 0.00	9.255 ± 0.002	$(0.80 \pm 0.10) \times 10^8$	1.051 ± 0.051
60.00	0.00 ± 0.00	9.255 ± 0.002	0.00 ± 0.00	8.954 ± 0.005

All tests were performed in duplicate.

As seen in Table 3.1, at all thymol concentrations, microbial log reduction values of *B. pumilus* were determined to be lower than the microbial log reduction values of *E. coli*. For example, at 60 mg/ml thymol concentration, average log reduction values were determined as 9.255 and 8.954 against *E. coli* and *B. pumilus*, respectively. Also the growth of *E. coli* was completely inhibited in liquid medium containing 50 mg/ml thymol and complete bacterial inactivation was observed against *B. pumilus* at 60 mg/ml thymol concentration. All these findings consequently indicated that *E. coli* showed higher sensitivity to thymol than *B. pumilus* in agreement with the results of Tippayatum and Chonhenchob (2007). Hydrophobicity characteristic of thymol enable it to penetrate the lipopolysaccharide part of the Gr(-) cell membrane, and the leakage of cell content occurred by lesion of the membrane and quick discharge of potassium ions (Burt, 2004 and Pina-Vaz *et al.*, 2004).

3.1.1.2. Carvacrol

For assessment of the antibacterial effect of carvacrol, different concentrations were tested on the growth of both *E. coli* and *B. pumilus* in liquid cultivation media. Carvacrol was used as positive control as thymol in further antimicrobial property investigation experiments. So, again major aim was to observe complete inhibition, not to determine the exact IC_{100} of carvacrol against both *E. coli* and *B. pumilus*. Concentrations of carvacrol & percentage of bacterial inactivation are given in Table 3.2.

Table 3.2 Antimicrobial property of carvacrol in liquid medium against both *E. coli* & *B. pumilus*

Carvacrol concentration range (mg/ml)	The number of *E. coli* cells (CFU/ml) after overnight incubation at 37°C	Microbial log reduction in the number of *E. coli* cells	The number of *B. pumilus* cells (CFU/ml) after overnight incubation at 30°C	Microbial log reduction in the number of *B. pumilus* cells
0.00	$(1.80 \pm 0.01) \times 10^{9}$	0.000 ± 0.000	$(9.00 \pm 0.10) \times 10^{8}$	0.000 ± 0.000
0.05	$(1.07 \pm 0.01) \times 10^{9}$	0.226 ± 0.004	$(5.10 \pm 0.10) \times 10^{8}$	0.247 ± 0.008
5.00	$(8.50 \pm 0.30) \times 10^{8}$	0.326 ± 0.015	$(4.10 \pm 0.20) \times 10^{8}$	0.341 ± 0.021
10.00	$(1.10 \pm 0.10) \times 10^{8}$	1.214 ± 0.040	$(5.50 \pm 0.15) \times 10^{7}$	1.214 ± 0.121
50.00	0.00 ± 0.00	9.255 ± 0.002	0.00 ± 0.00	8.954 ± 0.005

All tests were performed in duplicate.

The antimicrobial effect of carvacrol is associated with its lipophilic character, which affects both the lipid ordering and the bilayer stability of cell membranes, leads to the decrease of membrane integrity and depletion of intracellular ATP (Friedman, 2006 and Sikkema *et al.*, 1995). Plant phenolics, carvacrol and thymol possess similar mechanisms of antibacterial activity resulting from their similar chemical structures including systems of delocalized electrons and hydroxyl groups (Ultee *et al.*, 2002). As seen in Table 3.2., all microbial log reductions in the number of tested microorganisms are quite parallel for each carvacrol concentration.

Carvacrol exhibited very similar antibacterial effect in liquid medium against both Gr(+) and Gr(-) bacteria in agreement with the results of Burt (2004) and Ben Arfa *et al.* (2006). From Table 3.1 & Table 3.2 it can also be deduced that thymol exhibits weaker inhibitory activity towards both *E. coli* & *B. pumilus* than carvacrol by comparing microbial log reductions at different thymol & carvacrol concentrations, which is on par with literature (Michiels *et al*., 2007).

3.1.2. Solid Medium Tests

In solid medium tests, agar disc diffusion method described in section 2.7.2 was used. Definite amount of bacterial cells in exponential growth phase were transferred from liquid medium to Mueller-Hinton agar. Sterile discs were soaked into the solution of tested compounds at definite concentrations and then were placed onto inoculated Mueller-Hinton agar. Clear zones around discs were measured after 24 hours incubation to assess the antibacterial activity of tested compounds. The results of thymol, carvacrol and tannic acid are given and discussed.

3.1.2.1. Thymol, Carvacrol and Tannic acid

Thymol, carvacrol and tannic acid were characterized by means of their antimicrobial property via agar disc diffusion method. For thymol & carvacrol, the concentrations at which complete inhibition was observed in previous liquid medium tests were used in this part. No liquid medium test was performed for tannic acid owing to its inappropriateness that has mentioned in liquid medium tests part. For tannic acid, the concentration, 5 mg/ml was used as Chung *et al.* (1993) did. The major aim of this part is to observe clear zones that will be comparable with further solid medium experiments. Concentrations of tested compounds & zone of inhibition values for each bacterium are given in Table 3.3.

Table 3.3 The antimicrobial activity of thymol, carvacrol and tannic acid against both *E. coli* & *B. pumilus* in solid medium

		Zone of Inhibition (mm)	
Compound	Concentration (mg/ml)	*B. pumilus*	*E. coli*
Thymol	60	30 ± 1	37 ± 3
Carvacrol	50	22 ± 2	26 ± 1
Tannic acid	5	15 ± 1	19 ± 2

Sterile Disc Diameter was 10 mm.

From Table 3.3, it can be concluded that *B. pumilus* shows lesser sensitivity to thymol & carvacrol than *E. coli* by evaluating measured clear zones. Also Gr(+) bacterium, *B. pumilus* showed lesser sensitivity to tannic acid than Gr(-) bacterium, *E. coli* in agreement with the results of Chung *et al.* (1993). However, the antimicrobial property of these three plant-based compounds against tested microorganisms cannot be compared with one another. And also findings obtained from this part cannot be compared with the results of liquid medium tests part.

Because; agar disc diffusion method in itself is extremely dependent on water solubility and the diffusivity of tested compounds through agar. In this part, problems associated with the evaluation of the antimicrobial effects of tested compounds via diffusion method were originated from low water solubility of thymol & carvacrol and low diffusivity of tannic acid through agar.

3.2. Extraction of Low-cost Antimicrobial Compounds from Plant-Based Waste Materials

Lignocellulosic biomass refers to plant biomass that is composed of cellulose, hemicellulose, and lignin. Also, lignocellulosic biomass is affordable, renewable and environmentally acceptable resource with high potential for production of antioxidants and antimicrobial compounds as well. Different types of lignin were extracted from lignocellulosic biomass, cotton stalk via different extraction methods and were characterized by means of their antimicrobial properties via liquid and solid medium tests. Additionally, products such as garlic stalk juice and olive mill wastewater, which are isolated from non-lignocellulosic agricultural waste materials, were also characterized in the same way.

3.2.1. The Assessment of Antimicrobial Properties of Cotton Stalk Lignins

For the first type of alkali soluble lignin extracted from cotton stalk, lignin and xylan separation from cellulose was done via Zilliox & Debeire (1998) method. For the second type of alkali soluble cotton stalk lignin, lignin and xylan separation from cellulose was done by a modified version of Zilliox & Deberie (1998) method. Lignin was concentrated via evaporation and pH precipitation as Xu *et al.* (2005) did. Isolated lignins were evaluated for their antibacterial properties by liquid and solid medium tests against Gr(+) and Gr(-) bacteria.

3.2.1.1. Application without any post treatments

Two types of cotton stalk lignin and chemicals used during their whole extraction period were characterized by means of their antibacterial properties against both *B. pumilus* and *E. coli*. For both liquid and solid medium tests, 50 mg/mL concentration of isolated lignins was used, in agreement with Nelson *et al.* (1994). In liquid medium tests of this part, the pH values of all test media were adjusted to 8.5 owing to working with alkali soluble lignins and being able to observe the bacterial inactivation independently from pH. In this part, no post fragmentation treatments such as ultrasonication or photocatalytic oxidation were performed on lignin molecules.

Table 3.4 The antibacterial effects of cotton stalk lignins and their extraction chemicals against both *E. coli* & *B. pumilus* in liquid medium

Compounds & Concentrations (g/L)	The number of *E. coli* cells (CFU/ml) after overnight incubation at 37°C	Microbial log reduction in the number of *E. coli* cells	The number of *B. pumilus* cells (CFU/ml) after overnight incubation at 30°C	Microbial log reduction in the number of *B. pumilus* cells
Lignin A, 50 g/L	$(1.00 \pm 0.02) \times 10^9$	0.013 ± 0.009	$(9.20 \pm 0.30) \times 10^8$	0.014 ± 0.004
Lignin B, 50 g/L	0.00 ± 0.00	9.013 ± 0.004	0.00 ± 0.00	8.978 ± 0.010
A, -	0.00 ± 0.00	9.013 ± 0.004	0.00 ± 0.00	8.978 ± 0.010
B, -	$(1.03 \pm 0.01) \times 10^9$	0.000 ± 0.000	$(9.50 \pm 0.20) \times 10^8$	0.000 ± 0.000

All tests were performed in duplicate. Initial load of *E. coli* cells: $(1.03 \pm 0.01) \times 10^9$ CFU/ml, Initial load of *B. pumilus* cells: $(9.50 \pm 0.20) \times 10^8$ CFU/ml, Lignin A: Lignin isolated from cotton stalk via Zilliox & Debeire (1998) method, A: %24 KOH + %1 $NaBH_4$ + acetic acid + ethanol, Lignin B: Lignin isolated from cotton stalk by a modified version of Zilliox & Debeire (1998) method, B: %10 NaOH + %1 Boric acid + acetic acid + ethanol.

Isoeugenol, which is one of the antimicrobial lignin monomers, could be used as positive control. However, due to its lower antimicrobial activity with respect to generalized antimicrobial activities of thymol & carvacrol, it was not preferred in the study. At 50 g/l concentration, while thymol & carvacrol were completely inactivating the growth of *E. coli*, isoeugenol inhibited only the 40% of *E. coli* growth at the same concentration (Mastelic *et al.*, 2008). From Table 3.4, it can be indicated that lignin B demonstrates significant antibacterial effect against both *E. coli* and *B. pumilus*. At 50 g/l lignin B concentration, complete bacterial inactivation was observed against *E. coli* & *B. pumilus*. On the other hand in the case of lignin A, microbial log reductions in the number of both *E. coli* and *B. pumilus* were around 0.01 average. Though chemical mixture A includes a highly toxic compound called sodium borohydride ($NaBH_4$) which inhibits the growth of both *E. coli* and *B. pumilus* completely, lignin A isolated with the assist of this chemical mixture demonstrated very weak antibacterial effect against tested microorganisms.

Also, no significant *E. coli* or *B. pumilus* inactivation originated from chemical mixture B was observed. However, lignin B showed the strongest activity against tested bacteria. From these findings, it can be deduced that the purity of alkali cotton stalk lignins is very high after whole extraction procedure and chemicals A and B that were used during lignin extraction are separated from cotton stalk lignins in a fairly effective way. Inactivation caused by lignin B is independent from extraction chemicals and pH.

Table 3.5 The antimicrobial activity of Lignin A and Lignin B against both *E. coli* & *B. pumilus* in solid medium

		Zone of Inhibition (mm)	
Compound	Concentration (mg/ml)	*B. pumilus*	*E. coli*
Lignin A	50	X	X
Lignin B	50	17 ± 1	21 ± 1

Sterile Disc Diameter was 10 mm, X: No inhibition zone.

