XYLAN-BASED BIODEGRADABLE AND WHEAT GLUTEN-BASED ANTIMICROBIAL FILM PRODUCTION

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ABSTRACT

XYLAN-BASED BIODEGRADABLE AND WHEAT GLUTEN-BASED ANTIMICROBIAL FILM PRODUCTION

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In the first part of the study, birchwood xylan-lignin film formation was studied. After film forming effect of lignin on pure birchwood xylan was demonstrated, the minimum lignin concentration necessary to form films was determined as 1.1% (lignin/xylan). So, it was determined that keeping about one percent lignin in xylan (w/w) was sufficient for film formation.

Biodegradability of the lignin-birchwood xylan composite films was investigated enzymatically using 0.21 U / ml xylanase in an accelerated test. All the films containing lignin were hydrolyzed by xylanase showing biodegradability of the films.

Colors of the birchwood xylan-lignin composite films containing different lignin concentrations were compared. Deviations of the color from the reference color were similar between the films.

In the second part of the study, photocatalytic antimicrobial film production was investigated on wheat gluten-based films. In order to produce antimicrobial films, wheat gluten films were coated with a semiconductor, titanium dioxide (TiO_2), applying different procedures. Coated films were illuminated and photocatalytical inactivation of *Escherichia coli* on films were determined by antimicrobial tests. The coating procedure in which titanium dioxide (TiO_2) was produced from titanium tetraisopropoxide (TTIP) in aqueous-nitric acid and aqueous-hydrochloric acid solutions gave the best antimicrobial result but the films turned out to be deformed and brittle. Spreading TiO_2 sol-gel on semi-dried wheat gluten films resulted in flexible and undeformed films having about 40% antimicrobial activity.

Keywords: Agricultural Waste, Xylan, Lignin, Biodegradable, Photocatalytic, Wheat Gluten, Titanium Dioxide, Antimicrobial.

ÖZ

BİYOBOZUNUR KSİLAN VE ANTİMİKROBİK GLUTEN FİLM ÜRETİMİ

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Çalışmanın ilk kısmında, huş ağacı ksilanı-lignin film oluşturulması çalışılmıştır. Ligninin film oluşturma özelliği saf huş ağacı ksilanı üzerinde gösterildikten sonra film oluşturmak için gereken minimum lignin miktarı %1.1 (lignin/ksilan) olarak belirlenmiştir. Böylece, ksilandaki lignini yüzde bir kadar tutmanın film oluşturmak için yeterli olduğu saptanmıştır.

0.21 U / ml ksilanaz kullanılarak lignin - huş ağacı ksilanı kompozit filmlerinin biyobozunurluğu enzimatik olarak hızlandırılmış bir testle incelenmiştir. Lignin içeren tüm filmler ksilanaz tarafından hidrolize edilerek filmlerin biyobozunur olduğu gösterilmiştir.

Farklı lignin konsantrasyonları içeren lignin-huş ağacı ksilanı kompozit filmlerinin renkleri karşılaştırılmıştır. Renklerin referans renkten sapmaları filmler arasında benzerdir.

Çalışmanın ikinci kısmında, fotokatalitik antimikrobiyal film üretimi buğday gluteni bazlı filmler üzerinde araştırılmıştır. Antimikrobiyal film üretmek için buğday gluteni filmler, bir yarı-iletken olan titanyum dioksit ile (TiO_2) farklı yöntemler kullanılarak kaplanmıştır. Kaplı filmler ışığa tutulmuş ve film üstündeki *Escherichia coli'* nin fotokatalitik inaktivasyonu antimikrobik testle belirlenmiştir. Titanyum dioksitin sulu-nitrik asit ve sulu-hidroklorik asit solüsyonunda öncü titanyum tetraisopropoksitten (TTIP) üretildiği kaplama metodu en iyi antimikrobik sonucu vermiştir ama deforme olmuş ve kırılgan filmler elde edilmiştir. TiO_2 sol-jeli yarı-kurutulmuş buğday gluteni filmi üzerine yaymak %40 antimikrobik aktiviteli, elastik ve deforme olmamış filmler olarak sonuçlanmıştır.

Anahtar sözcükler: Zirai Atık, Ksilan, Lignin, Biyobozunur, Fotokatalitik, Buğday Gluteni, Titanyum Dioksit, Antimikrobiyal.

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LIST OF ABBREVIATIONS

CFU: Colony-Forming Unit

E. coli : *Escherichia coli*

EDX: Energy-Dispersive X-ray spectroscopy

h: Hour

LB medium: Luria Bertani medium

min: Minute

ml: Milliliter

μl : Microliter

sec: Second

SEM: Scanning Electron Microscopy

U/ml: Unit/mililiter

w/w: weight/weight

CHAPTER 1

INTRODUCTION

1.1 Food Packaging Materials

Food packaging is concerned mainly with containment and protection of food. As the consumer interests in health, food quality, convenience and safety continue to increase, it presents food processors to offer potential solutions in film concepts. Food scientists and engineers have isolated new materials that present new opportunities in the formation and properties of films to help meet the many challanges involved with the marketing of foods (Tharanathan, 2003; Krochta et al., 1994). Chemical structure, molecular weight, crystallinity and the processing conditions of the polymers used influence the physical characteristics of the packaging films (Chandra and Rustgi, 1998). Commonly used packaging films are mostly polyethylene, polyvinylidene, polyester, polyamide and cellophane (Tharanathan, 2003). Petrochemical-based polymers like polyolefins, polyesters, polyamides are mostly preferred because they are available in large quantities, cheap and they have favourable functionality characteristics, such as good tensile and tear strength, good barrier properties to O_2 and heat sealability and good elongation at break as synthetic polymers like polyethylene or plasticized PVC that have elongation at breaks from 150% to about 400% (Alves et al., 2006; Tharanathan, 2003; Audic and Chaufer, 2005). However, petrochemical-based polymers are harmful to environment as they are totally non-biodegradable (Tharanathan, 2003). As a consequence, the consumer demand has shifted to eco- friendly biodegradable materials,

especially from renewable agriculture by-products, food processing industry wastes and low cost natural resources (Alves et al., 2006).

1.2 Biodegradable Polymer Films As Food Packaging Materials

Biodegradation is the kind of degradation in which organic chemicals in the environment are broken down to simpler compounds by the enzymatic action of living organisms such as bacteria, yeasts and fungi, mineralized and redistributed through elemental cycles such as the carbon and nitrogen cycles. The ultimate end products of the degradation process are carbondioxide, water and biomass under aerobic conditions and hydrocarbons, methane, and biomass under anaerobic conditions (Chandra and Rustgi, 1998; Lim et al., 1999).

Biodegradable polymers or biopolymers are the special class of polymers, that are naturally biodegradable formed in nature and use of natural biopolymers for diversified applications in life sciences has several advantages, such as availability from replenishable agricultural or marine food resources, biocompatibility, biodegradability, therefore leading to ecological safety and the possibility of preparing a variety of chemically or enzymatically modified derivatives for specific end uses so that they are the new alternatives for biodegradable packaging materials especially in countries where landfill is the main waste management tool. (Chandra and Rustgi, 1998; Prashanth and Tharanathan, 2007; Petersen et al., 1999).

Naturally occuring biopolymers used in packaging are from animals like collagen, gelatin; from marine food processing industrial wastes like chitin, chitosan; from microbial sources like pullulan, polylactic acid, polyhydroxy alkanoates; and from agricultural feed stock like some lipids, proteins and polysaccharides (Tharanathan, 2003).

1.2.1 Polysaccharide Films

Polysaccharides, as a class of natural macromolecules, have the tendency to be extremely bioactive and are generally derived from agricultural feedstock or crustacean shell wastes.

Cellulose, starch, pectin, etc. are the biopolymers derived from agricultural feedstock, while chitin and chitosan are derived from crustacean shell wastes (Prashanth and Tharanathan, 2007).

Linear structure of, cellulose (1,4-β-D-glucan), amylose (a component of starch, 1,4-α-D-glucan), chitosan (1,4-β-D-glucosamine polymer), render their films tough, flexible and transparent. Their films are resistant to fats and oils. Cross-linking, for example, of chitosan with aldehydes make the film much tougher, water insoluble (or swellable) and highly resistant. Also chitin and pectin are used for food packaging materials (Tharanathan, 2003).

Cellulose is one of the components of plant cell wall along with hemicellulose and lignin. It is a highly uniform 1,4-β-linked polyglucan (Ebringerova and Heinze, 2000). The barrier and mechanical properties of cellulose-based films are dependent on the molecular weight of cellulose, higher the molecular weight better is the properties. Cellophane, a regenerated cellulose film is made by the viscose process. Some of the cellulose esters like cellulose acetate propionate and butyrate are thermoplastic products of commercial importance. The anionic cellulose ether, carboxymethyl cellulose (CMC), being water soluble and compatible with other (bio-)molecules, has excellent film forming properties (Tharanathan, 2003) .

In terms of availability, chitin is next to cellulose (Prashanth and Tharanathan, 2007). It is a β-1,4 linked polymer of 2-acetamido-2-deoxy-D-glucopyranosyl

residues. It occurs as the major organic skeletal substance of invertebrates and as a cell wall constituent of fungi and green algae. Fusion of chitin with alkalies give the product chitosan, a heterogeneous substance in various stages of deacetylation and depolymerization. One of the main applications of chitosan include coating of foods (Nisperos-Carriedo, 1994). Chitosan is also present in cell walls of some fungi such as Rhizopus species.

Pectin, occuring in land plants is a complex anionic polysaccharide composed of β-1,4-linked D-galacturonic acid residues, wherein the uronic acid carboxyls are either fully (HMP, high methoxy pectin) or partially (LMP, low methoxy pectin) methyl esterified. HMP forms excellent films. Plasticized blends of citrus pectin and high amylose starch give strong, flexible films (Tharanathan, 2003).

Starch is a raw material that is commonly used to produce biodegradable films. It is abundant especially from corn, having thermoplastic properties upon disruption of its molecular structure. Majority of amylose (>70%) in amylomaize starches gives stronger, more flexible films however, branched structure of amylopectin generally leads to films with poor mechanical properties for example, decreased tensile strength and elongation (Tharanathan, 2003).

Pullulan produced by several fungi as an extracellular secondary metabolite is comprised primarily of maltotriose units connected by α-1,6 linkages and has been approved for food coatings. It is a water-soluble polymer which produces clear, edible films (Chandra and Rustgi, 1998; Tharanathan, 2003).

Carrageenan is an extract from seaweed and its gels can be used as food coatings (Kester and Fennema, 1986).

1.2.2 Agricultural Wastes As Food Packaging Materials

Livestock and crops represent 90% of the agricultural sector in Turkey. The type and quantity of crops that form the basis of the agricultural sector in Turkey (cotton, wheat, barley, tobacco, rice, etc.) give rise to huge amounts of agricultural residues. If these residues are treated in an uncontrolled manner like burning them in open-air fires or disposing them to decay, they cause significant environmental impacts while at the same time useful resources are wasted (agrowaste-tr.org).

1.2.2.1 Cotton Stalk

Cotton (*Gossypium hirsutum*) from Malvaceae family is a hardwood. It is the most important fiber crop grown in 90 countries (Lee at al., 2006). Avarage cotton production in the world is about 19,60 million tons and Turkey is one of the major producers of cotton along with China, India, the United States, Pakistan, Brazil, Uzbekistan, Greece, Turkmenistan and Syria. Therefore, cotton stalk is an important agricultural product in Turkey that is about 2.474.868 tons and 2.554.418 tons is the total residue produced from it having 1.532.649 tons avaliable residue (agrowaste-tr.org; cotton.org; www.dtm.gov.tr). One of the main the sustainable exploitation of this agricultural waste is to use its cellulose, hemicellulose and lignin which are contained in its structure. Cellulose is about %36, hemicellulose is %21 and lignin is %28 of cotton stalk (Akpinar et al., 2007).

1.2.2.1.1 Cellulose

Cellulose has different industrial uses. For example it is the major constituent

of paper and textiles made of cotton, linen and other plant fibers. Also it is used as emulsifier, stabilizer, dispersing agent, thickener and films.

1.2.2.1.2 Hemicellulose

Hemicellulose, another component of plant cell wall, located primarily in the secondary cell walls, and together with cellulose and lignin, gives the best combination of mechanical support and transport properties in plants. Hemicelluloses, interlocking the cellulose scaffold through hydrogen bonding, are cross-linking glycans. They include xylan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan. It has a heterogenous, amorphous structure with little strength while cellulose is crystalline, strong, and resistant to hydrolysis.

Hemicelluloses, even though being as abundant as cellulose have not been commercially utilized more than as a sizing agent in paper to some extent and as a source of xylitol. However, recent research has begun to find new applications as food additives, thickeners, emulsifiers, gelling agents, adhesives, and adsorbents. Hemicelluloses have also been proved to be a good binder for charcoal/coal briquettes (Ebringerova and Heinze, 2000; Gabrielli and Gatenholm, 1998; Gabrielli et al., 2000; Kadla et al., 2006).

Hemicelluloses represent polysaccharides of different structure. The most commonly existing sugars are D-glucose, D-mannose, D-xylose, D-glucuronic acid, L-arabinose , 4-*O*-methyl-D-glucuronic acid, and D-galacturonic acid in various amounts or traces dependent on the natural source. The most prevalent sugar moieties are β -D-glucopyranose (β-D-Glc*p*), β-D-mannopyranose (β -D-Man*p*), and β -D-xylopyranose (β-D-Xyl*p*), which constitute the backbone structure of glucomannans and xylans, respectively, while various other

monosaccharides make up the short branches or side chains (Ebringerova and Heinze, 2000; Gabrielli and Gatenholm, 1998; Kadla et al., 2006).