From Table 3.5, it can be concluded that lignin A showed no antibacterial activity against both *E. coli* and *B. pumilus* in solid medium test. On the other hand, lignin B exhibited stronger inhibitory activity towards both *E. coli* & *B. pumilus* than lignin A. Inhibition zone values of lignin B were indicated that *B. pumilus* exhibited lesser sensitivity to lignin B than *E. coli*. The antimicrobial properties of lignin B were demonstrated via both liquid and solid medium tests. Lignin B is antibacterial, natural, renewable and not toxic to human health and can be integrated into xylan-based biodegradable films to render them antibacterial. Before commercialization of xylan-based biodegradable films, the antimicrobial activity of lignin B should be investigated against different microorganisms including pathogens that are more resistant than both *E. coli* and *B. pumilus*. Fragmentation treatments such as ultrasonication and photocatalytic oxidation will be applied on lignin A to break down lignin molecule and to isolate smaller phenolic fractions that demonstrate antibacterial activity.

3.2.1.2. Application of cotton stalk lignin after exposed to ultrasonication

In this part, the major aim is to break down lignin A to phenolic components that exhibit antibacterial effects. The phenolic components of lignin have been reported to inhibit growth of microorganisms such as *E. coli*, *Saccharomyces cerevisiae*, *Bacillus licheniformis* and *Aspergillus niger* (Zemek *et al*., 1979). Different concentrations of lignin A were exposed to ultrasonication at a frequency of 20 ± 0.4 KHz for different time periods. After ultrasonication treatment, the antibacterial effect of lignin A was evaluated against both *E. coli* & *B. pumilus* and was discussed.

Table 3.6 Antibacterial activity of ultrasonicated lignin A in liquid medium against both *E. coli* & *B. pumilus*

Compounds & Ultrasonication time (min.)	The number of *E. coli* cells (CFU/ml) after overnight incubation at 37°C	Microbial log reduction in the number of *E. coli* cells	The number of *B. pumilus* cells (CFU/ml) after overnight incubation at 30°C	Microbial log reduction in the number of *B. pumilus* cells
50 g/l Lignin A, 0	$(9.95 \pm 0.05) \times 10^8$	0.015 ± 0.002	$(8.70 \pm 0.10) \times 10^8$	0.038 ± 0.005
50 g/l Lignin A, 10	$(9.70 \pm 0.20) \times 10^8$	0.026 ± 0.010	$(8.60 \pm 0.30) \times 10^8$	0.043 ± 0.015
50 g/l Lignin A, 30	$(9.40 \pm 0.10) \times 10^8$	0.040 ± 0.005	$(8.90 \pm 0.10) \times 10^8$	0.028 ± 0.005
100 g/l Lignin A, 0	$(9.90 \pm 0.10) \times 10^8$	0.017 ± 0.005	$(8.85 \pm 0.05) \times 10^8$	0.031 ± 0.002
100 g/l Lignin A, 30	$(9.80 \pm 0.10) \times 10^8$	0.022 ± 0.005	$(8.60 \pm 0.10) \times 10^8$	0.043 ± 0.005

All tests were performed in duplicate. Initial load of *E. coli* cells: $(1.03 \pm 0.01) \times 10^9$ CFU/ml, Initial load of *B. pumilus* cells: $(9.50 \pm 0.20) \times 10^8$ CFU/ml, Lignin A: Lignin isolated from cotton stalk via Zilliox & Debeire (1998) method.

After liquid medium tests, as seen in Table 3.6 it can be concluded that no additional inhibition due to ultrasonication was observed against both *E. coli* & *B. pumilus* under the conditions used. The maximum microbial log reduction in the number of bacterial cells was 0.04 average. Also, in solid medium tests, no inhibition zone was observed for all post treated cotton stalk lignins used. As a result, in spite of altering the concentration and ultrasonication exposure time of lignin A, no positive effects of these parameters were observed by means of antimicrobial property.

In literature it has been reported that macromolecules such as potato starch can be degraded by ultrasonication similar to the process performed on lignin A in this part (Azhar and Hamdy, 1979). Due to the complexity of lignin molecule and the strength of C-C bonds and α and β-aryl ether bonds in lignin molecule (Besle *et al.*, 1994), depolymerization of lignin A may not be accomplished via ultrasonication. Alternative fragmentation methods such as photocatalytic oxidation should be applied on lignin A.

3.2.1.3. Application of cotton stalk lignin after exposed to TiO_2-assisted photocatalytic oxidation

The purpose of this part is to break down lignin A via TiO_2-assisted photocatalysis to smaller phenolic fragments that demonstrate antibacterial properties. Lignin A and TiO_2 concentrations were kept constant at 100 mg/l and 1 g/l, respectively because these concentrations were found to be the most effective ones for photocatalytic degradation of lignin molecule by Kansal *et al.* , 2008. 100 mg/l lignin A and 1 g/l TiO_2 were exposed to UV irradiation under constant mixing rate for different time periods to break down the lignin A. TiO_2 demonstrates antimicrobial activity under sunlight. TiO_2 was removed from samples to observe the antimicrobial activity of lignin independently from TiO_2. After removal of TiO_2 from oxidized lignin products, concentration was carried out via evaporation. Oxidized lignins were characterized by means of their antimicrobial properties against both *E. coli* & *B. pumilus* in liquid and solid media.

Table 3.7 The antimicrobial effect of lignin A oxidized by TiO_2 based photocatalysis against both *B. pumilus* and *E. coli* in liquid medium

Compounds & Oxidation time (h.)	The number of *E. coli* cells (CFU/ml) after overnight incubation at 37°C	Microbial log reduction in the number of *E. coli* cells	The number of *B. pumilus* cells (CFU/ml) after overnight incubation at 30°C	Microbial log reduction in the number of *B. pumilus* cells
Lignin A, 0.0	$(9.60 \pm 0.40) \times 10^8$	0.030 ± 0.018	$(8.70 \pm 0.30) \times 10^8$	0.038 ± 0.015
Lignin A, 1.0	$(9.70 \pm 0.30) \times 10^8$	0.026 ± 0.014	$(8.90 \pm 0.10) \times 10^8$	0.028 ± 0.005
Lignin A, 3.5	$(9.95 \pm 0.05) \times 10^8$	0.015 ± 0.002	$(9.00 \pm 0.00) \times 10^8$	0.023 ± 0.000

All tests were performed in duplicate. Initial load of *E. coli* cells: $(1.03 \pm 0.01) \times 10^9$ CFU/ml, Initial load of *B. pumilus* cells: $(9.50 \pm 0.20) \times 10^8$ CFU/ml, Lignin A: Lignin isolated from cotton stalk via Zilliox & Debeire (1998) method.

No significant antibacterial effect was observed against tested microorganisms for all oxidized lignins as seen in Table 3.7. As the photo-oxidation time increases, microbial log reductions in the number of both *E. coli* and *B. pumilus* cells decrease. Moreover, no microbial log reduction was observed more than 0.04 average. Also, no clear zones were observed for all oxidized lignins used against both *E. coli* and *B. pumilus*, respectively.

One reason for not observing any antimicrobial effect against tested microorganisms may be unsuccessful depolymerization of lignin A under the conditions used. Another possible reason may be the complete degradation of lignin instead of obtaining desired antibacterial fractions.

To understand whether lignin compound was completely broken down or not, molecular size distributions in oxidized and non-oxidized cotton stalk lignins were analyzed via dynamic light scattering (DLS).

Table 3.8 Molecular size distributions in both oxidized and non-oxidized lignin A obtained from DLS via cumulant fit method

Sample	Mean radius (nm)	Polydispersity index (PDI)
Non-oxidized Lignin A	56.05	0.1872
Lignin A oxidized via 1 hour UV irradiation	66.56	0.0958
Lignin A oxidized via 3.5 hours UV irradiation	59.35	0.1491

All samples were analyzed in a single DLS run. Lignin A: Lignin isolated from cotton stalk via Zilliox & Debeire (1998) method.

Vainio *et al.* (2004) have reported that the molecule size of lignin is affected by lignin type and solvent type and alkali soluble lignin molecule size range is between 44-170 nm (Gupta *et al.*, 1960). As seen in Table 3.8 the radii of non-

oxidized and oxidized lignins were determined within the alkali soluble lignin radius range of Gupta *et al.* (1960) by cumulant fit method. From Table 3.8 it can also be deduced that lignins exposed to TiO_2-assisted photocatalytic oxidation are not completely broken down under the conditions used. As the oxidation time increases, no significant change in mean radius values of photo-oxidized lignin A is observed with respect to non-oxidized lignin A. Also no substantial change in polydispersity indexes was observed. Polydispersity indexes gave information about the distribution of mean radii in lignin A samples. With respect to non-oxidized lignin A, 1-hour photo-oxidized lignin A sample demonstrated narrower molecular size distribution and lignin A sample photo-oxidized for 3.5 hours demonstrated wider molecular size distribution. Neither polymerization nor depolymerization of lignin A was observed originating from TiO_2-assisted photocatalytic oxidation.

Table 3.9 Molecular size distributions in both oxidized and non-oxidized lignin A obtained from DLS via distribution function fit method

Sample	Mean radius (nm)	Molecular size distribution in lignin (%)
Non-oxidized Lignin A	57.70	97.7
Lignin A oxidized via 1 hour UV irradiation	70.00	99.9
Lignin A oxidized via 3.5 hours UV irradiation	63.82	98.6

All samples were analyzed in a single DLS run. Lignin A: Lignin isolated from cotton stalk via Zilliox & Debeire (1998) method.

As seen in Table 3.9 the radii of non–oxidized and oxidized lignins were determined within the alkali soluble lignin radius range of Gupta *et al.* (1960) by distribution function fit method. Major part of all samples is lignin A with distribution percentage values close to 100%. Also from Table 3.9 it can be deduced that lignins exposed to TiO_2-assisted photocatalytic oxidation were not completely broken down under the conditions used. As the oxidation time

increases, first an increase in lignin mean radius from 57.7 nm to 70 nm, then a decrease in lignin mean radius from 70 nm to 63.82 nm is observed. However, these changes in mean radii of lignin A were not significant. From these findings obtained from DLS via both cumulant fit and distribution function fit methods, it can be concluded that lignin A undergoes neither depolymerization nor polymerization under the photocatalysis conditions used. Increasing the time of oxidation might be increased the yield of fragmentation of lignin A.

Moreover, no smaller phenolic products of lignin A that exhibit antimicrobial activities were isolated via both ultrasonication and photocatalysis experiments. Lignin B, which demonstrates significant antibacterial effects against both *E. coli* & *B. pumilus*, will be integrated into xylan-based biodegradable films to render them antimicrobial.

3.2.2. The Assessment of Antimicrobial Properties of Green Garlic Stalk Juice

The antimicrobial compounds such as allicin and ajoene, which are extracted from garlic bulbs and cloves, have proven to inhibit the growth of several Gr(+) and Gr(-) bacteria (Harris *et al.*, 2001). In literature, there is no study in which green garlic stalks are used as the source of antimicrobial compounds. In this part, green garlic stalks, which can be considered as non-edible and waste parts of garlic, were used for the extraction of antibacterial juice. The antibacterial effect of garlic stalk juice was evaluated against both *E. coli* and *B. pumilus* via liquid and solid medium tests and was discussed in this part.

Table 3.10 Antibacterial effect of garlic stalk juice in liquid medium against both *E. coli* & *B. pumilus*

Sample	The number of *E. coli* cells (CFU/ml) after overnight incubation at 37°C	Microbial log reduction in the number of *E. coli* cells	The number of *B. pumilus* cells (CFU/ml) after overnight incubation at 30°C	Microbial log reduction in the number of *B. pumilus* cells
Green garlic stalk juice	$(2.90 \pm 0.10) \times 10^8$	0.550 ± 0.015	$(3.70 \pm 0.30) \times 10^7$	1.409 ± 0.037

All tests were performed in duplicate. Initial load of *E. coli* cells: $(1.03 \pm 0.01) \times 10^9$ CFU/ml, Initial load of *B. pumilus* cells: $(9.50 \pm 0.20) \times 10^8$ CFU/ml, green garlic stalk juice was extracted from 60g of green garlic stalk.

As seen in Table 3.10 it can be deduced that garlic stalk juice exerts significant antibacterial effects towards both *B. pumilus* and *E. coli*. Microbial log reductions originated from garlic stalk juice were determined as 0.55 and 1.41 average against *E. coli* and *B. pumilus*, respectively. *B. pumilus* exhibited higher sensitivity to garlic stalk juice than *E. coli*.