In softwoods, the primary hemicellulose components are galactoglucomannans and arabinoglucuronoxylans. They are heteropolysaccharides with heterolinkages. Hardwoods contain hemicellulose polymers that are structurally similar to those found in softwoods but the principal hemicellulose polymer is a 4-*O*-methylglucuronoxylan accompanied by a lesser amount of glucomannans. This is in contrast to softwoods wherein the glucomannans are the major hemicellulose and xylans are the minor components. As with the galactoglucomannans in softwoods, the xylans in hardwoods are partially acetylated along the β -D-Xyl*p* backbone at the C2 and/or C3 hydroxyl groups (Kadla et al., 2006).

1.2.2.1.2.1 Xylan

Xylans are the most common hemicelluloses and they are considered to be the second most abundant biopolymer in the plant kingdom. Xylans are not only present in wood but also in various other plants such as grasses, cereals, and herbs. The xylan-type polysaccharides are known to occur in several structural varieties in terrestrial plants and algae, and even in different plant tissues within one plant (Ebringerova and Heinze, 2000). Main sugar of xylan is D-xylose.

Xylan is generally characterized by a relatively short backbone of β (1-4)-linked D-xylopyranosyl units with 4-*O*-methyl-D-glucuronopyranosyl units attached by α (1−2) bonds to some of the anhydroxylose units, although linkages to the C-3 position have been reported. The acids occur in the native state as carboxyls, esters, or possibly as salts. Arabinofuranosyl units,

mannose, rhamnose, glucuronic acid, galacturonic acid, and *O*-acetyl groups have also been found in xylan hydrolysates and may be a component in some instances. Glycosidic and ester linkages to lignin also occur (Thompson, 2000).

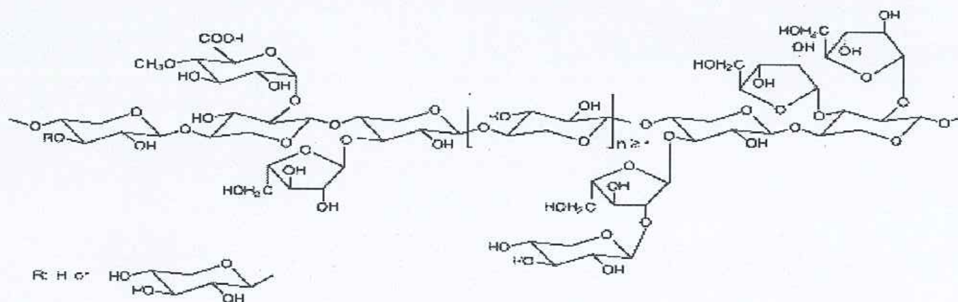


Figure 1.1 Xylan structure (Ebringerova and Heinze, 2000).

Homoxylans with β-1,3 glycosidic linkages, are known to substitute cellulose in the cell wall architecture of green algae (*Caulerpa* sp.), whereas homoxylans with mixed β-1,3 and 1,4 glycosidic linkages are known cell wall components of red seaweeds of the *Palmariales* and *Nemaliales*. Xylans of all higher plants possess β-1,4-linked xylopyranose units as the backbone, usually substituted with sugar units and *O*-acetyl groups. The only exception of heteroxylans with mixed linkages in the main chain, have been isolated from the seeds of *Plantago* species. The occurrence of homoxylans in higher plants is rather rare. However, a neutral linear xylan has been isolated from guar seed husks as the hemicellulose (Ebringerova and Heinze, 2000).

Film production from xylan is also possible; however, pure xylan did not form continuous and self-supporting films. Mixtures of xylan extracted from

birchwood and aspen and various amounts of chitosan were prepared and it was observed that at as low as 5% chitosan content, the solutions started to form films upon drying. Continuous, self-supporting films were achieved at 10%. Films with 5-30% chitosan formed hydrogels when immersed into water with a degree of swelling increasing with the increase in the chitosan content (Gabrielli and Gatenholm, 1998; Gabrielli et al., 2000).

Composite film production of cotton stalk xylan was also studied. Film formation was achieved using 8-14 % (w/w) xylan without complete removal of lignin during xylan isolation but the thickness and mechanical properties of the films obtained by using 8% xylan were found to be low in comparison with the ones containing 10-14% xylan. The water vapor transfer rates (WVTR) decreased by increasing xylan concentration which made the films thicker. The glycerol addition as an additional plasticizer resulted in more stretchable films having higher WVTR and lower water solubility values (Göksu, 2005).

Xylan was also incorporated into wheat gluten films to produce biodegradable composite films. It was found that up to 40% (w/w) xylan could be added to wheat gluten films without altering the gluten film properties significantly. Different properties of films were produced when different type of xylans like birchwood, corncob and grass xylan with different compositions were added into gluten film forming solutions. Tensile strength, strain at break and elastic modulus of the films found to be ranging between 1-8 MPa, 20-650 % and 5-140 MPa, respectively, depending on the type of the xylan used. The film solubility was between 40-60% depending on xylan content, and the water vapor transfer rate was not affected by the xylan additions (Kayserilioğlu et al., 2003a).

1.2.2.1.3 Lignin

Lignin is a necessary constituent in the cell walls of all vascular plants as well as cotton stalk, covalently linked to hemicellulose. It is one of the most abundant biopolymers, and a considerable part of the carbon fixed by photosynthesis is consumed by lignin biosynthesis. This polymer has many biological functions like they give stiffness and strength to the secondary wall of vascular plants. The lignified cell wall can be seen as a composite, with cellulose microfibrils as reinforcing fibers and lignin as a plastic matrix giving stiffness to the material. They also make the cell wall hydrophobic allowing the development of tissues for efficient water transport in vascular plants. They can also be considered as an obstacle to microbial attack (Önnerrud, 2002).

Lignin is a large amorphous, polyphenolic and racemic macromolecule. It is not possible to isolate lignin from wood without degradation, so that the true molecular weight of lignin in wood is not known. Light scattering and vapor phase pressure osmometry are the traditional methods of analysis. By using these methods, the weight-average molecular weight, Mw, of softwood milled wood lignin is estimated to be 20,000; lower values have been reported for hardwoods. Lignin arises from enzymatic dehydrogenative polymerization of three phenylpropanoid monomers which are coniferyl alcohol, sinapyl alcohol, and p-coumaryl alcohol (Lebo et al., 2001). These are incorporated into lignin in the form of the phenylpropanoids *p*-hydroxyphenyl, guaiacyl, and syringal respectively.

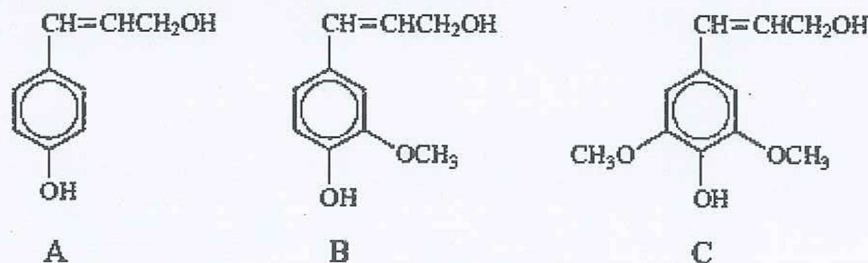


Figure 1.2 Phenylpropanoid monomers of lignin.: (A) p-coumaryl alcohol; (B) coniferyl alcohol; (C) sinapyl alcohol (Lebo et al., 2001).

Hardwood lignin (in dicotyledons) originates mainly from sinapyl alcohol and coniferyl alcohol, whereas softwood lignin (in coniferous trees and gingko) is made mainly from coniferyl alcohol. However, compression wood in coniferous trees also contains significant amounts of lignin that originates from the monolignol *p*-coumaryl alcohol (Önnerrud, 2002).

As a biomacromolecule, lignin is unusual in having a complex network–type structure. It is optically inactive. The traditionally held biosynthesis process, which consists essentially of random radical coupling reactions is sometimes followed by the addition of water, of primary, secondary, and phenolic hydroxyl groups to quinonemethide intermediates, so that lignin polymerization from monolignols is not a condensation, leads to the formation of a three-dimensional polymer that lacks the regular and ordered repeating units found in other natural polymers such as cellulose and proteins. Traditionally, no other enzymes or proteins are thought to be required and many different types of bonds connect the monolignol residues in a random pattern (Önnerrud, 2002; Lebo et al., 2001).

Those structural building blocks of lignin are linked by carbon–carbon and ether bonds. Units that are trifunctionally linked to adjacent units represent branching sites which give rise to the network structure characteristic of lignin. There are interunit linkages in the polymer like β-aryl ether or β—O—4, phenylcoumaran or β-5, resinol or β–β, biphenyl or 5—5, diphenyl ether or 4—O—5. Also there are hydroxycinnamyl alcohol end groups from the few initial dimerization reactions, structures such as non-cyclic α-aryl ethers (which arise from addition of a phenol to an intermediate β-aryl ether quinone methide) and various other structures (Ralph, 1999).

Table 1.1 Types and frequencies of interunitary linkages in softwood and hardwood lignins, number of linkages per 100 C_9 units (Lebo et al., 2001).

Linkage	Softwood lignin	Hardwood lignin
β-*O*-4	49–51	65
α-*O*-4	6–8	
β-5	9–15	6
β-1	2	15
5-5	9.5	2.3
4-*O*-5	3.5	1.5
β-β	2	5.5

Lignin behaves as a thermoplastic material undergoing a glass-transition at temperatures that vary widely depending on the method of isolation, sorbed water, and heat treatment. It is subject to oxidation, reduction, discoloration, hydrolysis, and other chemical and enzymatic reactions (Lebo et al., 2001).

The principal commercially available lignin types are the lignosulfonates which are hydrophilic derived from sulfite pulping of wood and kraft lignins which are hydrophobic derived from kraft pulping of wood. Lignin is generally used as dispersants, emulsifiers, additives and binders (Lignin.org).

Lignin was also used as an additive in film forming solutions of pure xylan extracted from cotton stalk and was found to be a binding agent to form continuous and self-supporting films (Göksu, 2005).

1.2.3 Lipid Films

Use of lipids in coatings for food products has been practiced for centuries. In the past, waxes and oil were used alone, but now they are often blended with solvents, emulsifiers, surfactants, plasticizers, resins, etc. Lipids are compounds including neutral lipids of glycerides which are esters of glycerol and fatty acids, and the waxes that are esters of long-chain monohydric alcohols and fatty acids. From this group acetylated monoglycerides, natural waxes and surfectants are commonly utilized in edible coatings (Hernandez, 1994; Kester and Fennema, 1986). Although lipid based films have proved to be good water vapour barriers, they lack sufficient strength to form self-supporting films (Morillon et al., 2002).

1.2.4 Protein Films

The film forming ability of several proteinaceous substances has been utilized in industrial applications for a long time. The food industry recognized proteins possessed film forming properties (Gennadios et al., 1994).

Proteins are polymers formed by various amino acids capable of promoting intra- and inter-molecular bonds, allowing the resultant materials to have a large variation in their functional properties and the macroscopic properties of agro-packaging materials based on proteins and macromolecular three dimensional networks are largely dependent on interactions between polymers (Moore et al., 2006; Cuq et al., 1998). Due to their renewable and biodegradable nature, proteins are attractive for innovative uses. They have abundant resources, low cost, good biodegradability and are relatively easy to handle. Many animal proteins such as gelatin, milk proteins, and collagen; and cereal and vegetable proteins such as corn and soy proteins and wheat gluten are commonly used to form agricultural packaging materials (Cuq et al., 1998).

Gelatin is an animal protein extracted from the collagen. Gelatin-based films are biodegradable, thin, flexible and transparent (Bergo and Sobral, 2007). Film forming applications of gelatin in the pharmaceutical and food industry include microencapsulation and manufacture of tablet and capsule coating (Gennadios et al., 1994).

Casein, milk protein is used as surface coating material in leather, paper and textile industries (Somanathan et al., 1992). Casein films are mainly used on food products because they are transparent, flavourless and flexible (Gennadios et al., 1994).

Whey protein is the part milk proteins that is globular and can be isolated from whey. The formation of edible films and coatings composed of whey is also possible. When appropriately processed, whey proteins produce transparent, flavourless and flexible edible films similar to films produced from caseinates (Gennadious, 1994).

Collagen, a fibrous protein, is the main protein of connective tissue and

the most abundant protein of animal origin (Fernandes et al., 2008). The film forming ability of collagen has been traditionally utilized in the meat industry for production of edible sausage casing (Gennadios et al,1994).

Keratin is the protein containing insoluble cystine extracted from epidermal tissues of vertebrates such as hair, nails, feathers, hoofs, scales and claws (Fraser et al., 1972). Stability of the keratin in the solid state is due to cross-linking produced by the formation of cystine disulphide bonds, hydrogen bonds and salt linkages. Keratins are known to have a great ability to form films (Moore et al., 2006).

Corn zein is a plant protein which can produce protein edible film. Zein is a class of prolamine protein found in corn that is usually manufactured as powder. It is used for formulation of coating for nutmeats, candy and pharmaceutical tablets (Gennadios et al., 1994).

Soy proteins are comercially available as soy flour, soy concentrate and soy isolate, all in different in protein content (Weber, 2001). Film forming ability of soy protein has been utilized in Far East and films obtained from soymilk are called yuba or tou-fu-pi (Gennadios et al., 1994). Soy films have been developed mainly coating materials to help preserve of numerous foods (Baker et al., 1972). Soymilk, necessary to make the films is prepared from soybeans by subjecting them successively to washing, soaking in water, draining and wet grinding (Smith and Circle, 1972; Wang, 1981).

1.2.4.1 Wheat gluten

Gluten, which is another plant protein, is the major storage protein in wheat and corn (Weber, 2001). Wheat gluten is the most important by-product of wheat starch production. It is consisted of proteins, carbohydrates,

lipids and fibers (Kersting et al., 1994).

Gluten is a mixture of gliadin and glutenin proteins which comprise about 80% of the protein contained in wheat seed. Glutenin, glutelin fraction of wheat protein, is responsible for elasticity and many of its subunits are linked by intermolecular disulfide bonds. Gliadin makes up the prolamin fraction of wheat endosperm. It is a single-chained molecule and responsible of extensibility. It mainly has intramolecular disulfide bonds. As they are insoluble in water, they can be purified by washing away the associated starch. The distinctive feature of gluten proteins is that they contain high glutamic acid and prolin as amino acid compozition (Kasarda et al., 1978). Proline has amino groups that are involved in a ring structure, so that peptide bonds are not flexible (Wu and Dimler, 1963).

There are a large number of nonpolar side chains resulting in an increase in the possibility of apolar bonding. Also, gluten proteins have low ionic characters. 35% of the total amino acids have hydrophobic side chains indicating that the apolar residues are not accomodated in the hydrophobic core of the protein. Therefore, it is believed that hydrophobic interactions between the proteins have a significant role in structure stabilization (Kasarda et al., 1976).

Upon hydration, gliadin and glutenin form a tenacious colloidal complex known as wheat gluten (Pomeranz, 1987). The cohesiveness and elasticity of hydrated gluten provide dough-forming ability of wheat flour (Krull and Wall, 1969). These properties also generate interest for film formation from gluten protein (Gennadios, 1994).

Wheat gluten is an excellent edible film forming agent because of the unique cohesive and elastic properties of gluten as well as being a major functional food ingredient especially in baked goods (Gontard et al., 1993; 1992).

A film forming solution of whole gluten in an ethanol/ lactic acid/ water solvent was prepared but films were found to be brittle. A method of solubilizing wheat gluten in a heated alcoholic and alkaline solution with glycerol as plasticizer was also casted into a film. Formation of homogeneous film-forming solution of gluten require use of alkaline (pH > 10.5) or acidic (pH<5) conditions (Gontard et al., 1992). Intramolecular and intermolecular disulfide bonds in the gluten complex are cleaved and reduced to sulfhydryl groups when dispersing gluten in alkaline environments (Okamoto, 1978).

Addition of plasticizers in wheat gluten film-forming solutions is necessary to induce film flexibility because film cast without plasticizer are very brittle because of the extensive intermolecular associations (Wall and Beckwith, 1969).

In overall, characterization of films was studied in previous studies. Generally tensile strength expressesing the maximum stress developed in a film during a tensile test, offering a measure of integrity and heavy-duty use potential of films; strain at break, film's ability to stretch (Gennadios et al., 1993); and elastic modulus, ratio of stress to strain over the linear range and measures the intrinsic stiffness of the film (Chen, 1995), were measured as given in Table 1.2.

In general essential prerequisites of a good packaging film are:
1. Allow for a slow but controlled respiration (reduced O_2 absorption) of the commodity;
2. Allow for a selective barrier to gases (CO_2) and water vapour;
3.Creation of a modified atmosphere with respect to internal gas composition, thus regulating the ripening process and leading to shelf-life extension;
4. Retard oil and fat migration;

5. Maintain structural integrity and improve mechanical handling;

6. Serve as a vehicle to incorporate food additives like flavour, colours, antioxidants, and antimicrobial agents and;

7. Prevent or reduce microbial spoilage during extended storage. As a consequence, as a food additive, antimicrobials can be incorporated into films (Tharanathan, 2003; Donhowe and Fennema, 1994).

Table 1.2 Tensile strengths, strain at break and elastic modulus of various films.

Component	Tensile Strength, MPa	Strain at Break, %	Elastic Modulus, MPa	Reference
Cotton stalk xyan	1.3	51	0.4	Göksu (2005)
Corncob xylan-wheat gluten	1.3	650	10.0	Kayserilioğlu et al. (2003a)
Grass xylan-wheat gluten	2.0	20	25.0	
Wheat gluten	4.0	250	70.0	Kayserilioğlu et al. (2003b)
Methyl cellulose	55.6-61.2	17		Tharanathan
LDPE	13.1-27.6	100-965		(2003)
Feather keratin	16.6	2.00	10.0	Moore et al. (2006)

1.3 Antimicrobial Films

Development of antimicrobial materials for food packaging is an important issue in food applications because microbial growth on the surface of food is a major cause of food spoilage and food-borne illness (Kandasamy, 2007).

There are two basic categories of antimicrobial films. One involves the direct incorporation of the antimicrobial additive into the packaging film, while the second type of film is coated with a material which is antimicrobial or acts as a carrier for the additive (Cooksey, 2001).

So far chitosan, nisin, potassium sorbate, imazalil, triclosan have been used as antimicrobial agents in food packages.

Chitosan derived from various fungi or shells of crustaceans through repeated washes in alkaline and acid solutions is an inherently antimicrobial biodegradable polysaccharide inhibiting the growth of a wide variety of fungi, yeasts, and bacteria and has been used in films and coatings. It is a cationic polymer like poly-L-lysine and it promotes cell adhesion since charged amines interact with negative charges on the cell membrane, causing leakage of intracellular constituents and leading to cell death. (Appendini and Hotchkiss, 2002; Cooksey, 2001; Bégin, 1999). Chitosan has been used as a coating and appears to protect fresh vegetables and fruits from fungal degradation. Although the antimicrobial effect is attributed to antifungal properties of chitosan, it may be that the chitosan acts as a barrier between the nutrients contained in the produce and microorganisms. A low concentration of chitosan (0.1% w/v) caused nearly total inhibition of *Aspergillus niger* growth in either film-forming solution or film (Sebti, 2005). Antimicrobial films were also prepared by dissolving chitosan into hydrochloric, formic, acetic, lactic and

citric acid solutions (Bégin, 1999).

Others have also considered the importance of the permeation of potassium sorbate in bio-polymer films. Low but not too low potassium sorbate permeability is important to maintain microbial stability (Cooksey, 2001). Sorbic acid and its more water soluble salts are widely used as preservatives in various food products, such as cheeses and beverages. They inhibit or delay the growth of numerous microorganisms, including yeasts, molds,and selective bacteria with effective concentrations in the range of 0.05–0.30 g/100 ml potassium sorbate constitutes the most soluble form of sorbate. Good solubility, stability, and ease of manufacture make potassium sorbate the most widely used form in food systems. (Choi et al., 2005).

Imazalil is an anti-fungal agent and used for reducing surface-spoilage in cheese (Cooksey, 2001).

Triclosan is an inorganic antimicrobial has been considered for use in packaging. A coating made of a styrene-acrylate copolymer containing triclosan inhibited growth of *Enterococcus faecalis* (Chung, 2003).

There are also some examples of ionic and covalent immobilization of antimicrobials onto polymers or other materials have been published. Those films are aimed to control or even prevent the growth of undesired bacterial species responsible for the packed foodstuff degradation. This type of immobilization requires the presence of functional groups on both the antimicrobial and the polymer. Antimicrobials with functional groups can be peptides, enzymes, polyamines and organic acids (Appendini and Hotchkiss, 2002; Conte, 2007).

Lysozyme, a single peptide protein, is a promising antimicrobial compound

which possesses enzymatic activity against beta 1–4 glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine found in peptidoglycan of the bacteria. Lysozyme was immobilized onto polyvinyl alcohol (PVOH) matrix. Lysozyme acts directly from the film without being released into the packed foodstuff. The antimicrobial activity of the films were found to increase as the amount of enzyme incorporated increases (Conte, 2007).Also antimicrobial properties of chitosan films were increased by incorporating lysozyme into chitosan in which lysozyme is released from the film matrix (Park, 2004).

Nisin is a natural antimicrobial peptide produced by *Lactococcus lactis*. While it is effective against Gram (+) bacteria, it does not inhibit Gram (-) bacteria. Nisin was incorporated into heat pressed corn zein films and found to have a bacteriocidal effect when used on agar plates inoculated with *Listeria monocytogenes*. EDTA, used in conjunction with nisin, increased the antimicrobial effectiveness of nisin against Gr (-) bacteria (Cooksey, 2001).

Coating or adsorbing antimicrobials to polymer surfaces can also be used to produce antimicrobial films.

Fungicides were incorporated into waxes to coat fruits and vegetables and shrink films coated with quaternary ammonium salts to wrap potatoes were in early developments in antimicrobial packaging (Shetty and Dwelle, 1990). Other early developments included coating wax paper and cellulose casings with sorbic acid for wrapping sausages and cheeses (Labuza and Breene, 1989). Cast edible films have been used as carriers for antimicrobials and applied as coatings onto packaging materials and/or foods. Examples include nisin/methylcellulose coatings for polyethylene films (Cooksey, 2000; Appendini and Hotchkiss, 2002).

Proteins have an increased capacity for adsorption for example nisin adsorbed onto silanized silica surfaces inhibited the growth of *L. monocytogenes* (Bower, McGuire and Daeschel, 1995).

1.3.1 Preparation of Antimicrobial Surfaces by Photocatalytic Semiconductor Films

Antimicrobial surfaces can be produced by coating those surfaces like glass or ceramics with semiconductors and antimicrobial property can be gained by photocatalysis on surfaces coated by semiconductors.

The photocatalytic process which is based on interaction between light and semiconductor particles, does oxidation/degradation of organic contaminants in environmental control. In this process a semiconductor activated by ultraviolet (UV) radiation is used as a catalyst to destroy organic contaminants.

The photocatalytic process has several advantages when compared to biological and traditional chemical oxidation processes. First, the photocatalytic reaction is not specific so that it destroys a wide spectrum of organic chemicals. Also, the process is very powerful, often achieving a complete mineralization of organics. Another advantage is that the process is immune to organic toxicity. This advantage makes the photocatalytic process particularly attractive for the degradation of recalcitrant and toxic xenobiotic compounds. Also, the process can be applied equally well to liquids like wastewater and contaminated groundwater, and gaseous streams like VOC - volatile organic compounds- emission. Finally, there is a potential to utilize sunlight instead of an artificial light as an UV source, thereby reducing the energy cost for the process (Chang et al., 2000).

Large band gap semiconductors, such as TiO_2, SnO_2, and ZnO, are

suitable photocatalytic materials with their higher wavelength UV absorption (UV-A, 320–400 nm). Semiconductors' electronic structure is characterized by a filled valance band and an empty conduction band and due to this structure semiconductors with their reasonable band gap energies find great attraction through this purpose. The energy difference between the lowest energy level of the conduction band and the highest energy level of the valance band is the so-called band gap energy Eg. It corresponds to the minimum energy of light required to make the material electrically conductive (Erkan, 2006; Benedix et al., 2000).

The most widely used catalyst is TiO_2, because of its very important photoactivity, its lack of toxicity, and its high stability. TiO_2 mediated photocatalysis has been widely investigated for a large number of organic contaminants (Benabbou, 2007) .

The photocatalytic reaction mechanism is initiated by the absorption of UV light which, results in the promotion of an electron from the valence band to the conduction band. When the electron is transferred to the empty conduction band, electron/hole (e^-/h^+) pairs are formed within the semiconductor materials. The electron/hole pairs move to the surface as a result of charge separation where, electrons reduce oxygen to superoxide radicals ($\bullet O2^-$) and further reaction with H_2O molecules yield OH^- ions. Both OH^- ions and H_2O molecules are converted to •OH radicals by reacting with the holes. Both hydroxyl and oxygen radicals are capable of performing many selective/nonselective oxidation reactions. Cell membrane and the wall damage, physicochemical alteration of the cell membrane, promotion of cell membrane permeability to Ca^{2+}, reduction of intracellular superoxide dismutase activity (a protective enzyme from oxidative stress) and abnormal cell division can be used to explain photocatalytic microbial inactivation reported in different studies. (Erkan, 2006).

In first repoted photocatalytic microbial inactivation study, *Lactobacillus acidophilus, Saccharomyces cerevisiae* and *Escherechia coli* were completely inactivated with the use of Pt-loaded TiO_2 powder and the cell inactivation mechanism was explained with the oxidation of coenzyme A and the inhibition of respiration (Matsunaga et al., 1985).

Transmission electron microscopy studies carried out by Saito et al. (1992) showed that the cell walls of *Streptococcus sobrinus* appear broken after photocatalysis. In addition, they were able to demonstrate that illuminated cells release potassium, protein and ribonucleic acid into the medium during the reaction. The increase in the concentration of these indicates changes in the permeability of the cell envelope go parallel to inactivation, and occur as soon as the reaction commences.