Figure 3.1 The antimicrobial activity of garlic stalk juice against *B. pumilus* in solid medium. 1: Garlic juice extracted from 60g green garlic stalk, 2: Non-concentrated olive mill wastewater

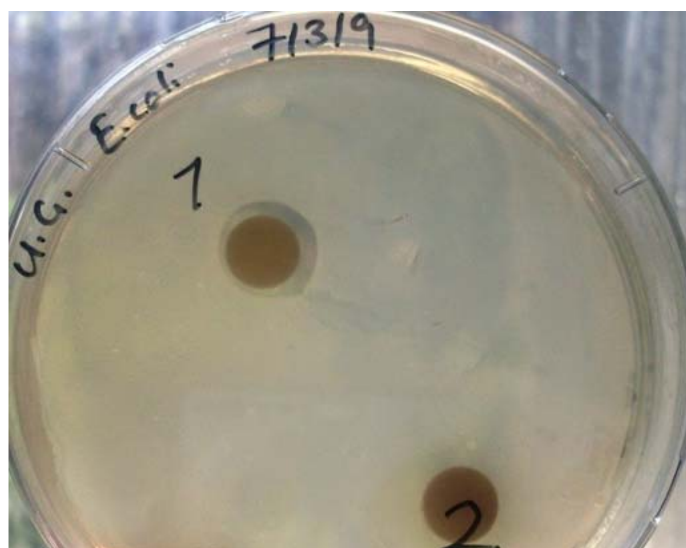

Figure 3.2 The antimicrobial activity of garlic stalk juice against *E. coli* in solid medium. 1: Garlic juice extracted from 60g green garlic stalk, 2: Non-concentrated olive mill wastewater

As seen in Figure 3.1 & Figure 3.2, zone of inhibition values were measured as 19mm and 15mm averages against *B. pumilus* and *E. coli*, respectively. Also, it can be deduced that *E. coli* exhibits lesser sensitivity to garlic stalk juice than *B. pumilus*. Significant antibacterial activity of garlic stalk juice was demonstrated via both solid and liquid medium tests against *E. coli* & *B. pumilus*. So, garlic stalk juice will be integrated into xylan-based biodegradable films to render them antibacterial. On the other hand, no inhibition zones were observed for non-concentrated olive mill wastewater against both *E. coli* & *B. pumilus*.

3.2.3. The Evaluation of Antibacterial Activities of Olive Mill Wastewater

In literature it is pointed out that olive mill wastewater (OMW) possesses antibacterial activity against *Bacillus megaterium* due to its high simple phenolics content (Gonzalez *et al.*, 1990). Olive mill wastewater (OMW) was obtained from Marmara Birlik, olive plant located in Bursa. Before evaluation of its antibacterial effect, it was 10-fold concentrated via evaporating its water and its pH was adjusted to 7 to prevent bacterial deaths originating from pH. In this part of the study, OMW was characterized against both *E. coli* and *B. pumilus* by means of its antimicrobial properties in solid and liquid media.

Table 3.11 The antibacterial activity of OMW against both *E. coli* & *B. pumilus* in liquid medium

Sample	The number of *E. coli* cells (CFU/ml) after overnight incubation at 37°C	Microbial log reduction in the number of *E. coli* cells	The number of *B. pumilus* cells (CFU/ml) after overnight incubation at 30°C	Microbial log reduction in the number of *B. pumilus* cells
10-fold concentrated OMW	$(5.70 \pm 0.20) \times 10^7$	1.257 ± 0.015	$(9.00 \pm 1.00) \times 10^6$	2.023 ± 0.052

All tests were performed in duplicate. Initial load of *E. coli* cells: $(1.03 \pm 0.01) \times 10^9$ CFU/ml, Initial load of *B. pumilus* cells: $(9.50 \pm 0.20) \times 10^8$ CFU/ml.

Findings at Table 3.11 indicate that olive mill wastewater is capable of inhibiting the growth of tested bacteria. Microbial log reductions were determined as 1.26 and 2.02 average against *E. coli* and *B. pumilus*, respectively. *E. coli* exhibited lesser sensitivity to OMW than *B. pumilus*.

Table 3.12 The antimicrobial effect of green OMW against both *E. coli* & *B. pumilus* in solid medium

	Zone of Inhibition (mm)	
Sample	*B. pumilus*	*E. coli*
10 – fold concentrated olive mill wastewater	16 ± 1	11 ± 2

Sterile Disc Diameter was 10 mm.

As seen in Table 3.12, inhibition zones were measured as 16mm and 11mm average against *B. pumilus* and *E. coli*, respectively. So, it can be deduced that *E. coli* exhibits lesser sensitivity to OMW than *B. pumilus* in agreement with liquid medium test results. This natural waste water, which exerts substantial antibacterial effects towards both *E. coli* & *B. pumilus*, will be further utilized in the production of antibacterial xylan-based biodegradable films.

3.3. Natural Antimicrobial Compounds and Their Utilization in Xylan-Based Biodegradable Films

Tannic acid is well known plant phenolic that is capable of inhibiting the growth of several Gr(+) & Gr(-) bacteria (Chung *et al.*, 1993). The antimicrobial activities of lignin B, green garlic stalk juice and olive mill wastewater (OMW) have been demonstrated against both *E. coli* & *B. pumilus* via solid and liquid medium tests. Birchwood xylan biodegradable films were prepared as described in chapter 2.8. Natural antimicrobial compounds were added to the film forming solutions at different percentages. Antimicrobial compound integrated films were characterized against both *E. coli* & *B. pumilus* by means

of their antimicrobial properties after 8-hour interaction between films and tested bacteria. Except alkali soluble lignin B-integrated films, pH values of all films were adjusted to 7 to prevent the bacterial deaths originating from pH. In the case of alkali soluble lignin B-integrated films, the pH values of both blank (means film not contain lignin B) & lignin B-integrated xylan-based films were adjusted to 9 in order to investigate the antimicrobial effects of solubilized lignin B within film in a pH-independent manner.

In this part of the study, tannic acid integrated films were used as positive control films due to the well known antibacterial activities of tannic acid (Chung *et al.*, 1993). Other positive control compounds such as thymol and carvacrol used in previous parts of the study cannot be integrated into xylan-based films owing to their insolubility in water.

3.3.1. The Assessment of the Antibacterial Activity of Tannic acid-integrated Birchwood Xylan Biodegradable Films

Different percentages of tannic acid were added to film forming solutions. Tannic acid-integrated xylan films were characterized against both *E. coli* & *B. pumilus* by means of their antimicrobial activities after 8-hour interaction between films and tested bacteria.

Table 3.13 The antimicrobial activity of tannic acid-integrated birchwood xylan biodegradable films against both *E. coli* & *B. pumilus*

Film type	Tannic acid content (%)	The number of *E. coli* cells (CFU/ml) after 8-hour bacteria-film interaction	Microbial log reduction in the number of *E. coli* cells	The number of *B. pumilus* cells (CFU/ml) after 8-hour bacteria-film interaction	Microbial log reduction in the number of *B. pumilus* cells
Blank xylan film	0.0	$(9.45 \pm 0.05) \times 10^8$	0.037 ± 0.002	$(8.30 \pm 0.10) \times 10^8$	0.059 ± 0.004
Tannic acid-integrated film	0.8	$(5.95 \pm 0.05) \times 10^8$	0.238 ± 0.004	$(5.60 \pm 0.20) \times 10^8$	0.229 ± 0.016
Tannic acid-integrated film	2.0	$(3.30 \pm 0.10) \times 10^8$	0.494 ± 0.013	$(3.45 \pm 0.05) \times 10^8$	0.440 ± 0.006
Tannic acid-integrated film	4.0	$(1.15 \pm 0.05) \times 10^8$	0.952 ± 0.019	$(1.75 \pm 0.05) \times 10^8$	0.735 ± 0.012

All tests were performed in duplicate. Initial load of *E. coli* cells: $(1.03 \pm 0.01) \times 10^9$ CFU/ml, Initial load of *B. pumilus* cells: $(9.50 \pm 0.20) \times 10^8$ CFU/ml.

As seen in Table 3.13 there are around 0.04 and 0.06 bacterial log reductions on blank (means not containing tannic acid) xylan film against *E. coli* and *B. pumilus*, respectively. These losses can be considered as normal because of the difficulty of collecting bacteria from films after 8-hour interaction time. Maximum bacterial inactivation was observed on xylan films containing 4% tannic acid against both *E. coli* & *B. pumilus*. In the case of xylan films containing 4% tannic acid, microbial log reductions were determined as 0.952 and 0.735 average against *E. coli* and *B. pumilus*, respectively.

No more tannic acid is added in film forming solutions because continuous film formation cannot be observed above 4% tannic acid content. Continuous xylan-based film containing 4% (w/w) tannic acid is given in Figure 3.3.

Figure 3.3 Continuous xylan-based biodegradable film containing 4% (w/w) tannic acid

3.3.2. The Evaluation of Antimicrobial Effect of Birchwood Xylan-based Biodegradable Films Containing Green Garlic Stalk Juice

Green garlic stalk juice (GGSJ), which was extracted from 60g green garlic stalk, was used at different percentages to produce antibacterial xylan-based films. Garlic stalk juice-integrated films were evaluated for their antimicrobial effects against *E. coli* & *B. pumilus*.

Table 3.14 The antibacterial activity of birchwood xylan biodegradable films containing GGSJ against both *E. coli* & *B. pumilus*

Film type	GGSJ content (%)	The number of *E. coli* cells (CFU/ml) after 8-hour bacteria-film interaction	Microbial log reduction in the number of *E. coli* cells	The number of *B. pumilus* cells (CFU/ml) after 8-hour bacteria-film interaction	Microbial log reduction in the number of *B. pumilus* cells
Blank xylan film	0.0	$(9.65 \pm 0.25) \times 10^{8}$	0.028 ± 0.011	$(8.55 \pm 0.15) \times 10^{8}$	0.046 ± 0.008
GGSJ-xylan film	0.8	$(5.50 \pm 0.20) \times 10^{8}$	0.272 ± 0.016	$(3.65 \pm 0.05) \times 10^{8}$	0.415 ± 0.006
GGSJ-xylan film	2.0	$(2.90 \pm 0.10) \times 10^{8}$	0.550 ± 0.015	$(1.55 \pm 0.03) \times 10^{8}$	0.787 ± 0.008
GGSJ-xylan film	4.0	$(7.70 \pm 0.20) \times 10^{7}$	1.126 ± 0.011	(0.00 ± 0.00)	8.978 ± 0.010

All tests were performed in duplicate. Initial load of *E. coli* cells: $(1.03 \pm 0.01) \times 10^{9}$ CFU/ml, Initial load of *B. pumilus* cells: $(9.50 \pm 0.20) \times 10^{8}$ CFU/ml.

From Table 3.14 it can be deduced that *B. pumilus* exhibits higher sensitivity to xylan films containing garlic stalk juice than *E. coli*. In the case of xylan films containing 4% garlic stalk juice, microbial log reductions were determined as 1.126 and 8.978 average against *E. coli* and *B. pumilus*, respectively.

3.3.3. The Assessment of Antimicrobial Activity of Birchwood Xylan-based Biodegradable Films Containing Olive Mill Wastewater

Different percentages of 10-fold concentrated olive mill wastewater (OMW) were added in xylan film forming solutions to render them antibacterial. The antibacterial property of xylan-based films containing OMW was investigated against both *E. coli* & *B. pumilus*.