It was found that TiO_2 photocatalysis promotes the peroxidation of the *E. coli* membrane phospholipids and induces major disorders in the cell membrane (Maness et al., 1999).

In another study to understand the killing mechanism(s) underlying the TiO_2 photocatalytic reaction cellular damage sites and their contribution to cell death were investigated. A sensitive approach using o-nitrophenol β-D–galactopyranosideside (ONPG) as the probe and *E. coli* as model cells has been developed. Treatment of *E. coli* with TiO_2 and near-UV light resulted in an immediate increase in permeability to small molecules such as ONPG, and the leakage of large molecules such as β-D–galactosidase after 20 min. Kinetic data showed that cell wall damage took place in less than 20 min, followed by a progressive damage of cytoplasmic membrane and intracellular components. The results from the ONPG assay correlated well with the loss of cell viability. Cell wall damage followed by cytoplasmic membrane damage leading

to a direct intracellular attack has therefore been proposed as the sequence of events when microorganisms undergo TiO_2 photocatalytic attack. It has been found that smaller TiO_2 particles cause quicker intracellular damage (Huang et al., 2000).

Also the change in survival of intact *E. coli* cells on illuminated glass plate coated by TiO_2 film demonstrated a two-step decay dynamics. The first step of the photokilling process is the disordering of the outer membrane of *E. coli* cells on illuminated TiO_2 film. This process was necessary for the inner membrane penetration of reactive species produced by photocalysis. The second step of the process was the disordering of the inner membrane (the cytoplasmic membrane), resulting in cell death (Sunada et al., 2003).

Slices of UVA-transparent Plexiglas were coated with TiO_2 and *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecium* and *Candida albicans* were illuminated on the sample specimen in different concentrations for 1 h. Light and scanning electron microscopic examinations suggested that the germ destruction achieved takes place through direct damage to cell walls caused by OH-radicals (Kühn et al., 2003).

When illuminated by near-UV light, titanium dioxide (TiO_2) exhibits excellent bactericidal activity which was investigated by atomic force microscopy (AFM) in conjugation with some other techniques and the results showed that the cell death was caused by the decomposition of the cell wall and the cell membrane and the resultant leakage of intracellular molecules (Lu, 2003).

In conventional photocatalytic processes, powder TiO_2 was used as the catalyst for organic destruction. These fine particulates were added to the waste stream that was placed under an UV irradiation. The powder catalysts, however, suffer two serious drawbacks which limit its practical applications. First, the

settling velocity of powder TiO_2 is very slow and as the dosage of TiO_2 is increased in order to increase the photocatalytic rate, the high turbidity created by the high TiO_2 concentration which can actually decrease the depth of UV penetration. In order to avoid the drawbacks of powder, techniques were developed to immobilize TiO_2 on a solid carrier such as glass and ceramic tiles (Chang et al., 2000; Mills et al., 2003).

TiO_2 has been used in advanced oxidation applications for the destruction of organic compounds in water (Hand et al., 1995), and as a synergistic disinfectant for microorganisms (Ryu, 2007).

An evaluation of a photocatalytic process in the presence of titanium dioxide for water purification to destroy hazardous organic contaminants was done. By the solar detoxification tests, blank experiments (light with no titanium dioxide) have confirmed that loss of the contaminants was only due to the photocatalytic process. Although in the same study it was found that natural UV component of sunlight was able to destroy micro-organisms at a rate similar to that of illuminated TiO_2 particles, photocatalytic oxidation is considered to be a preferred method than UV near disinfection, because OH radicals are able to destroy biological contaminants, with the oxidation of proteins, lipids or nucleic acids, thus resulting in inhibition of respiration or growth of the microorganisms (Vidal et al.,1999).

The inactivation of coliform bacteria and poliovirus 1 was studied in secondary waste water effluent containing suspensions of titanium dioxide irradiated with either F40BL fluorescent lights or sunlight and it was found that the poliovirus 1 was effectively inactivated by titanium dioxide photocatalysis, and the rates were more rapid than for the inactivation of coliform bacteria but it was also pointed out that the photocatalytic disinfection of effluents using titanium

dioxide under sunlight may be limited due to the relatively low inactivation rates and resulting long contact times compared to conventional disinfection methods (Watts et al., 1995).

When a glass plate was coated by a layer of TiO_2 in a flow-through reactor allowing either artificial light or sunlight as a source of radiation it was found that UV irradiation of *E. coli* in the photoreactor for 6 h decreased the number of viable bacteria to one-half of its original value because the number of bacteria in the blank, an experiment without UV radiation, remained constant with the experimental error (Belhacova, 1999).

Photo induced bactericidal capacity of TiO_2 based films was evaluated, using as model organism *Pseudomonas aeruginosa.* Thin films were obtained by spray pyrolysis; they included undoped, Cu doped, and Al doped TiO_2. Scanning electron microscopy was used to observe the final effect of the irradiated films upon the bacteria. Depending on the composition and characteristics of the films, quantitative experiments show that bacterial inhibition varies between 28 and 96% (Amézega, 2002).

Titanium dioxide covered with apatite, was developed for as an antimicrobial, antifungal, and antifouling coating where apatite was used to adsorb contaminants and titanium dioxide was used as a photocatalys by decomposing the material adsorbed by the apatite on exposure to light. It was suggested that prototype coatings produced by using the apatite-coated titanium dioxide particles could be applied not only to concrete structures and ceramics/earthenware, just like ordinary coats/paints, but also to metal, wood, textile and paper (Nonami, 2004).

The photocatalytic antimicrobial activity over TiO_2, SnO_2 and their Pd doped thin film samples were determined against *Escherichia coli*, *Staphylococcus*

aereus, *Saccharomyces cerevisiae* and *Aspergilus niger* spores. Higher antimicrobial activity was observed with TiO_2 than SnO_2 thin films and, Pd addition was found to contribute to an increase in the activity of both semiconductor oxides. The highest microbial inactivation was achieved with 1% PdO/TiO2 against *E. coli* with a 98% decrease in survival after 2 h illumination (Erkan, 2006).

The antimicrobial activity of nanoparticles consisting of a mixture of silver nitrate and titanium dioxide was also searched and 100% reduction in viable *E. coli* and *S. aureus* was observed in the coated mask materials after 48 h of incubation (Li, 2006).

1.4 Aim of the Study

Food packaging is an important field in food industry and studies related to food packaging is mainly concerned with containment, protection, quality and safety of food. Biodegradable film production has gained attention in the last years produced mainly from lipids, proteins and polysaccharides.

Agricultural wastes cause significant environmental impacts if they are treated in an uncontrolled manner. Cotton stalk is an important agricultural waste in Turkey containing cellulose, hemicellulose and lignin. Unlike cellulose and lignin, hemicellulose does not have many areas to be used in, although it is the most widely distributed component in the nature after cellulose. However, in a recent study, xylan, the hemicellulose part of cotton stalk was extracted with lignin and used in producing biodegradable film production (Göksu, 2005).

Antimicrobial property is another important issue in food packaging because microbial growth on the food surfaces is a major cause of food spoilage and food-borne illness (Kandasamy, 2007). So far, chitosan, nisin, potassium

sorbate, imazalil, triclosan have been used as antimicrobial agents in food packages. There are also some examples of ionic and covalent immobilization of antimicrobials onto polymers or other materials have been published. Those films are aimed to control or even prevent the growth of undesired bacterial species responsible for the packed foodstuff degradation.

There has been studies on development of photocatalytic antimicrobial surfaces like glass or ceramics by coating those surfaces with semiconductors and antimicrobial property has been gained by photocatalysis on those surfaces coated by semiconductors. However, there has not been a study on producing antimicrobial biodegradable packaging films coated with semiconductors.

This thesis is composed of two parts. In the first part, pure xylan was extracted from cotton stalk and separated from lignin. Then, lignin was used as an additive to birchwood xylan films, which normally do not form continuous films, to find the minimum amount of lignin to form continuous and self supporting films. Afterwards, characterization of these xylan films were performed.

The aim of the second part of the study is to produce photocatalytic antimicrobial gluten films by addition and/or coating the films with TiO_2.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Cotton stalk was brought from a local producer in Urfa, Turkey. Wheat gluten was obtained from Kröner Starke (Germany).

Birchwood xylan and titanium tetraisopropoxide (TTIP) were purchased from Sigma (Germany). Titanium dioxide was bought from Aldrich (Germany). Acetic acid (CH_3COOH), ethanol (C_2H_5OH), 35% hydrochloric acid (HCl_{aq}), nitric acid (HNO_3), isopropyl alcohol (C_3H_7OH), potassium hydroxide (KOH), sodium hydroxide (NaOH), sodium borohydride ($NaBH_4$), sodium bromide (NaBr), potassium bromide (KBr) and sulphric acid (H_2SO_4) were purchased from Merck (Germany). Shearzyme was given by Novo Nordisk (Denmark). All the other chemicals used were analytical grade and bought either from Sigma or Merck.

2.2 Microorganisms and Growth Conditions

Escherichia coli was maintained on LB agar (Appendix A). Plates were incubated at 35°C overnight and then stored at 4°C until use. *E.coli* (XL1-blue) was cultured in Luria-Bertani (LB) broth (Appendix A) at 35°C on a rotary shaker (Minitron) at 175 rpm overnight.

2.3 Xylan and Lignin Extraction

The method used by Zilliox and Debeire (1998) was followed for the xylan and lignin extraction. 100 ml of distilled water was added to 2 g of milled cotton stalk and the sample was swelled at 60°C for 16 h in an incubator (Nüve, Turkey). The swollen sample filtered by a filter paper (Whatman 41) and the pellet was mixed with solution containing 17 ml of 24 %w/v KOH + 1 %w/v $NaBH_4$ solution and stirred for 3 hours at room temperature. Afterwards, the suspension was filtered and the supernatant was mixed with 5 ml cold ethanol solution containing 10% acetic acid. This suspension was shaken for several minutes and a centrifugation step was performed at 8300xg for 20 minutes to recover xylan. The pellet was dried at 60°C for 24 hours and used as xylan source containing lignin. In order to remove the lignin completely, the volume of the washing solution was increased to 110 ml (cold ethanol solution containing 10% acetic acid). Afterwards, lignin containing washing solution was separated from the remaining pellet which is the cotton stalk xylan containing no lignin, by filtration. This solution was concentrated in a vacuum evaporator (BÜCHI Rotavapör R-200) 10 times and used as lignin source.

2.4 Film Production

2.4.1 Preparation of Birchwood Xylan Films with Addition of Lignin

Film forming solutions were prepared by dissolving 10 g of birchwood xylan in distilled water (10% w/w) and also by dissolving 10 g birchwood xylan with the addition of various quantities of concentrated extra washing solution of cotton stalk xylan (in the range of 28%-52% w/w) in pure water. After 8 h of stirring, the beakers were placed into a water bath at 70°C for 10 min to prevent bubble formation in the films upon casting. Then, 20 g of the solution was drop casted into petri disks with diameter of 9.0 cm. The films were dried

in a controlled environment at a temperature of 20±2°C and a relative humidity of 40±4% into a condition that they could be peeled from the petri plates.

2.4.2 Wheat Gluten Films

Film forming solutions were prepared by dissolving 10 g of wheat gluten in distilled water (10% w/w) with the addition of different quantities of glycerol (in the range of 2% - 20%, w/w). Their pH was adjusted to 11 either by NaOH or KOH. After 30 min of stirring, the beakers were placed into a water bath at 70°C for 10 min to prevent bubble formation in the films upon casting. Then, 20 g of the solution was drop casted into petri disks with diameter of 9.0 cm. The films were dried in a controlled environment at a temperature of 20±2°C and a relative humidity of 40±4% into a condition that they could be peeled from the petri plates.

2.5 Determination of Lignin Content

A filtering crucible was dried in an oven for 2 h at 105 °C. Then it was cooled in a dessicator for 1 h and weighed. Until reaching the constant value of the filtering crucible, the drying procedure was continued considering three digits after the point in the balance.

A 1-g lignin containing film sample was placed in a small beaker with a glass cover and 15 ml of cold (12 to 15 °C) H_2SO_4 (%72) was added slowly while stirring. For at least 1 min, the sample was mixed well with the acid by continuous stirring. At a temperature of 18 to 20 °C, the frequent stirring was applied for approximately 2 h. Then the material was washed into a 500 ml erlenmeyer flask and diluted to a 3% concentration of H_2SO_4 by adding 360 ml of distilled water. As the next step, the material was boiled for 4 h under a

reflux condenser. After allowing the insoluble material to settle, the material was filtered into the tared filtering crucible. After that, the residue was washed with 500 ml of hot water in order to get rid of the acid, and the crucible and contents were dried in an oven for 2 h at 105 ° C. Then the crucible and its content were transferred into a dessicator and weighed for 1 h. Lastly, the corresponding weight was recorded. Until the related weight was constant, the drying procedure was repeated taking into account three digits after the point.

2.6 Antimicrobial Film Preparation

2.6.1 Production of Titanium Dioxide As an Antimicrobial Additive

Acetic acid (0.5 ml) was added to 100 ml water and 2.5 ml titanium tetraisopropoxide (TTIP) was added slowly to this solution. Then, 0.350 ml nitric acid (HNO_3) was added and the material was boiled for half an hour under a reflux condenser at 80°C. As the next step, this mixture was stirred at room conditions on a magnetic stirrer for 24 h. Next day, solution was conditioned in room conditions for 24 h. As the final step, solution was put in 100 °C until it dried and TiO_2 obtained as in powder form. This powder was pestled well and mixed with 2 ml of pure water and placed in sonic water bath. Then, it was used (either 0.8% or 1.6% (w/w)) as an additive in wheat gluten films, containing 2 g glycerol.