Table 3.15 The antibacterial effect of birchwood xylan biodegradable films containing OMW against both *E. coli* & *B. pumilus*

Film type	OMW content (%)	The number of *E. coli* cells (CFU/ml) after 8-hour bacteria-film interaction	Microbial log reduction in the number of *E. coli* cells	The number of *B. pumilus* cells (CFU/ml) after 8-hour bacteria-film interaction	Microbial log reduction in the number of *B. pumilus* cells
Blank xylan film	0.0	$(9.50 \pm 0.20) \times 10^8$	0.035 ± 0.009	$(8.80 \pm 0.10) \times 10^8$	0.033 ± 0.005
OMW-xylan film	0.8	$(4.30 \pm 0.40) \times 10^8$	0.379 ± 0.039	$(3.60 \pm 0.20) \times 10^8$	0.421 ± 0.024
OMW-xylan film	2.0	$(1.90 \pm 0.10) \times 10^8$	0.734 ± 0.023	$(1.40 \pm 0.00) \times 10^8$	0.832 ± 0.000
OMW-xylan film	4.0	(0.00 ± 0.00)	9.013 ± 0.004	(0.00 ± 0.00)	8.978 ± 0.010

All tests were performed in duplicate. Initial load of *E. coli* cells: $(1.03 \pm 0.01) \times 10^9$ CFU/ml, Initial load of *B. pumilus* cells: $(9.50 \pm 0.20) \times 10^8$ CFU/ml.

E. coli exhibits lesser sensitivity to xylan films containing olive mill wastewater than *B. pumilus*. In the case of xylan films containing 4% OMW, microbial log reductions were determined as 9.013 and 8.978 average against *E. coli* and *B. pumilus*, respectively. Complete bacterial inactivation was observed on the xylan films containing 4% OMW against both *E. coli* and *B. pumilus* as seen in Table 3.15.

3.3.4. The Characterization of Cotton Stalk Lignin-Integrated Birchwood Xylan-based Biodegradable Films by Means of Antibacterial Activity

Antibacterial Lignin B, which was extracted from cotton stalk via Boric acid-assisted alkaline extraction, was used at different percentages in film forming solutions to produce antibacterial xylan-based films. Cotton stalk lignin-integrated films were assessed for their antimicrobial effects against *E. coli* & *B. pumilus*. Xylan film containing 4% (w/w) lignin B is given in Figure 3.4.

Figure 3.4 Continuous xylan-based biodegradable film containing 4% (w/w) antibacterial lignin B

Table 3.16 The antibacterial effect of cotton stalk lignin B-integrated birchwood xylan-based biodegradable films against both *E. coli* & *B. pumilus*

Film type	Lignin B content (%)	The number of *E. coli* cells (CFU/ml) after 8-hour bacteria-film interaction	Microbial log reduction in the number of *E. coli* cells	The number of *B. pumilus* cells (CFU/ml) after 8-hour bacteria-film interaction	Microbial log reduction in the number of *B. pumilus* cells
Blank xylan film	0.0	$(9.25 \pm 0.25) \times 10^8$	0.047 ± 0.012	$(8.30 \pm 0.10) \times 10^8$	0.059 ± 0.005
Lignin B-integrated film	2.0	$(2.53 \pm 0.02) \times 10^8$	0.610 ± 0.004	$(1.23 \pm 0.01) \times 10^8$	0.888 ± 0.004
Lignin B-integrated film	4.0	$(8.00 \pm 0.20) \times 10^7$	1.110 ± 0.010	$(4.80 \pm 0.30) \times 10^7$	1.296 ± 0.027

All tests were performed in duplicate. Initial load of *E. coli* cells: $(1.03 \pm 0.01) \times 10^9$ CFU/ml, Initial load of *B. pumilus* cells: $(9.50 \pm 0.20) \times 10^8$ CFU/ml. The pH value of all films was adjusted to 9.

As seen in Table 3.16 microbial log reductions in blank xylan film are determined as 0.05 and 0.06 average against *E. coli* and *B. pumilus*, respectively. These log reductions are calculated via comparing the number of bacteria cultivated in liquid medium at pH 7 (refers to blank bacteria concentration) with the number of bacteria collected on blank xylan film (pH=9) after 8-hour interaction between film & bacteria. The reasons for these microbial log reductions are errors originating from human and working with alkali pH which is needed to solubilize alkali lignin B. These log reductions were considered while calculating all bacterial inactivation percentages caused by lignin B.

From Table 3.16 it can be concluded that *B. pumilus* exhibits stronger sensitivity to lignin B-integrated xylan films than *E. coli*. Xylan films containing lignin B exhibit more antibacterial effect against tested microorganisms than the positive control film, tannic acid-integrated film. Maximum log reduction in tested films was observed on xylan films containing 4% lignin B against both *E. coli* & *B. pumilus*. In the case of xylan films containing 4% lignin B, microbial log reductions were determined as 1.110 and 1.296 average against *E. coli* and *B. pumilus*, respectively. No more lignin B was added in film forming solutions because; continuous film formation was not observed above 4% lignin content as in the case of tannic acid-integrated xylan films. *B. pumilus* exhibits stronger sensitivity to all xylan films than *E. coli*. Xylan-based biodegradable films containing OMW, GGSJ and cotton stalk lignin B are natural, antibacterial, easy and cheap to produce, environmental friendly and not toxic to human health. Natural compounds, which were isolated from agricultural biomass, were used to render the xylan biodegradable films antimicrobial. Before commercialization of these antimicrobial xylan-based biodegradable films, mechanical properties of these films must be further investigated.

3.4. Molecular Characterization of Cotton Stalk Lignins via Different Methods

In previous parts of the study, lignin B isolated from cotton stalk via H_3BO_3-assisted alkaline extraction has demonstrated significant antibacterial effects against both *E. coli* and *B. pumilus*. On the other hand, lignin A, which is also isolated from cotton stalk via $NaBH_4$-assisted alkaline extraction, has not exhibited substantial antimicrobial activity against tested microorganisms.

Baurhoo *et al.* (2008) have reported that lignins may possess different biological activities depending on extraction methods. Gosselink *et al.* (2004) reported that the major chemical functional groups in lignin include the hydroxyl, methoxyl, carbonyl, and carboxyl groups in various numbers and proportions, depending on origin and extraction processes. So, depending on method of isolation and chemical treatment, new functional groups not present in natural lignin may appear.

To elucidate the structure of different types of lignin, and to investigate the differences in the structure of lignin A, lignin B and commercial lignin, different molecular characterization methods such as FTIR (Fourier transform infrared spectroscopy) and LC-MS (Liquid chromatography-mass spectrometry) were used.

3.4.1. FTIR Spectra of Different Types of Lignins

In this part of the study, to characterize cotton stalk lignin B, cotton stalk lignin A and commercial lignin by means of their chemical structure, FTIR spectroscopy was used. The dried samples were embedded in KBr pellets in the concentrations of about 1mg/100mg KBr. The FTIR spectra were recorded in the absorption band mode in the range of 4000-400 cm^{-1}. FTIR spectra of different lignins are given in Figure 3.5.

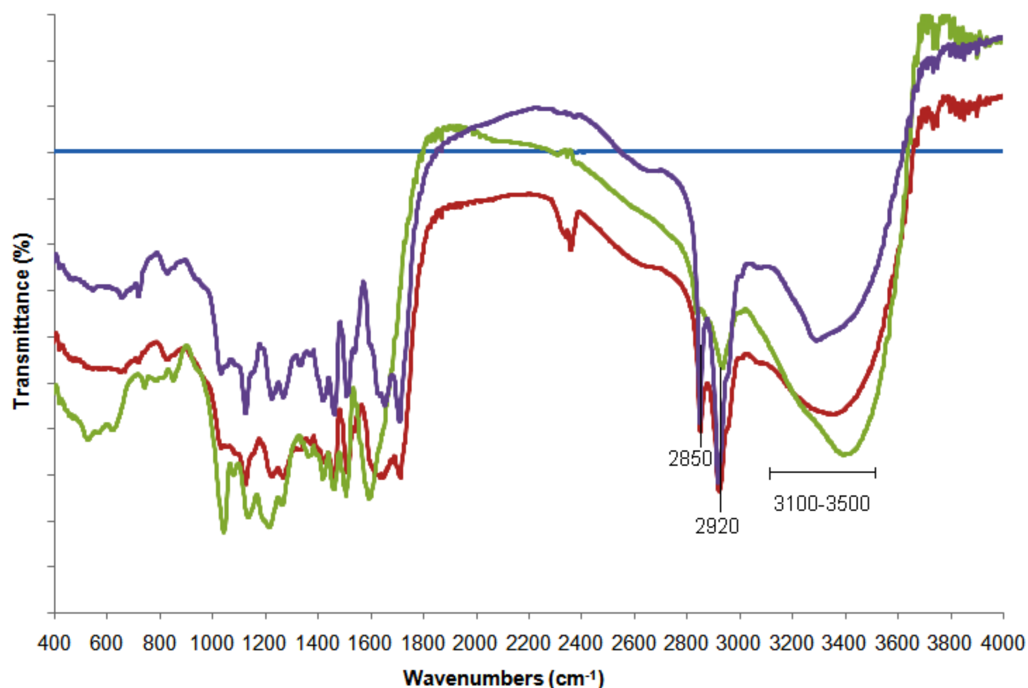

Figure 3.5 FTIR spectra of three different lignins in the 400-4000 cm^{-1} range ▬ : Blank (only KBr pellet), ▬ : Lignin A (Lignin isolated from cotton stalk via Zilliox & Debeire (1998) method), ▬ : Commercial lignin, ▬ : Lignin B (Lignin isolated from cotton stalk via Boric acid-assisted alkaline extraction)

As seen in Figure 3.5, for three different lignins, the region from 4000 to 1800 cm^{-1} did not give any useful information since it is responsible for the O-H and aliphatic C-H stretching frequencies. Every lignin IR spectrum has a strong wide band between 3500-3100 cm^{-1} assigned to O-H stretching vibrations. Absorption bands at 2920 and 2850 cm^{-1} assigned to aliphatic C-H stretching vibrations. Spectra of lignin show no absorption bands in the 2800-1800 cm^{-1} (Stewart and Morrison, 1992).

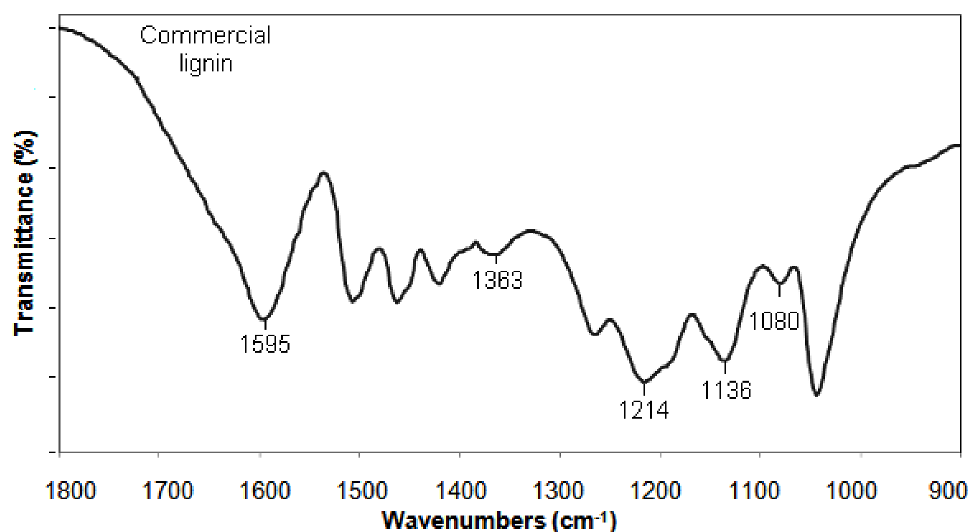

Figure 3.6 FTIR spectrum of commercial lignin in the 1800-900 cm^{-1} region

The remaining region, 1800-900 cm^{-1} was far informative for three different lignins with respect to the 4000-1800 cm^{-1} region. The FTIR spectrum of commercial lignin is shown in Figure 3.6. The bands at 1080 and 1136 cm^{-1} were due to the vibrations of C-O bonds in primary, secondary alcoholic groups and ether groups. The band at 1214 cm^{-1} was assigned to the asymmetric vibrations of the C-O-C linkages in ethers and esters. The band at 1363 cm^{-1} was due to the vibrations of phenolic hydroxyls. The band at 1595 cm^{-1} was assigned to aromatic rings conjugated with carbonyl groups (Bhat *et al.*, 2009).

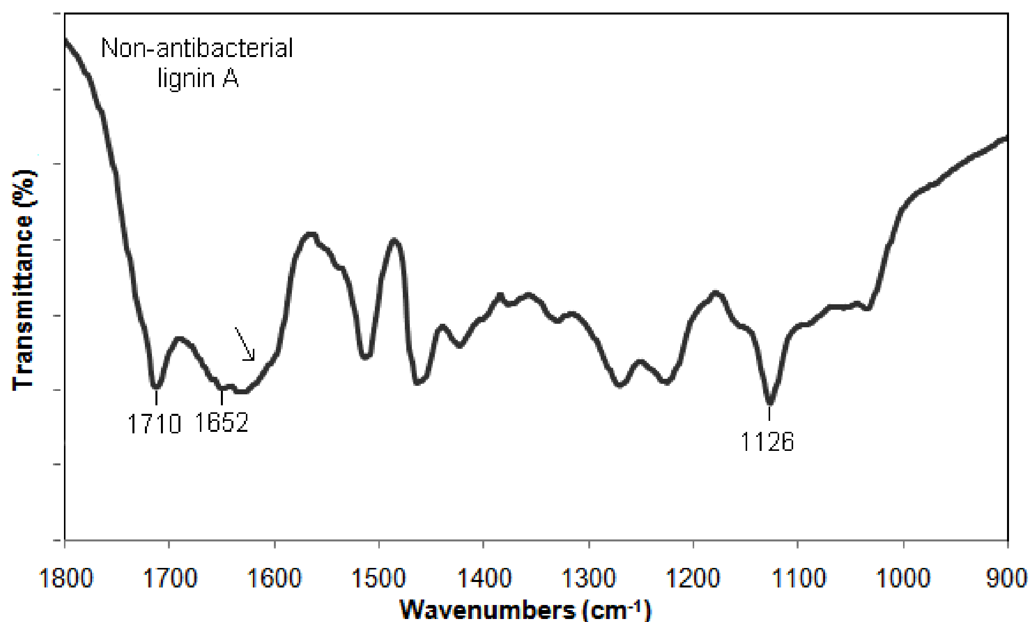

Figure 3.7 FTIR spectrum of non-antibacterial lignin A in the 1800-900 cm^{-1} region

The FTIR spectrum of non-antibacterial lignin A is shown in Figure 3.7. The band at 1126 cm^{-1} was assigned to the methoxyl groups. The band at 1652 cm^{-1} was due to the stretching of carbonyl groups (C=O) conjugated with aromatic ring. The band at 1710 cm^{-1} was assigned to non-conjugated carbonyl groups (C=O). The remaining bands in spectrum were main asymmetric absorption bands of lignins which were common with commercial lignin.

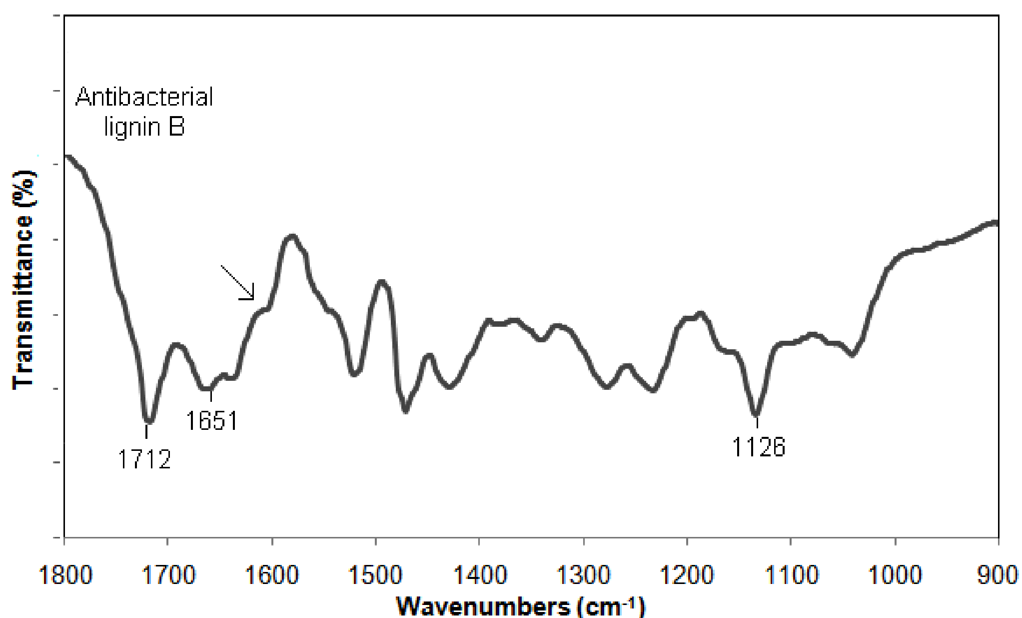

Figure 3.8 FTIR spectrum of antibacterial lignin B in the 1800-900 cm^{-1} region

The FTIR spectrum of antibacterial lignin B is shown in Figure 3.8. The band at 1126 cm^{-1} was assigned to the methoxyl groups. The band at 1651 cm^{-1} was due to the stretching of carbonyl groups (C=O) conjugated with aromatic ring. The band at 1712 cm^{-1} was assigned to non-conjugated carbonyl groups (Tejado *et al.*, 2007).

All these findings consequently indicated that both non-antibacterial lignin A and antibacterial lignin B were quite different from commercial lignin. The non-conjugated carbonyl, carboxylic acid group bands, 1710 and 1712 cm^{-1} were missing in the FTIR spectrum of commercial lignin. The presence of these bands in the spectra of cotton stalk lignins (CSLs) revealed that CSLs were significantly broken down by alkaline treatment. The breakdown of CSLs via alkaline treatment resulted in the formation of new carboxyl groups, which resulted from the oxidation of aromatic ring, oxidation of quinones and other conjugated carbonyl structures and direct oxidation of lignin side chains (Stewart and Morrison, 1992).

Band at 1363 cm^{-1} which was assigned to phenolic –OH groups in commercial lignin spectrum did not appear in the spectrum of both lignin A and lignin B and the bands at 1226 cm^{-1} in spectra of two CSLs, which were assigned to methoxyl groups, were missing in the spectrum of commercial lignin. From these findings it was deduced that CSLs were methylated with respect to commercial one. During methylation of lignin, the O-H bonds were splited and H was replaced by CH_3 group and amount of phenolic hydroxyl groups decreased (Sarkanen and Ludwig, 1971).

Absorbance at 1735 cm^{-1} is due to ester carbonyl vibration in acetyl, feruloyl, p-coumaroyl, etc. groups in lignin and hemicelluloses (Sun and Lawther, 1998). In FTIR spectra of both antibacterial lignin B and non-antibacterial lignin A 1080, 1136, 1214 and 1735 cm^{-1} bands were missing. The increase in carboxylic acid content, the decrease in phenolic hydroxyl content and the absence of 1080, 1136, 1214 and 1735 cm^{-1} bands in CSLs have revealed that the ester and ether bonds between hydroxycinnamic acids and lignin were cleaved during the alkaline treatments of both lignin A and lignin B (Buranov and Mazza, 2008).

By comparing the spectrum in Figure 3.7 with the spectrum in Figure 3.8, it was deduced that the spectra appeared to be rather similar. Except some slight band shifts, the only difference between non-antibacterial lignin A and antibacterial lignin B was observed in the region that was marked in both Figure 3.7 & Figure 3.8. A shoulder at 1603 cm^{-1}, which was observed in the spectrum of antibacterial lignin, was missing in the spectrum of non-antibacterial lignin. To clarify the difference between antibacterial and non-antibacterial lignin, the FTIR spectrum of antibacterial lignin was subtracted from the spectrum of non-antibacterial lignin. The resulting spectrum is shown in Figure 3.9.

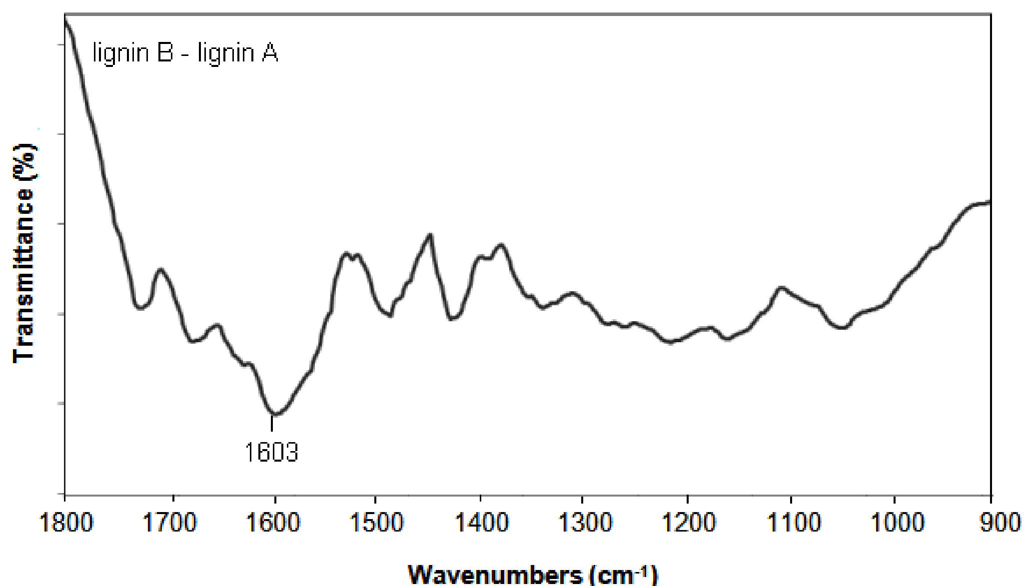

Figure 3.9 FTIR spectrum obtained by the subtraction of antibacterial lignin spectrum from non-antibacterial lignin in the 1800-900 cm^{-1} region

Except the band at 1603 cm^{-1}, the remaining bands in spectrum were common asymmetric absorption bands of lignins which were not under consideration while elucidating the antibacterial activity of lignin B. The band at 1603 cm^{-1} was assigned to the stretching vibrations of C=C bonds.

The antimicrobial activity of lignin is highly dependent on its side chain structure and the nature of the functional groups of its phenolic fragments. The antimicrobial effect of lignin monomer, isoeugenol was explained by the presence of both C=C bond and methyl group in its side chain (Jung and Fahey, 1983).

FTIR results revealed that cotton stalk lignins were significantly broken down via alkaline treatment and this breakdown resulted in the formation of new fractions. Moreover, it was also concluded that the ester and ether bonds between antimicrobial hydroxycinnamic acids and lignin were cleaved during the alkaline treatments of both lignin A and lignin B and cotton stalk lignins

were methylated with respect to commercial lignin. The antimicrobial activity of lignin B sample can be explained by the presence of both C=C bonds and methyl groups in the structure of newly formed fractions of lignin B and/or lignin B itself.

Comparison of changes of the lignin spectra shows that it is insufficient to use only FTIR to characterize the functional structure changes in lignin molecules. To understand the reason of antimicrobial activity of lignin B sample, smaller fractions of both lignin A and lignin B should be characterized by means of chemical structure via different method than FTIR.

3.4.2. Molecular Characterization of the Fragmentation Products of Cotton Stalk Lignins by LC-MS

The objective of this part is to investigate the differences of smaller fractions formed during alkaline extractions of both lignin A and lignin B. For this purpose, qualitative mass analysis was performed via LC-MS. Lignin A and lignin B were dissolved in tetrahydrofuran (THF) and were filtered through filters (0.45 μm pore size). After filtered lignin solutions were injected to LC-MS, mass spectroscopy spectra were recorded for fractions of both cotton stalk lignin A and cotton stalk lignin B. LC-MS qualitative mass analysis was performed in both positive and negative polarity mode in the m/z range of 55-1500. Abundance vs. m/z ratios of the fragments of analyzed cotton stalk lignins are given in Figure 3.10 and Figure 3.11.