Commercial TiO_2 powder (Aldrich) was directly mixed with 4 ml of pure water and placed in sonic water bath. Then, TiO_2, 3.2% (w/w), was added to wheat gluten films, containing 2 g glycerol.

2.6.2 Addition of TiO_2 into Film Forming Solution

Film forming solutions were prepared by dissolving 10 g of wheat

gluten in distilled water (10% w/w) with the addition of 2% glycerol and 0.8% or 1.6%, w/w titanium dioxide if produced from a precursor, TTIP, or 3.2% (w/w) if commercial titanium dioxide was used. Films' pH was adjusted to 11 by NaOH. After 30 min of stirring, the beakers were placed into a water bath at 70°C for 10 min to prevent bubble formation in the films upon casting. Then, 20 g of the solution was drop casted into petri disks with diameter of 9.0 cm. The films were dried in a controlled environment at a temperature of 20±2°C and a relative humidity of 40±4% into a condition that they could be peeled from the petri plates.

2.6.3 Film Coating by Sol-gel Method

Wheat gluten films of pH 11 adjusted by NaOH were coated with TiO_2 by using five different sol-gel methods.

2.6.3.1 First Procedure, Ethanol-Nitric Acid Method

90 ml ethanol mixed with 3 ml titanium tetraisopropoxide (TTIP) that was added slowly to avoid precipitation and pH was adjusted to 2 by using nitric acid (HNO_3). Afterwards, films were dipped in this solution for 30 sec and padded for 20 sec. Then, coated film samples were dried at 60°C for 20 min.

2.6.3.2 Second Procedure, Aqueous-Nitric Acid Method

0.5 ml acetic acid was added to 100 ml water. 2.5 ml titanium tetraisopropoxide (TTIP) was added slowly to this solution. 0.350 ml nitric acid (HNO_3) was also added and the material was boiled for half an hour under a reflux condenser at 80°C. As the next step, this mixture was stirred at room conditions in magnetic stirrer for 24 h. Next day, film samples were dipped in this coating solution for 30 min and dried at room conditions for 24 h. As the

final step, coated film samples were dried at 60°C for an hour.

2.6.3.3 Third Procedure, Aqueous-Hydrochloric Acid Method

0.5 ml acetic acid was added to 100 ml water. Titanium tetraisopropoxide (TTIP) of 2.5 ml was added slowly to this solution. 0.7 ml of 35% hydrochloric acid, (HCl_{aq}), was also added and the material was boiled for half an hour under a reflux condenser at 80°C. As the next step, this mixture was stirred at room conditions on a magnetic stirrer for 24 h. Next day, film samples were dipped in this coating solution for 30 min and dried at room conditions for 24 h. As the final step, coated film samples were dried at 60°C for an hour.

2.6.3.4 Fourth Procedure, Isopropyl Alcohol-Hydrochloric Acid Method

100 ml isopropyl alcohol (C_3H_7OH), 5 ml acetic acid, 0.5 ml 35% hydrochloric acid, (HCl_{aq}), and 2.5 ml TTIP were mixed for half an hour as the beaker was tightly closed. 1.8 ml pure water was added slowly (40 μl in every 1 min). Then, it was put in reflux condenser at 80°C for half an hour. As the next step, this mixture was stirred at room conditions on a magnetic stirrer for 24 h. Next day, film samples were dipped in this coating solution for 30 min and dried at room conditions for 24 h. As the final step, coated film samples were dried at 60°C for an hour.

2.6.3.5 Fifth Procedure, Aqueous-Nitric Acid Spreading Method

This procedure was carried out by using the same coating solution as in second procedure which was prepared by adding 0.5 ml acetic acid to 100 ml water. 2.5 ml titanium tetraisopropoxide was added slowly to this solution and 0.350 ml nitric acid (HNO_3) was also added and the material was boiled for half an hour under a reflux condenser at 80°C. After stirring this mixture in

magnetic stirrer for 24 h, 3 ml of this solution was added and spread on surface of semi-dried wheat gluten films. The pH of films was adjusted either by KOH or NaOH to 11. Drying of the wheat gluten films were continued after applying the coating solution.

2.7 Antimicrobial Test

Escherechia coli was cultured in Luria-Bertani (LB) broth (Appendix A) at 35°C on a rotary shaker (Minitron) (175 rpm) overnight.

An overnight *E. coli* culture of 1.5 ml/film sample was put in eppendorf tube and a centrifugation step was performed at 10,000 rpm for 4 min. Supernatant was separated and the pellet was mixed with 1 ml of peptone water which was then put on ¼ of film samples. Both sides of the film samples were contaminated with *E. coli* in petri dishes with diameter of 4.0 cm. Both sides of the film samples were illuminated from above using a glass filter with artificial solar light simulator (Osram, ultra-vitalux, 300W) for 1 hour. Then, a serial dilution in peptone water (0.1%) was performed. Therefore, film samples after illumination were well mixed with 9 ml of peptone water from which 1 ml of suspension was taken and mixed with 9 ml of peptone water. After vortex mixing, this suspension was diluted with peptone water for counting. 200μl of suitably diluted (10^6 dilution) microbial suspension was inoculated on Luria-Bertani (LB) agar plates (Appendix A) and incubated at 35°C overnight. Next day, number of *E. coli* cells survived were counted.

2.8 Scanning Electron Microscopy (SEM) Analysis

Differences in surface and cross sectional areas of film morphologies were investigated using a scanning electron microscope (SEM). Both uncoated and coated wheat gluten films, which were coated with different procedures, and

TiO_2 added films were investigated. Before the examination, the samples were coated with gold in order to achieve sufficient conductivity for analysis.

2.9 Biodegradation Test

Two test species of lignin-birchwood xylan composite film having different amounts of lignin were put into petri disks. Then, 5 ml of 0.21 U/ml Shearzyme, (a commercial xylanase), in 0.05 M citrate buffer (pH 4.6) was added and kept at 40°C for 5 days to check the film biodegradation by xylanase. During that time, 2 ml of 0.21 U/ml enzyme solution was added in every two days considering the possible enzyme deactivation. Also samples of each composite film were put in 5 ml pure water as control.

3.0 Color Test

Color of the birchwood xylan-lignin composite films containing different amounts of lignin was measured using a Minolta color reader (CR-10, Japan) using the CIE L*, a*, and b* color scale to determine the effect of lignin on film color. Readings were carried out at room temperature on four different locations of each sample, and the mean value was recorded. The L* value represents 'lightness', from zero (black) to 100 (white). The a* value represents, 'redness' or 'greenness' ranging from +60 to –60 and b* value represents 'yellowness' or 'blueness' ranging from +60 to –60. Total color change (ΔE) was calculated from the following equation in which white color was used as the reference point, which was denoted by L_0, a_0 and b_0.

$$\Delta E = [(L^*-L_0)^2 + (a^*-a_0)^2 + (b^*-b_0)^2]^{1/2} \quad (2.1)$$

CHAPTER 3

RESULTS AND DISCUSSION

In the first part of the study, minimum quantity of lignin in xylan (w/w) was determined for continuous self-supporting film formation. Normally pure xylan does not form films, for this purpose, lignin was completely removed from cotton stalk xylan, then predetermined lignin quantities were added and its effect on continuous film formation was investigated. Films were casted and minimum lignin concentration was investigated.

In the second part of the study, antimicrobial wheat gluten films were prepared either by coating the films with titanium dioxide (TiO_2) or adding TiO_2 into film forming solutions. Then, photocatalytic microbial inactivation characteristics were investigated.

3.1 Film Formation from Xylan

Birchwood is a hardwood and up to 30% of the mass of it consists of hemicellulose (Gabrielli and Gatenholm, 1998). Non-film forming property of pure commercial birchwood xylan when added to water, was observed in previous studies as well in this study as shown in Figure 3.1 (Gabrielli and Gatenholm, 1998; Kayserilioglu, 2003a; Göksu, 2005). There are various studies in literature for production of continuous films from xylan such as addition of chitosan and gluten. Addition of as little as 5% chitosan resulted formation of flakes and at 10% and above chitosan, continuous, self-

supporting composite films of birchwood xylan-chitosan were achieved (Gabrielli and Gatenholm, 1998). Xylan was added to wheat gluten films up to 40% (w/w) without worsening its mechanical properties (Kayserilioğlu, 2003a).

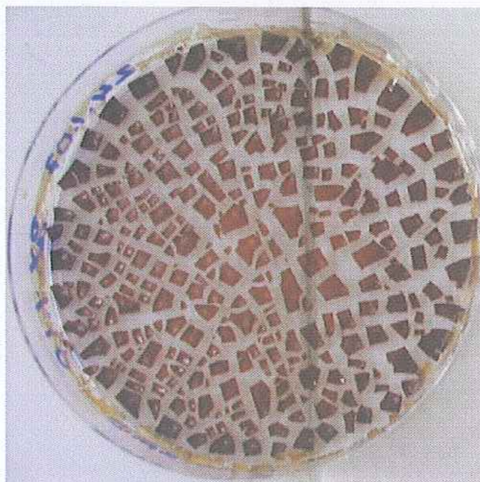
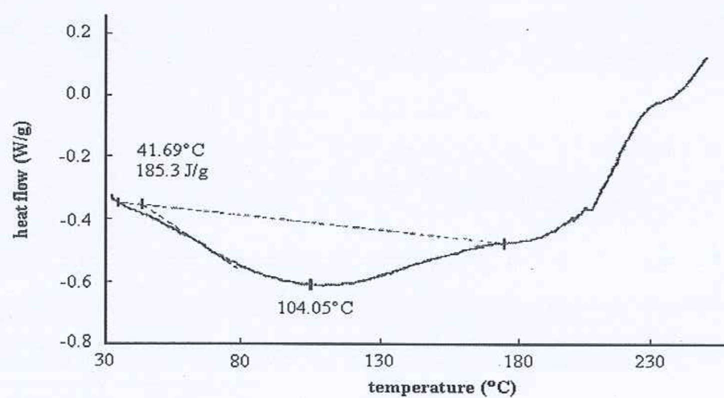


Figure 3.1 A cracked structure obtained using 10% pure birchwood xylan.

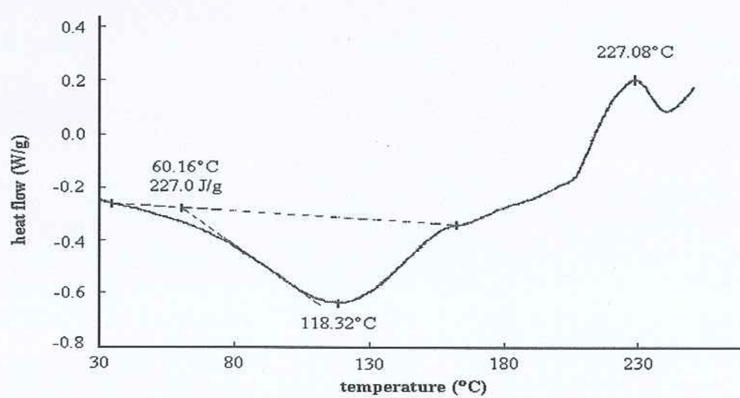
In a previous study undertaken in our laboratory, lignin was determined to be a film forming agent for xylan based films, in which extra washed pure cotton stalk xylan (EWX) and commercial birchwood xylan did not form films however when extra washing solution was added to commercial birchwood xylan and EWX, films were produced from both of them. The presence of lignin was shown by DSC.

Similarities between EWX and commercial birchwood xylan were observed in DSC diagrams verifying both complete removal of the film forming impurity, and structural similarity between birchwood and cotton stalk xylans.

When DSC diagram of xylan exracted only by 5 ml cold ethanol solution with 10% acetic acid (EX) was investigated, its diagram was found to be more different than EWX and commercial birchwood xylan proving that there was a different compound in EX as shown in Figure 3.2. According to this, unknown compound had relatively low molecular weight and two characteristic melting peaks. Other than lignin, this phenolic compound may be tannin but there was dissimilarity between DSC of tannic acid and the unknown compound. So that, the unknown compound was characterized as lignin (Göksu et al., 2007).



A



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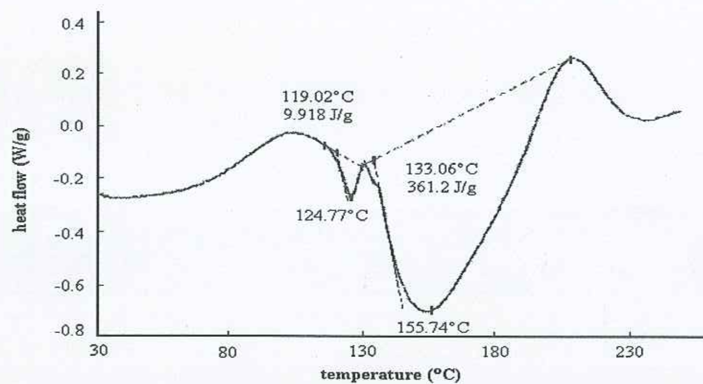


C

Figure 3.2 DSC diagrams of (A) pure cotton stalk xylan (EWX) (B) birchwood xylan (C) lignin containing cotton stalk (EX) (Göksu et al., 2007).

Xylan was extracted from cotton stalk by alkali method because generally hemicelluloses are typically defined as the material that can be extracted from the cell wall by alkali extraction (Thompson, 2000). When the swollen cotton stalk was stirred with KOH, cellulose in the form of pellet was separated from hemicellulose containing lignin suspension. $NaBH_4$, a reducing agent, was added to the alkaline solution to prevent glycosidic bond depolymerization.

Extracted cotton stalk xylan (EX) containing lignin was produced by washing the suspension only with 5 ml cold ethanol solution containing 10% acetic acid to keep some of the lignin in the xylan fraction and a centrifugation step was done to have the pellet as cotton stalk xylan source containing lignin after drying it at 60°C for a day.

At first, xylan without removal of lignin, produced by performing no washing step with ethanol during extraction, was casted to see if a continuous film was formed, however, film formation could not be formed as seen in Figure 3.3. The surface was wrinkled and it was hard to peel from the petri as it was brittle and was not self-supporting. The reason for this result could be insufficient quantity of xylan. Another reason might be high pH of the solution. So, its pH was adjusted to 7 by HCl addition. Afterwards, film formation was observed. However this time, salt formation was observed in the films as shown in Figure 3.4. After adjusting pH to 7, salt ions can be removed by ultrafiltration, and then remaining solution can be casted for the film formation.

Figure 3.3 Photograph of film casted from cotton stalk xylan-lignin solution at pH 11 without removal of lignin and adjusment of pH.

Figure 3.4 Film casted from xylan-lignin solution at pH 7 without removal of lignin, but with pH adjusment.

When cotton stalk xylan was extra washed with lignin removal solution, resulting supernatant was separated by filtration from pellet, pure xylan, and used as lignin source after evaporation of ethanol.

3.1.1 Determination of Minimum Lignin/Xylan Ratio for Film Formation

In order to find the minimum lignin concentration to form continuous films, at first, ethanol was evaporated from the lignin removal solution of cotton stalk xylan. Then, 10 times concentrated. The resulting lignin containing solution was added to 10% birchwood xylan solutions in different amounts. When (lignin solution/xylan solution) ratio is between at 28%-36%, a continuous film did not form however, sizes of films patches increased as the lignin concentration was increased and continuous film formation was observed in film forming solutions containing 38% lignin solution as shown in Figure 3.5.

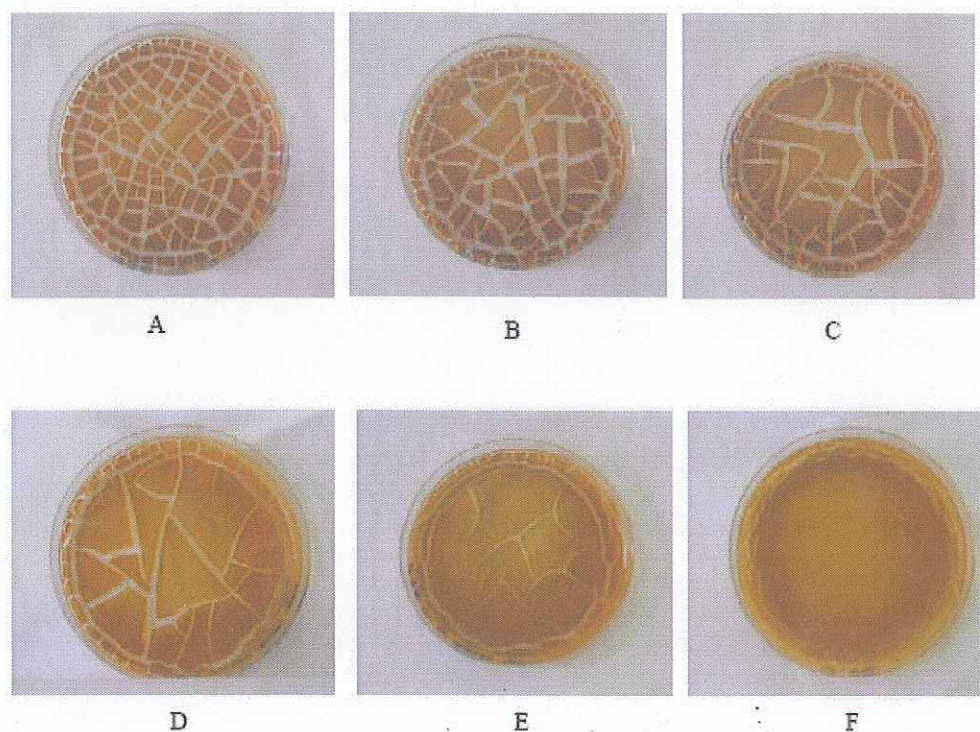
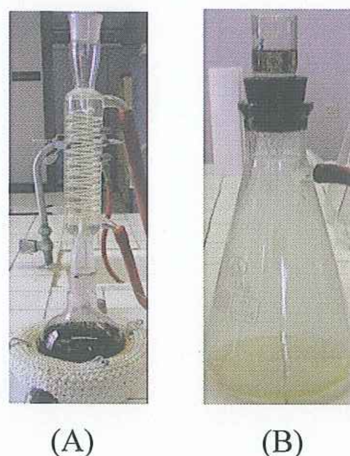


Figure 3.5 10% (w/w) birchwood xylan having increasing amounts of extra washing solution containing lignin (A) 28% (B) 30% (C) 32% (D) 34% (E) 36% (F) 38%.

In order to estimate the exact lignin concentration, acid insolubility of lignin was used. Samples containing lignin were treated with strong acids, where the carbohydrates were hydrolyzed, leaving an insoluble lignin residue. Therefore, 1 g lignin containing film samples were dissolved in H_2SO_4, and boiled in reflux condenser as illustrated in Figure 3.6 (A). The material was filtered into the tared filtering crucible as shown in Figure 3.6 (B). Finally, the crucible and contents were dried in an oven at 105°C until a constant dry weight was achieved.



(A) (B)

Figure 3.6 Lignin determination equipments (A) reflux condenser (B) filtering crucible on the top of filtering equipment.

At the end, lignin concentration was found to be 1.13 g (lignin/xylan) in the films for which 38% (w/w) washing solution was used. All the lignin concentrations for the films containing 28%-38% (w/w) washing solution are given in Table 3.1. Also lignin quantity in 1 g film forming cotton stalk xylan

obtained with 5 ml lignin removal solution was measured experimentally as 1.12% lignin/xylan.

Although 36% (w/w) washing solution containing birchwood xylan had the same amount of lignin, 1.12±0.001 % lignin/xylan, with 1 g cotton stalk xylan, a continuous film formation could not be observed. That might be due to differences in xylan structures. In addition; for cotton stalk xylan, 1.12% (lignin/xylan) ratio was obtained from 1 g cotton stalk xylan. However, in birchwood cases, 1 g film was used. So, the presence of minute quantities of soluble ions in distilled water may affect the result.

To conclude, the necessary quantity of lignin on xylan based film formation was determined as about 1.1% (w/w lignin/xylan) for both cotton stalk and birchwood xylan. So, keeping about one percent lignin in xylan (w/w) was determined sufficient for film formation. The resulting lignin from 1 g film sample before and after filtering is shown in Figure 3.7.

(A)

(B)

Figure 3.7 Lignin from 1 g film sample (A) before filtering (B) after filtering and drying.

As in control, only evaporated ethanol containing 10% acetic acid was added to 10% (w/w) birchwood xylan solution. As observed in Figure 3.8, film formation was not observed showing that lignin is the necessary additive for continuous film formation.

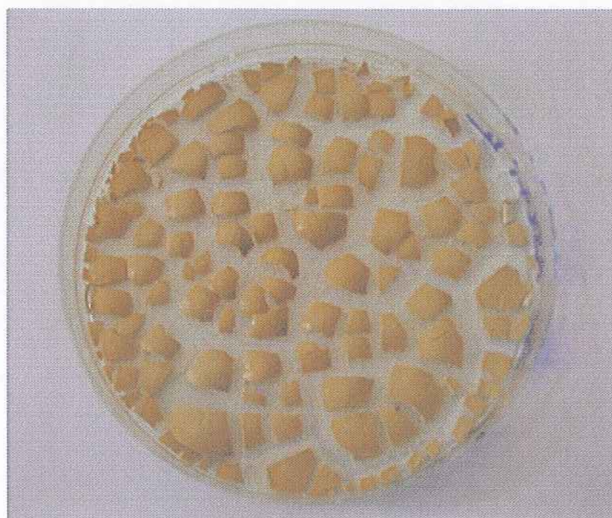


Figure 3.8 10% (w/w) birchwood xylan containing evaporated ethanol and acetic acid.

Table 3.1 Lignin concentration and lignin/xylan ratio in birchwood xylan films containing different amounts of extra washing solution containing lignin.

Extra washing solution amount in films, w/w	*Lignin, w/w, %*	*Lignin/xylan, w/w, %*
28%	0.86±0.005	0.87 ± 0.005
30%	0.93±0.004	0.94 ± 0.004
32%	0.99±0.002	1.00 ± 0.002
34%	1.05±0.000	1.06 ± 0.000
36%	1.11±0.001	1.12 ± 0.001
38%	1.12±0.003	1.13 ± 0.003

3.1.2 Biodegradation of Birchwood Xylan-Lignin Composite Films

For biodegradability test, firstly birchwood xylan-lignin composite films, containing 1.1-1.6 % lignin/xylan in 10% birchwood xylan were prepared as shown in Figure 3.9.

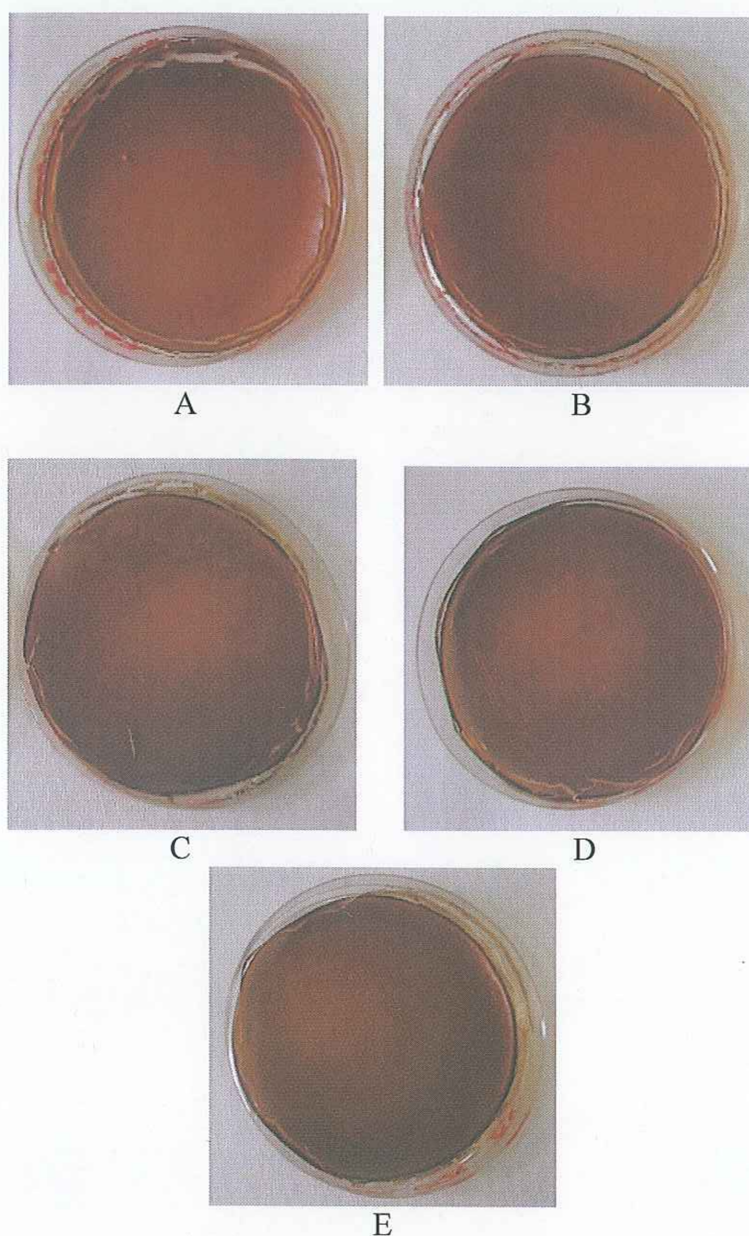
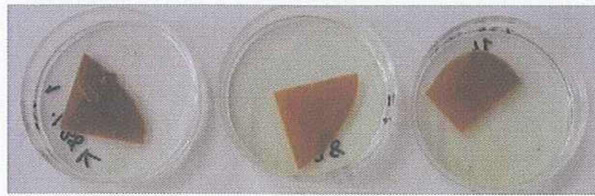


Figure 3.9 Birchwood xylan-lignin composite films, containing (A) 1.13% (B) 1.25% (C) 1.38% (D) 1.50% (E) 1.63% lignin/xylan.

An accelerated biodegradability test was performed enzymatically using 0.21 U/ml xylanase, (Shearzyme) in citrate buffer at pH 4.6. Films before the

degredation is shown in Figure 3.10. The aim of this experimental set is to observe solubilization of the lignin-xylan complex by xylanase treatment. Xylanase is an enzyme which hydrolyzes ß-1-4 glycosidic bonds in xylan structure. If the enzyme can hydrolyze these bonds and the film becomes soluble as a mixture of xylooligosaccharides, films can be accepted as biodegradable. In nature, the microorganisms which degrade xylan contain xylanases. When xylan is hydrolyzed to xylooligosaccharides, these sugars can be consumed by microorganisms.