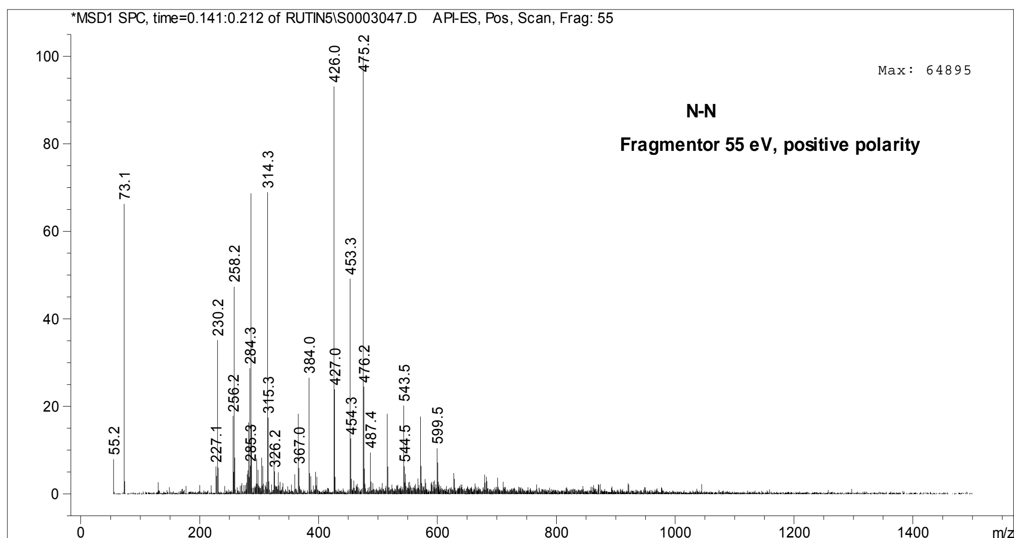

Figure 3.10 Mass spectrum of fragmentation products isolated from lignin A Lignin A: (Non-antibacterial lignin isolated from cotton stalk via Zilliox & Debeire (1998) method).

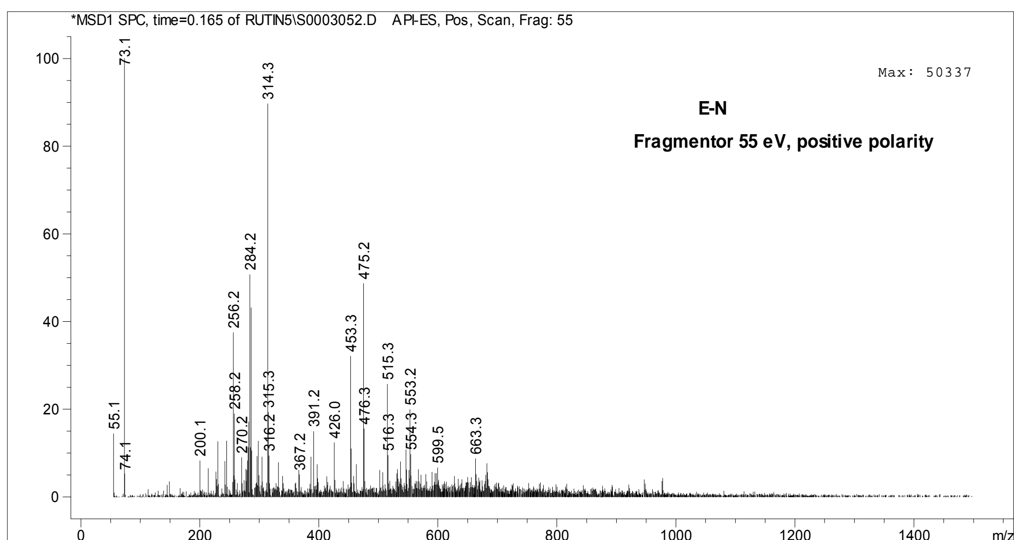

Figure 3.11 Mass spectrum of fragmentation products isolated from lignin B Lignin B: (Antibacterial lignin isolated from cotton stalk via modified version of Zilliox & Debeire (1998) method).

Cotton stalk lignin A, which was isolated from cotton stalks via Zilliox & Debeire (1998) method, have not demonstrated significant antibacterial effect against both *E. coli* & *B. pumilus*. Conversely, cotton stalk lignin B, which was extracted via modified version of Zilliox & Debeire (1998) method, has exhibited significant activity towards both *E. coli* & *B. pumilus*.

FTIR results revealed that cotton stalk lignins were significantly broken down via alkaline treatment and this breakdown resulted in the formation of new fractions and it was also concluded that the ester and ether bonds between antimicrobial hydroxycinnamic acids (such as ferulic acid, p-coumaric acid) and lignin were cleaved during the alkaline treatments of both lignin A and lignin B. Because of the usage of different chemicals and conditions during lignin extraction, some of phenolic fragments must be characteristic for each lignin. By comparing the peaks in both Figure 3.10 and Figure 3.11, common and characteristic peaks were observed for the fractions of both lignin A and lignin B. Several common peaks with different abundances were observed in the mass spectra of cotton stalk lignins. Also 11 characteristic peaks were observed in the spectra of fragmentation products isolated from antibacterial lignin sample and 6 characteristic peaks were observed for fractions isolated from non-antibacterial lignin sample.

Table 3.17 LC/MS results of fragmentation products isolated from both lignin A & lignin B.

Origin Of Fragments	m/z (mass to charge ratio)	Abundance
Non – antibacterial Lignin A sample	325.15	10.6
	384.00	26.5
	427.00	23.8
	487.40	9.4
	543.50	20.1
	571.55	17.6
Antibacterial Lignin B sample	200.10	8.2
	214.20	6.5
	242.20	8.1
	245.00	12.8
	270.20	8.9
	316.20	9.4
	391.20	14.9
	537.30	8.0
	553.20	19.9
	554.30	9.7
	663.30	8.7

The fractions of both lignin A and lignin B differ in abundance percentage and mass to charge ratio as seen in Table 3.17. These characteristic peaks refer to different fractions and/or smaller phenolics formed via breakdown of lignin molecule during alkaline lignin extraction process. As expected, the fragments of antibacterial lignin are found to be different from the fractions of non-antibacterial lignin so, the basis of the antimicrobial effect of cotton stalk lignin B is elucidated. Via previous LC/MS studies, the m/z ratio range of antimicrobial lignin monomers including ferulic acid, p-coumaric acid, caffeic acid, sinapic acid, vanilic acid and para hydroxyphenyloacetic acid (p-HPA) was found 79-179 (Biesaga and Pyrzynska, 2009). As seen in Table 3.17, m/z ratios of characteristic fragments of both lignin A and lignin B are not within the m/z range of lignin monomers.

So, it was deduced that the fractions formed during extraction of lignins were longer than lignin monomers. For antibacterial lignin B fractions, m/z ratio range is between 200-663. In literature, this m/z ratio range is appropriate for compounds including trimers and/or tetramers of monomeric phenolics of lignin molecule (Mandal and Dey, 2008), flavonoids (Stobiecki *et al.*, 2006). So, antimicrobial lignin fractions formed via fragmentation of lignin B during modified alkaline extraction may be oligomers derived from lignin monomers or may be flavonoids. To explain the contribution of extraction chemicals and extraction conditions in the fragmentation of cotton stalk lignin and to identify the exact structures of fractions that demonstrate antimicrobial effect, each characteristic oligomer of lignin B should be analyzed via LC-MS individually or cotton stalk lignin samples should be firstly analyzed via HPLC then determined HPLC fractions might be analyzed via LC-MS. It can be concluded that characterization of lignins is an extremely tough task owing to their diversity with respect to both their origin and extraction method.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

Cotton stalk lignin extractions were performed via alkaline methods at different conditions. Crude and post treated cotton stalk lignins, olive mill wastewater and garlic stalk juice were examined in terms of antimicrobial activity. Antimicrobial lignin was isolated depending on alkaline extraction conditions. Lignin extracted at 60°C exhibited significant antimicrobial effect towards both *Escherichia coli* and *Bacillus pumilus*. However different post treatments such as ultrasonication and TiO_2-assisted photocatalytic oxidation did not result in antimicrobial compounds. Olive mill wastewater and garlic stalk juice exerted substantial antimicrobial effects towards tested microorganisms.

Xylan-based biodegradable films containing lignin, garlic stalk juice, tannic acid and olive mill wastewater were characterized against both *B. pumilus* and *E. coli* by means of their antimicrobial activities. *E. coli* exhibited lesser sensitivity to all tested antimicrobial xylan films except tannic acid-integrated xylan film than *B. pumilus*. Antimicrobial lignin integrated-xylan film exhibited stronger effect towards tested microorganisms than the positive control film, tannic acid-integrated film. In the case of both antimicrobial lignin and tannic acid integrated xylan films, 4% was found to be the maximum antimicrobial compound percentage in film forming solutions to observe continuous film formation.

Lignin samples with/without antimicrobial activity were characterized by means of their chemical structure via FTIR and LC-MS. FTIR results revealed that cotton stalk lignins were significantly broken down via alkaline treatment and this breakdown resulted in the formation of new fractions and also ester & ether bonds between antimicrobial hydroxycinnamic acids and lignin were cleaved during the alkaline treatments of cotton stalk lignins. By FTIR results, C=C bonds were found to be characteristic for antimicrobial lignin sample and

it was suggested that these bonds might be the reason of the antimicrobial activity. By LC-MS qualitative mass analysis, antibacterial lignin fractions were found to be quite different from non-antibacterial lignin fractions. LC-MS results indicated that the antimicrobial lignin fractions might be lignin-derived oligomers and/or might be flavonoids. Cotton stalk lignin fractions demonstrated different antimicrobial activities depending on the method of isolation and chemical treatment.

To explain the contribution of extraction chemicals and extraction conditions in the fragmentation of cotton stalk lignin and to identify the exact structures of fractions that demonstrate antimicrobial effect, each characteristic oligomer of antimicrobial lignin should be analyzed via LC-MS individually or cotton stalk lignin samples should be firstly analyzed via HPLC then determined HPLC fractions might be analyzed via LC-MS. The extraction method developed in this study may be applied on other lignocellulosic agricultural wastes instead of cotton stalk and could also be improved by altering the extraction chemicals and conditions. Before commercialization of antimicrobial xylan-based biodegradable films, the antimicrobial activity of lignin should be further investigated against different microorganisms including pathogens that are more resistant than both *E. coli* and *B. pumilus* and mechanical properties of the films must also be investigated.

REFERENCES

Akpınar, O., Ak, O., Kavas, A., Bakır, U., Yılmaz, L., 2007. Enzymatic production of xylooligosaccharides from cotton stalks. Journal of Agricultural and Food Chemistry, 55: 5544-5551.

Amen-Chen, C., Pakdel, H., Roy, C., 2001. Production of monomeric phenols by thermochemical conversion of biomass: a review. Bioresource Technology, 79: 277-299.

Appendini, P. and Hotchkiss, J.H., 2002. Review of antimicrobial food packaging. Innovative Food Science & Emerging Technologies, 3: 113-126.

Azhar, A. and Hamdy, M. K., 1979. Sonication effect on potato starch and sweet potato powder. Journal of Food Science, 44: 801-804.

Balat, M., 2009. Gasification of biomass to produce gaseous products. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects, 31: 516-526.

Baurhoo, B., Ruiz-Feria, C. A., Zhao, X., 2008. Purified lignin: Nutritional and health impacts on farm animals-A review. Animal Feed Science and Technology, 144: 175-184.

Belgacem, M. N., 2000. Characterization of lignocellulosics by inverse gas chromatography: a review. Cellulose Chemistry and Technology, 34: 357-383.

Ben Arfa, A., Combes, S., Preziosi-Belloy, L., Gontard, N. and Chalier, P., 2006. Antimicrobial activity of carvacrol related to its chemical structure. Letters in Applied Microbiology, 43: 149–154.

Benedix, R., Dehn, F., Quaas, J., Orgass, 2000. M. Application of titanium dioxide photocatalysis to create self-cleaning building materials. LACER, Leipzig Annual Civil Engineering Report, 5:157–168.

Besle, J. M., Cornu, A., Jouany, J. P., 1994. Roles of structural phenylpropanoids in forage cell wall digestion. Journal of the Science of Food and Agriculture, 64: 171.

Bhat, R., Khalil, H. P. S. A., Karim, A. A., 2009. Exploring the antioxidant potential of lignin isolated from black liquor of oil palm waste. Comptes Rendus Biologies, 332: 827-831.