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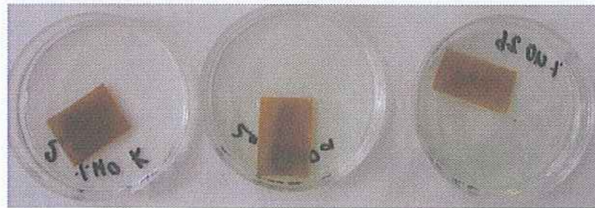

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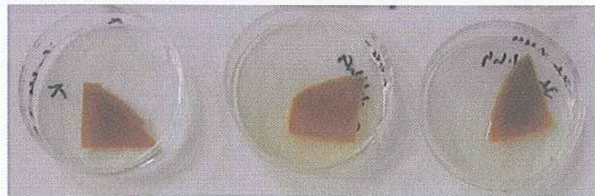

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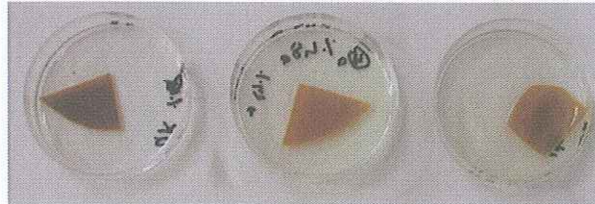

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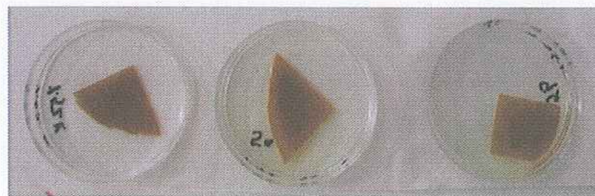
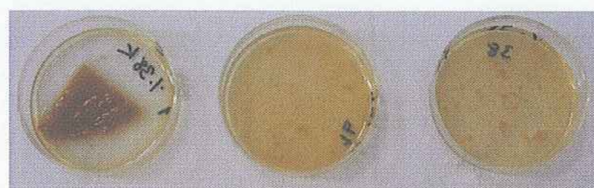


E

Figure 3.10 Before enzymatic biodegradation of films with (A) 1.13% (B) 1.25% (C) 1.38% (D) 1.50% (E) 1.63% lignin/xylan. In every picture, the one on the left shows the control films along with two parallels on the right.

After 24 h, films started to degrade to smaller fragments compared to the control films. The control composite films in the pure water without xylanase did not degrade. During the second day smaller fragments were observed as seen in Figure 3.11.



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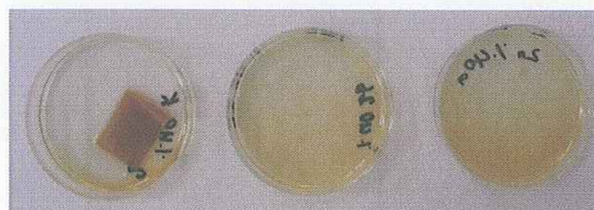

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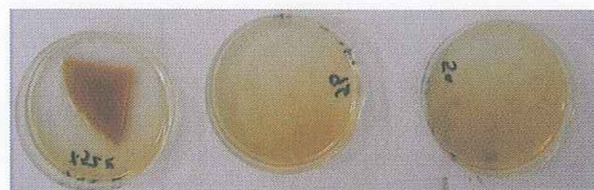


E

Figure 3.11 In the second day of degradation of films with (A) 1.13% (B) 1.25% (C) 1.38% (D) 1.50% (E) 1.63% lignin/xylan. In every picture, the one on the left shows the control films along with two parallels on the right.

In the fifth day, films were almost completely degraded as shown in Figure 3.12. In all the films, xylan composite was hydrolyzed into xylooligosaccharides and became soluble in water. Increasing amount of lignin did not affect the biodegradability significantly.

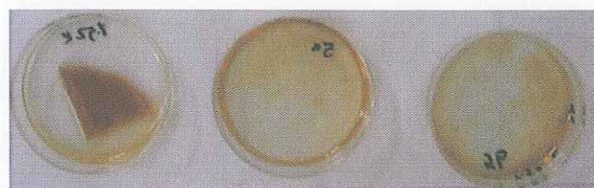
In another study, performed by Hosokawa, (1990) accelerated biodegradability test of chiotosan-cellulose composite films were performed by Meicelase, cellulase, for 1 week. Degradation of the composite film to fine particles was also observed. Increase of chitosan, and temperature prolonged the degradation periods for attack of cellulase as the cellulose was coated with chitosan becoming resistant against cellulase. Also increase in glycerol content in composite films shortened the degradation because high glycerol content made the composite films swollen so that their polymer-chain network was loose making the enzyme easily invade into films.

A

B

C

D



E

Figure 3.12 In the fifth day of degradation of films with (A) 1.13% (B) 1.25% (C) 1.38% (D) 1.50% (E) 1.63% lignin/xylan. In every picture, the one on the left shows the control films along with two parallels on the right.

3.1.3 Color Test of Birchwood Xylan-Lignin Composite Films

Color of birchwood xylan-lignin composite films having 1.1 to 1.6% lignin was measured using a colorimeter (Minolta color reader, CR-10, Japan) taking white paper as reference.

L* values, representing the lightness from zero (black) to one hundred (white) is given in Figure 3.13. Lightness slightly increased as 26.25; 26.26; 26.73 and 27.72 corresponding to films having 1.13%, 1.25%, 1.38% and 1.50% lignin/xylan. However, an insignificant decrease in 1.63% lignin/xylan containing film was observed as the L* value was 26.08. As a result, not a net effect was observed by increasing lignin from 1.1% to 1.6%. The average lightness of birchwood xylan-lignin composite films is lower than the lightness of the cotton stalk xylan film containing 10% xylan (w/w) (Göksu, 2005).

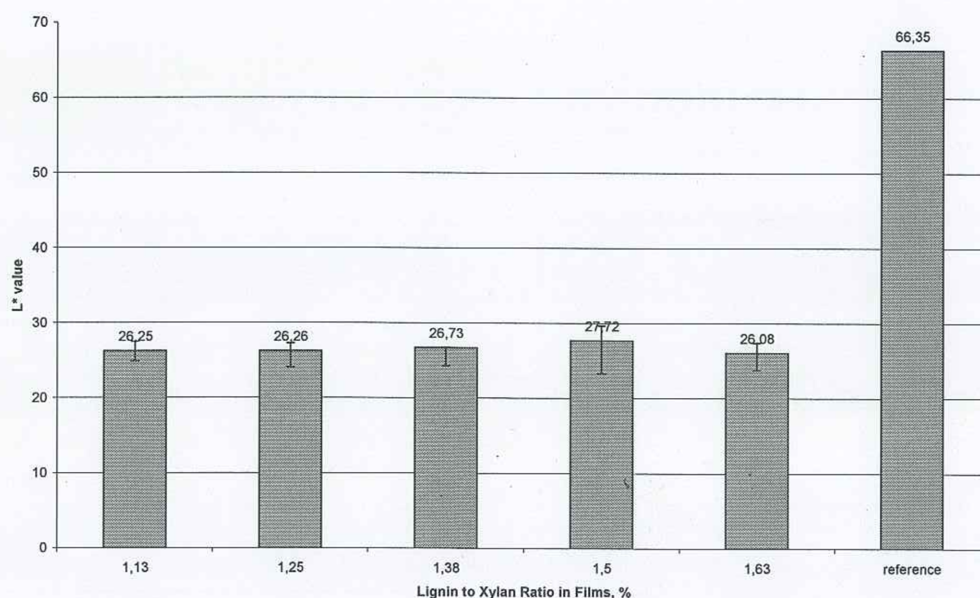


Figure 3.13 Lightness of birchwood xylan-lignin composite films as a function of lignin content.

a* values, representing the redness or greenness ranging from +60 to -60 is given in Figure 3.14. a* values were increased as 12.10; 12.76; 14.34, 15.28 corresponding to films having 1.13%, 1.25%, 1.38%, and 1.50% lignin/xylan. The a* value of the film containing 1.63% lignin/xylan was 13.24 which was slightly lower than the films containing 1.38% and 1.50% lignin/xylan. This result may be due to the lower thickness of the 1.63% lignin/xylan containing film because the thickness of the film containing 1.63% lignin/xylan was 0.383 mm as the films having 1.13%, 1.25%, 1.38% and 1.50% lignin/xylan were slightly thicker than this film. All the thickness values are given in Table 3.2. So that the decrease in a* value might be due to the less layer of xylan. However the effect was slight. The average a* value of birchwood xylan-lignin composite films is lower than the average a* value of the cotton stalk xylan film containing 10% xylan (w/w) (Göksu, 2005).

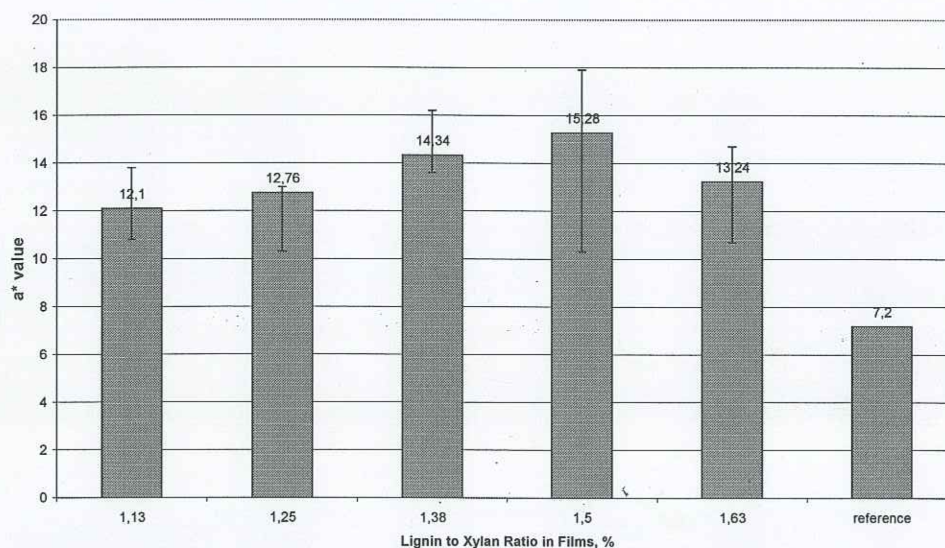


Figure 3.14 a* values of birchwood xylan-lignin composite films as a function of lignin content.

Table 3.2 Thickness of the lignin-xylan films.

Lignin/xylan ratio, %	*Thickness, mm*
1.13	0.507
1.25	0.464
1.38	0.465
1.50	0.436
1.63	0.383

b* values, representing the yellowness or blueness ranging from +60 to -60 is given in Figure 3.15. b* values increased as 25.34; 26.97; 29.75; 31.26 and corresponding to films having 1.13%, 1.25%, 1.38% and 1.50% lignin/xylan. The b* value of the film containing 1.63% lignin/xylan was 28.76 which was slightly lower than the films containing 1.38% and 1.50% lignin/xylan. This again may be due to the lower thickness of the 1.63% lignin/xylan containing film. The average b* value of birchwood xylan-lignin composite films is lower than the average b* value of the cotton stalk xylan film containing 10% xylan (w/w) (Göksu, 2005).

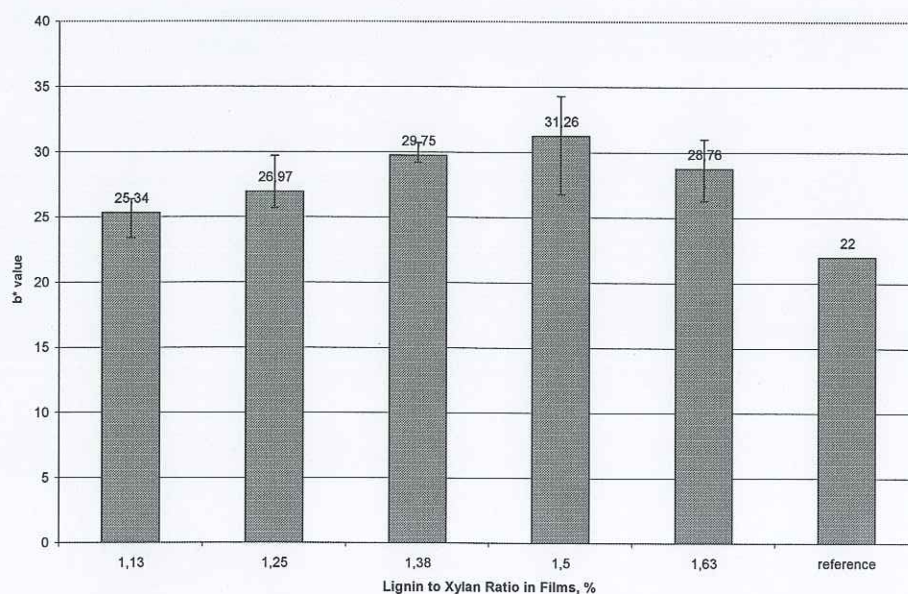


Figure 3.15 b* values of birchwood xylan-lignin composite films as a function of lignin content.