Bidlack, J., Malone, M., Benson, R., 1992. Molecular structure and component integration of secondary cell walls in plants. Proceedings Oklahoma Academy of Sciences, 72: 51–56.

Biely, P., 1993. Biochemical aspects of the production of microbial hemicellulases in Hemicellulose and Hemicellulases. pp.29-52. Coughlan M. P. and Hazlewood G. (Eds). Portland Press Ltd, NC, U.S.A.

Biesaga, M. and Pyrzynska, K., 2009. Liquid chromatography/tandem mass spectrometry studies of the phenolics compounds in honey. Journal of Chromatography A, 1216: 6620–6626.

Boudet, A. M. and Grima-Pettenati, J., 1996. Lignin genetic engineering. Molecular Breeding, 2: 25-39.

Brown, R. M., 2004. Cellulose structure and biosynthesis: What is in store for the 21st century?. Journal of Polymer Science, Part A: Polymer Chemistry, 42: 487-495.

Buranov, A. U. and Mazza, G., 2008. Lignin in straw of herbaceous crops: A review. Industrial Crops and Products, 28: 237–259.

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods. International Journal of Food Microbiology, 94: 223-253.

Burt, S.A., Vlielander, R., Haagsman, H.P. and Veldhuizen, E.J., 2005. Increase in activity of essential oil components carvacrol and thymol against *Escherichia coli* O157:H7 by addition of food stabilizers. Journal of Food Protection, 68: 919-926.

Carpita, N. C., 1996. Structure and biogenesis of the cell walls of grasses. Annual Review of Plant Physiology and Plant Molecular Biology, 47: 445-476.

Catignani, G. L., Carter, M. E., 1982. Antioxidant properties of lignin. Journal of Food Science, 47: 1745-1748.

Cha, D. S. and Chinnan, M. S., 2004. Biopolymer-based antimicrobial packaging: a review. Critical reviews in food science & nutrition, 44: 223-237.

Chandra, R. and Rustgi, R., 1998. Biodegradable polymers. Progress in Polymer Science, 23:1273-1335.

Chen, M. C., Yeh, G. H. and Chiang, B. H., 1996. Antimicrobial and physicochemical properties of methylcellulose and chitosan films containing a preservative. Journal of Food Processing and Preservation, 20(5): 279–390.

Chung K. T., Stevens S. E., Lin W. F. and Wei C. I., 1993. Growth inhibition of selected foodborne bacteria by tannic acid, propyl gallate and related compounds. Letters in Applied Microbiology, 17: 29-31.

Chung, K. T., Wong, T. Y., Wei, C. I., Huang, Y. W. and Lin, Y., 1998. Tannins and human health: A review. Critical Reviews in Food Science and Nutrition, 38: 421–464.

Coma, V., Martial-Gros, A., Garreau, S., Copinet, A. and Deschamps, A., 2002. Edible antimicrobial films based on chitosan matrix. Journal of Food Science, 67(3): 1162–1169.

Corzo-Martinez, M., Corzo, N., Villamiel, M., 2007. Biological properties of onions and garlic. Trends in Food Science and Technology, 18: 609–625.

Cowan, M. M., 1999. Plant products as antimicrobial agents. Clinical Microbiology Reviews, 12: 564–582.

Daneshvar, N., Salari, D., Khataee, A. R., 2004. Photocatalytic degradation of azo dye acid red 14 in water on ZnO as an alternative catalyst to TiO_2. Journal of Photochemistry and Photobiology A: Chemistry, 162: 317–322.

Davis, G. and Song, J. H., 2006. Biodegradable packaging based on raw materials from crops and their impact on waste management. Industrial Crops and Products, 23: 154-159.

Demirbaş, A., 2005. Bioethanol from cellulosic materials: a renewable motor fuel from biomass. Energy Sources, 27: 327–337.

Dış Ticaret Müsteşarlığı, www.dtm.gov.tr, Last accessed date, March 19, 2008.

Elegir, G., Kindl, A., Sadocco, P., Orlandi, M., 2008. Development of antimicrobial cellulose packaging through laccase-mediated grafting of phenolic compounds. Enzyme and Microbial Technology, 43: 84-92.

Evtuguin, D. V., Tomas, J. L., Silva, A. M. S. and Neto, C. P., 2003. Characterization of an acetylated heteroxylan from *Eucalyptus globulus* Labill. Carbohydrate Research, 338: 597-604.

Fidan, M. S., 2005. Production and application of phenol formaldehyde type resins from some lignocellulosic waste, Kahramanmaraş Sütçü İmam University, MSc Thesis.

Fox, M. A. and Dulay, M. T., 1993. Heterogeneous photocatalysis. Chemical Reviews, 93: 341-357.

Franta, J., Helmreich, B., Pribyl, M., Adamietz, E., Wilderer, P. A., 1994. Advanced biological treatment of paper mill wastewaters: Effects of operation conditions on COD removal and production of soluble organic compounds in activated sludge systems. Water Science and Technology, 30: 199.

Friedman, M., 2006. Antibiotic activities of plan compounds against non-resistant and antibiotic-resistant foodborne human pathogens. American Chemical Society Symposium Series, 961: 167–183.

Friedman, M., Henika, P. R., Mandrell, R. E., 2002. Bactericidal activities of plant essential oils and some of their isolated constitutents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. Journal of Food Protection, 65: 1545-1560.

Gabrielii, I. and Gatenholm, P., 1998. Preparation and properties of hydrogels based on hemicellulose. Journal of Applied Polymer Science, 69: 1661–1667.

Gabrielii, I., Gatenholm, P., Glasser, W. G., Jain, R. K., Kenne, L., 2000. Separation, characterization and hydrogel-forming of hemicellulose from aspen wood. Carbohydrate Polymers, 43: 367–374.

Göksu, E. I., 2005. Hemicellulose based biodegradable film production, METU, MSc Thesis.

Gonzalez, M. D., Moreno, E., Quevedo-Sarmiento, J., Ramos Cormenzana, A., 1990. Studies on antibacterial activity of waste waters from olive oil mills (alpechin); Inhibitory activity of phenolic and fatty acids. Chemosphere, 20: 423-432.

Goring, D. A. I., 1989. The lignin paradigm. American Chemical Society Symposium Series, 1: 2-10.

Gosselink, R. J. A., Snijder, M. H. B., Kranenbarg, A., Keijsers, E. R. P., De Jong, E., Stigsson, L.L., 2004. Characterization and application of Nova Fiber lignin. Industrial Crops and Products, 20: 191–203.

Gröndahl, M., Eriksson, L., Gatenholm, P., 2004. Material properties of plasticized hardwood xylans for potential application as oxygen barrier films Biomacromolecules, 5: 1528-1535.

Grönroos, A., Pentti, P., Hanna, K., 2008. Ultrasonic degradation of aqueous carboxymethylcellulose: Effect of viscosity, molecular mass, and concentration. Ultrasonics Sonochemistry, 15: 644–648.

Gross, R. A. and Kalra, B., 2002. Biodegradable polymers for the environment. Science, 297: 803–807.

Gross, R. A., Kumar, A., Kalra, B., 2001. Polymer synthesis by in vitro enzyme catalysis. Chemical Reviews, 101: 2097.

Gupta, P. R. and Goring D. A. I., 1960. Physicochemical studies of alkali lignins. Canadian Journal of Chemistry, 38: 270-279.

Hammel, K. E., 1997. Fungal degradation of lignin. In: Cadish, G., Giller, K. E. (Eds.), Driven by nature: plant litter quality and decomposition, CAB International, Wallingford, England, UK, pp. 33–45.

Han, J. H., 2005. Innovations in Food Packaging. Elsevier Academic Press, London, p. 517.

Han, J. H., Castell-Perez, M. E., Moreira, R. G., 2007. The influence of electron beam irradiation of antimicrobial-coated LPDE/polyamide films on antimicrobial activity and film properties. LWT-Food Science and Technology, 40: 1545-1554.

Harris, J. C., Cottrell, S. L., Plummer, S., 2001. Antimicrobial properties of Allium sativum (garlic). Applied Microbiology and Biotechnology, 57: 282-286.

Hashimoto, K., Irie, H., Fujishima, A., 2005. TiO_2 photocatalysis: A historical overview and future prospects. Japanese Journal of Applied Physics, 44: 8269-8285.

Jahan, M. S., Chowdhury, D. A. N., Islam, M. K., Moeiz, S. M. I., 2007. Characterization of lignin isolated from some nonwood available in Bangladesh. Biosource Technology, 98: 465-469.

Jung, H. G., Fahey, G. C., 1983. Nutritional implications of phenolic monomers and lignin: a review. Journal of Animal Science, 57: 206-219.

Kalemba D. and Kunicka A., 2003. Antibacterial and antifungal properties of essential oils. Current Medicinal Chemistry, 10: 813–829.

Kamm, B., Gruber, P. R., Kamm, M., 2006. Biorefineries-Industrial processes and products, Wiley-VCH, Weinheim, p.13.

Kansal, S. K., Singh, M., Sud, D., 2008. Studies on TiO_2/ZnO photocatalysed degradation of lignin. Journal of Hazardous Materials, 153: 412-417.

Karamanlıoğlu, M., 2008. Xylan-based biodegradable and wheat gluten-based antimicrobial film production, METU, MSc Thesis.

Khodja, A. A., Sehili, T., Pilichowski, J. F., Boule, P., 2001. Photocatalytic degradation of 2-phenylphenol on TiO_2 and ZnO in aqueous suspensions. Journal of Photochemistry and Photobiology A: Chemistry, 141: 231.

Kobayakawa, K., Sato, Y., Nakamura, S. and Fujishima, A., 1989. Photodecomposition of Kraft lignin catalyzed by TiO_2. Bulletin of the Chemical Society of Japan, 62 : 3433.

Lathasree, S., Nageswara, R., Sivasankar, B., Sadasivam, V., Rengaraj, K., 2004. Heterogeneous photocatalytic mineralization of phenols in aqueous solutions. Journal of Molecular Catalysis A: Chemical, 223: 101.

Lee, J., 1997. Biological conversion of lignocellulosic biomass to ethanol. Journal of Biotechnology, 56: 1–24.

Lignin.org, http://www.lignin.org, Last accessed date, March 19, 2008.

Mandal, S. M. and Dey, S., 2008. LC-MALDI-TOF MS-Based rapid identification of phenolic acids. Journal of Biomolecular Techniques, 19: 116–121.

Mason, T., Lorimer, J., Applied sonochemistry: The uses of power ultrasound in chemistry and processing, Wiley, VCH, Weinheim, Germany: Chapter 5, ”Polymers”, pp. 161-163.

Mastelic, J., Jerkovic, I., Blazevic, I., Poljak-Blazi, M., Borovic, S., Ivancic-Bace, I., Smrecki, V., Zarkovic, N., Brcic-Kostic, K., Vikic-Topic, D. and Müller, N., 2008. Comparative study on the antioxidant and biological activities of carvacrol, thymol, and eugenol derivatives. Journal of Agricultural and Food Chemistry, 56: 3989-3996.

Michiels, J., Missotten, J., Fremaut, D., De Smet, S. & Dierick, N., 2007. In vitro dose-response of carvacrol, thymol, eugenol and transcinnamaldehyde and interaction of combinations for the antimicrobial activity against the pig gut flora. Livestock Science, 109: 157–160.

Nada, A. M. A., El-Diwany, A. I., Elshafei, A. M., 1989. Infrared and antimicrobial studies on different lignins. Acta Biotechnologica, 9: 295-298.

Nakamura, Y., Sawada, T., Kobayashi, F., and Godliving, M., 1997. Microbial treatment of Kraft pulp wastewater pretreated with ozone. Water Science and Technology, 35: 277.

Nelson, J. L., Alexander, J. W., Gianotti L., Chalk C. L., Pyles T., 1994. Influence of dietary fiber on microbial growth in vitro and bacterial translocation after burn injury in mice. Nutrition, 10: 32-36.

Ou, S. Y., Luo, Y. L., Huang, C. H., Jackson, M., 2009. Production of coumaric acid from sugarcane bagasse. Innovative Food Science & Emerging Technologies, 10: 253-259.

Perez, J., Munoz-Dorado, J., De La Rubia, R. T. & Martinez, J., 2002 Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. International Microbiology, 5: 53–63.

Pina-Vaz, C., Rodrigues, A. G., Pinto, E., Costa-de-Oliveira, S., Tavares, C., Salgueiro, L. R., Cavaleiro, C., Gonçalves, M. J. & Martinez-de-Oliveira, J., 2004. Antifungal activity of Thymus oils and their major compounds. Journal of the European Academy of Dermatology & Venereology, 18: 73–78.

Puls, J. and Schuseil, J., 1993. Chemistry of hemicelluloses: relationship between hemicellulose structure and enzymes required for hydrolysis in hemicellulose and hemicellulases. pp.1-28. Coughlan M. P. and Hazlewood G. (Eds). Portland Press Ltd, NC, U.S.A.

Rousu, P., Rousu, P., Anttila, J., 2002. Sustainable pulp production from agricultural waste. Resources, Conservation and Recycling, 35: 85–103.

Sarkanen K. V. & Ludwig C. H., 1971. Lignins, Occurrence, Formation, Structure and Reactions, Wiley-Interscience, New York, 1971: 55.

Scalbert, A., Monties, B., Lallemand, J. Y., Guittet, E., Rolando, C., 1985. Ether linkage between phenolic acids and lignin fractions from wheat straw. Phytochemistry, 24(6): 1359.

Sikkema, J., De Bont, J. A., & Poolman, B., 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews, 59(2): 201–222.

Sivropoulou, A., Papanikolaou, E., Nikolaou, C., Kokkini, S., Lanaras, T. and Arsenakis, M., 1996. Antimicrobial and cytotoxic activities of origanum essential oils. Journal of Agricultural and Food Chemistry, 44: 1202-1205.

Stewart, D. and Morrison, I. M., 1992. FT-IR spectroscopy as a tool for the study of biological and chemical treatments of barley straw. Journal of the Science of Food and Agriculture, 60: 431-436.

Stobiecki, M., Skirycz, A., Kerhoas, L., Kachlicki, P., Muth, D., Einhorn, J. and Mueller-Roeber, B., 2006. Profiling of phenolic glycosidic conjugates in leaves of *Arabidopsis thaliana* using LC/MS. Metabolomics, 4: 197-219.

Sun, R. C. and Lawther, J. M., 1998. Fractional and structural characterization of ball-milled and enzyme lignins from wheat straw. Journal of Applied Polymer Science, 68: 1633-1641.

Tanaka, K., Calanag, R. C. R., Hisanaga, T., 1999. Photocatalyzed degradation of lignin on TiO_2. Journal of Molecular Catalysis A: Chemical, 138: 287-294.

Tejado, A., Penaa, C., Labidia, J., Echeverria, J. M. and Mondragon, I., 2007. Physico-chemical characterization of lignins from different sources for use in phenol-formaldehyde resin synthesis. Bioresource Technology, 98: 1655-1663.

Tharanathan, R. N., 2003. Biodegradable films and composite coatings: past, present and future. Food Science & Technology, 14:71-78.

Thiruvenkatachari, R., Vigneswaran S., Moon, I. S., 2008. A review on UV/TiO_2 photocatalytic oxidation process. Korean Journal of Chemical Engineering, 25: 64-72.

Thompson, N. S., 2000. Hemicellulose. Kirk-Othmer Encyclopedia of Chemical Technology. John Wiley & Sons, Inc.

Tippayatum, P. and Chonhenchob, V., 2007. Antibacterial activities of thymol, eugenol and nisin against some food spoilage bacteria. Natural Science, 41: 319-323.

Ultee, A., Bennik, M. H. J. and Moezelaar, R., 2002. The phenolics hydroxyl group of carvacrol is essential for action against the foodborne pathogen *Bacillus cereus*. Applied and Environmental Microbiology, 68: 1561–1568.

Vainio, U., Maximova, N., Hortling, B., Laine, J., Stenius, P., Simola, L. K., Gravitis, J., Serimaa, R., 2004. Morphology of dry lignins and size and shape of dissolved Kraft lignin particles by x-ray scattering. Langmuir, 20: 9736-9744.

Xu, F., Sun, R., Sun, J., Liu, C., He, B. & Fan, J., 2005. Determination of cell wall ferulic and p-coumaric acids in sugarcane bagasse. Analytica Chimica Acta, 552: 207–217.

Zemek, J., Kosikova, B., Augustin, J., Joniak, D., 1979. Antibiotic properties of lignin components. Folia Microbiologica, 24: 483-486.

Zheng, Y., Pan, Z., Zhang R., 2009. Overview of biomass pretreatment for cellulosic ethanol production. International Journal of Agricultural and Biological Engineering, 2(3); 51-68.

Zilliox, C. and Debeire, P., 1998. Hydrolysis of wheat straw by a thermostable endoxylanase: Adsorption and kinetic studies. Enzyme and Microbial Technology, 22: 58-63.

APPENDIX A

MEDIUM AND AGAR BASES

1. Luria-Bertani (LB) Broth:

Product Company: Merck
Basis: 1L (in distilled water)
Tryptic soy broth, 10g
Yeast extract, 5g
NaCl, 10g

2. Luria-Bertani (LB) Agar:

Product Company: Sigma-Aldrich
Basis: 1L (in distilled water)
Agar, 15g
Tryptic soy broth, 10g
Yeast extract, 5g
NaCl, 10g

3. Nutrient Broth (NB):

Product Company: Merck
Basis: 1L (in distilled water)
Peptones, 15g
Yeast extract, 3g
NaCl, 6g
D(+)glucose, 1g

4. Mueller Hinton Agar (MHA):

Product Company: Sigma-Aldrich

Basis: 1L (in distilled water)

Agar, 17.0g

Beef heart infusion, 2.0g

Casein acid hydrolysate, 17.5g

Starch, soluble, 1.5g

APPENDIX B

BACTERIAL CELL CALIBRATION CURVES

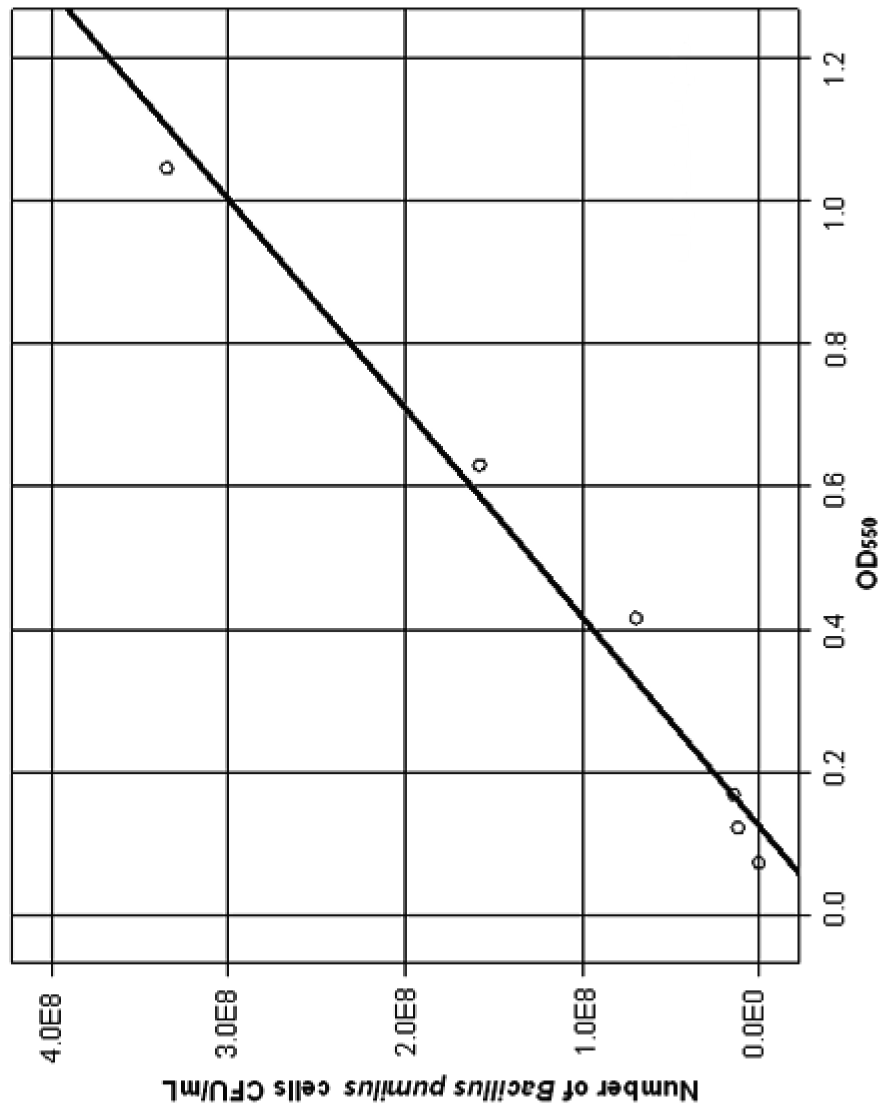

Figure B.1 Calibration curve for *Bacillus pumilus*

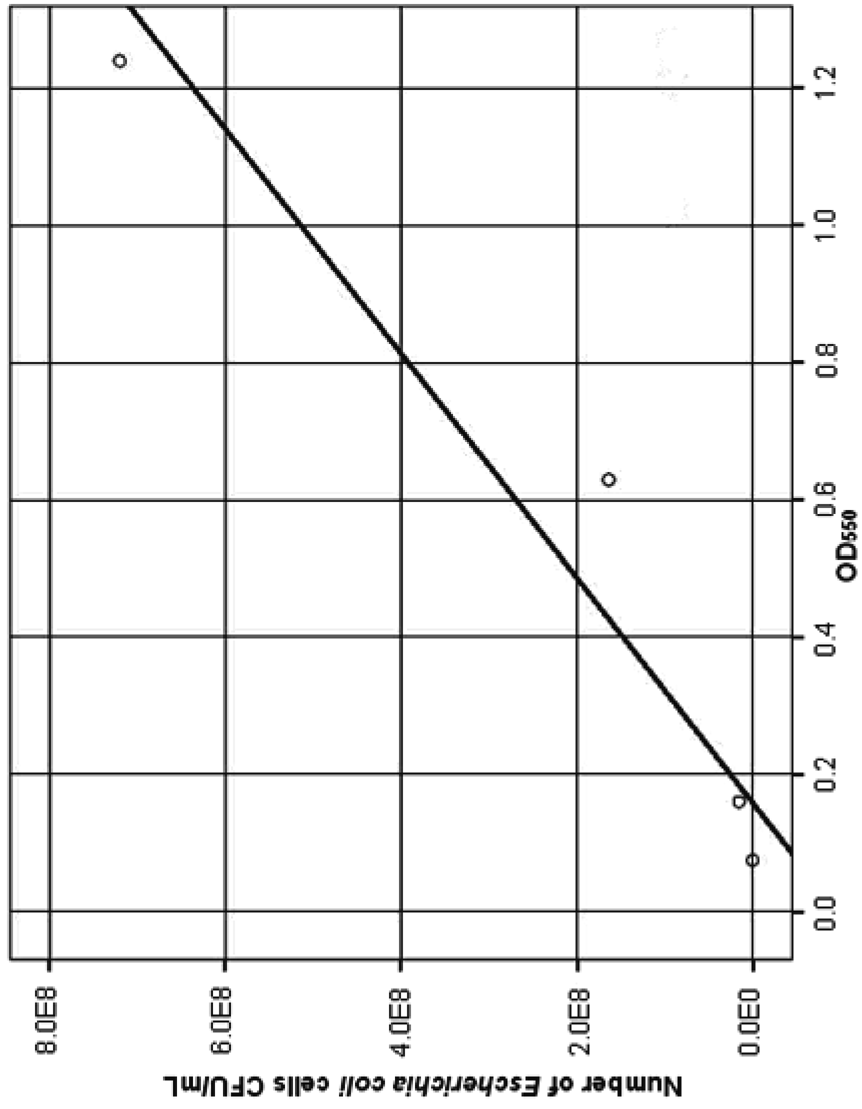

Figure B.2 Calibration curve for *Escherichia coli.*