Total color change from white (ΔE) was calculated from the equation given in 2.1 in Chapter 2, section 3.0. Total color change, shown in Figure 3.16, was determined as 40.54; 40.78; 41.00; 40.53 and 41.29 corresponding to to films having 1.13%, 1.25%, 1.38%, 1.50%, 1.63% lignin/xylan. There was no significant difference between them. As the film with 1.63% lignin/xylan had lower lightness, its total color change was slightly higher than the other films.

Since lignin is a brown-colored compound an increase in color indexes with respect to white paper was expected by increasing lignin content in the films. This trend was observed increasing lignin content from 1.1% to 1.5%. The change in color indexes were slight because the change in lignin concentration was slight.

Although the film having 1.63% lignin/xylan expected to have the highest a*, b* values, they were slightly in the middle as the thickness of this film was lower than the other films. As thickness affects color indexes, all the color indexes were normalized by dividing them to thickness as given in Table 3.3.

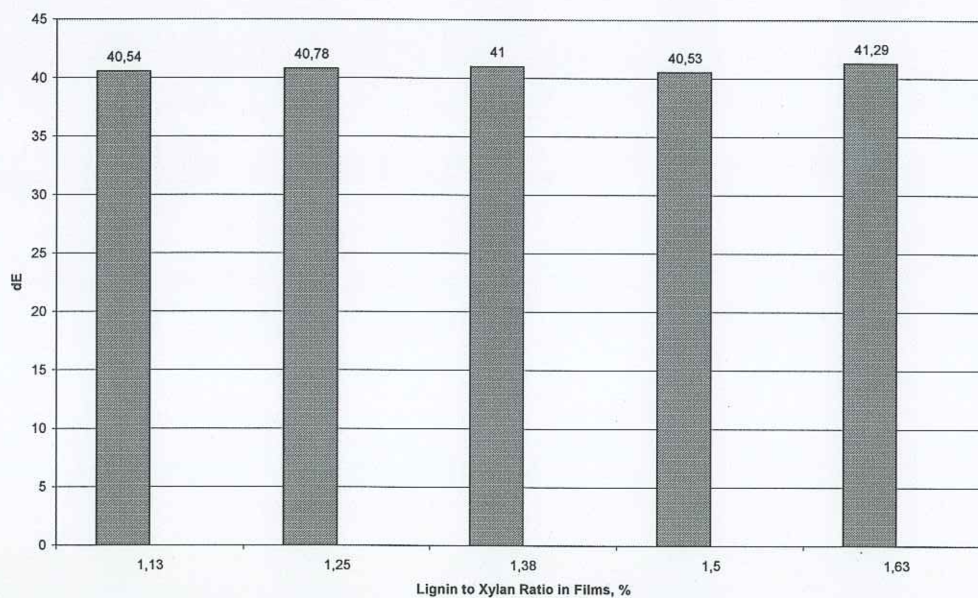


Figure 3.16 Total color change, (ΔE), values of birchwood xylan-lignin composite films as a function of lignin content.

Table 3.3 Normalized color indexes of the lignin-xylan films.

Lignin/ xylan ratio, %	*Normalized L**	*Normalized a**	*Normalized b**	*Normalized dE*
1.13	51.78	23.87	49.98	79.96
1.25	56.59	27.5	58.12	87.89
1.38	57.48	30.84	63.98	88.17
1.5	63.58	35.05	71.7	92.96
1.63	67.52	34.57	75.09	107.8

3.2 Photocatalytic Antimicrobial Films

3.2.1 Antimicrobial Gluten Film Formation by TiO_2

For photocatalytic antimicrobial film production, wheat gluten based films were chosen considering better mechanical characteristics of gluten films compared to cotton stalk xylan films. Tensile strength of wheat gluten films (4.0 MPa) is higher than cotton stalk xylan films (1.3 MPa) so that gluten films have more heavy-duty use potential than xylan films. Gluten films are also more stiff than xylan films because gluten films' elastic modulus is 70 MPa and xylan films' is 0.4 MPa. Gluten films can stretch more than xylan films as the former one's strain at break is 250% while the latter one's is about 51% (Göksu, 2005; Kayserilioğlu, 2003b).

First of all, 10% (w/w) gluten containing film forming solution was prepared with the addition of 2% (w/w) glycerol as explained in Chapter 2 in section 2.4.2. A wheat gluten film is shown in Figure 3.17.

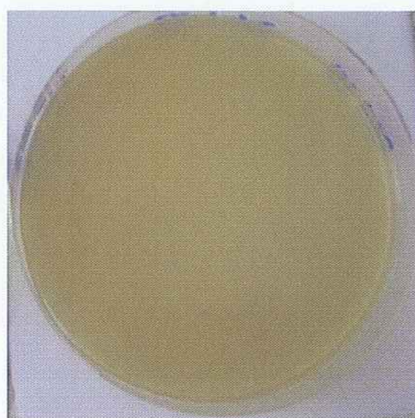


Figure 3.17 10% (w/w) wheat gluten film containing 2% glycerol.

Afterwards, gluten films were either coated with titania using different procedures or titania was added to film forming solutions and their antimicrobial activities were investigated.

TiO_2 was used to give an antimicrobial property to gluten films by photocatalysis because it has strong oxidation/reduction property results in oxidation of all organic compunds to carbondioxide and water including microorganisms.

3.2.1.1 Antimicrobial Gluten Film Formation by Direct Addition of TiO_2

As explained in Chapter 2, section 2.6.2, direct addition of TiO_2 to film forming solutions was used to obtain antimicrobial films. The concentration of TiO_2 was changed in range of, 0.8-1.6% (w/w).

At first, TiO_2 was formed from a precursor, TTIP. Films containing 0.8% TiO_2 (w/w) were formed well as shown in Figure 3.18.

For the antimicrobial test, which was done according to Chapter 2, section 2.7. overnight cultured, fresh and centrifuged *Escherichia coli* cells suspended in 1 ml peptone water were put on both TiO_2 added and non-TiO_2 added film samples and illuminated for 1 h using an artificial sunlight, Osram, ultra-vitalux, 300W.

Then, 9 ml peptone water was added to the illuminated film sample as the 10^1 dilution, and this suspension serially diluted until 10^6 dilution was achieved. 200 μl of microbial suspension was inoculated on LB agar plates.

The number of *E. coli* cells were counted and according to the results, films were found to be slightly antimicrobial, 17%, because TiO_2 containing film samples had 274±97 CFUs and no TiO_2 containing samples had 331±57 CFUs based on viable counting method.

Figure 3.18 A film containing 0.8% titanium dioxide (w/w).

Films containing 1.6% TiO_2 (w/w) were formed again well but they were not antimicrobial, in which antimicrobial test was done according to Chapter 2, section 2.7. In addition, wheat gluten films were prepared by adding 3.2% (w/w) commercial TiO_2 which is equal to 1.6% TiO_2 prepared from TTIP, because commercial TiO_2 was prepared by mixing this powder with 4 ml water instead of 2 ml which was used to prepare TiO_2 formed from TTIP. Although films formed well, they were not antimicrobial, either as TiO_2 containing film samples had 315±56 CFUs and no TiO_2 containing samples (blanks) had 274±11 CFUs based on viable counting method.

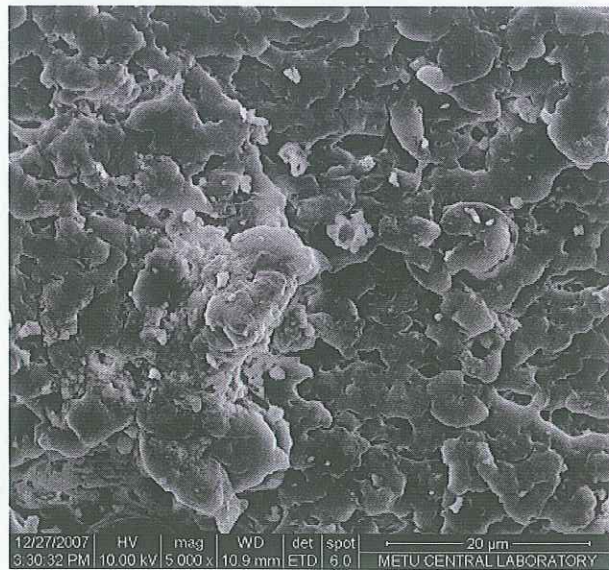
To characterize the films, SEM and EDX analysis were performed and no titanium was observed on the surface of these films according to the EDX of the surface area of wheat gluten film including 1.6% titanium dioxide, as shown in Figure 3.19. EDX result shows carbon having the highest weight followed by oxygen. So that, electron/hole pair could not move to surface and therefore could not react anything on the surface, as a consequence hydroxyl radicals and oxygen radicals could not be formed to destroy *E. coli* cells. So that, there was no antimicrobial effect.

Figure 3.19 EDX spectra of surface area of wheat gluten film containing 1.6% TiO_2.

According to the SEM image of the surface area in Figure 3.20, there was no titanium because the image was similar to the SEM image of the control wheat gluten films containing no titanium dioxide shown in Figure 3.21. Titanium was not observed either on surface or cross-sectional area of the EDX spectra as expected in control films. The dark parts in the SEM image of uncoated film show the presence of carbon, as wheat gluten is an organic material having carbon in its structure and it is about 56% of its weight in the cross sectional area followed by oxygen at about 20%.

Figure 3.20 SEM image of surface area of wheat gluten film containing 1.6% TiO_2.

(A)



(B)

Figure 3.21 SEM images of uncoated wheat gluten film (A) surface area (B) cross sectional area.

However, titanium was observed in the EDX spectra of the cross sectional area of the film containing 1.6% TiO_2 as seen in Figure 3.22. SEM image of the cross sectional area is given in Figure 3.23.

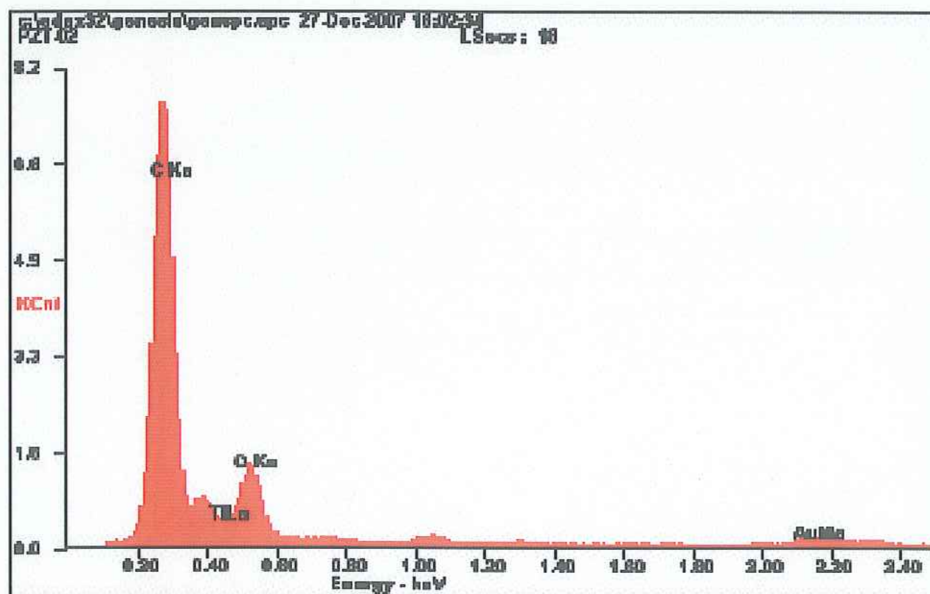


Figure 3.22 EDX spectra of cross sectional area of wheat gluten film containing 1.6% TiO_2.

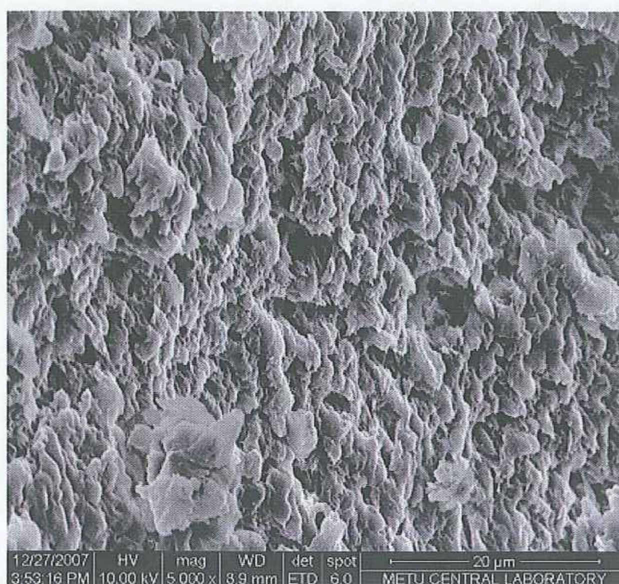


Figure 3.23 SEM image of cross sectional area of wheat gluten film containing 1.6% TiO_2.

All results having the viable cell counts of different amount of TiO_2 addition after antimicrobial tests including photocatalytic inactivation are given in Table 3.4.

Table 3.4 Antimicrobial activity of the films prepared by TiO_2 addition.

TiO_2 amount in films, %	*Blank, CFUs*	*TiO_2 added films, CFUs*	*Antimicrobial activity, (%)*
0.8	331±57	274±97	17
1.6	274±11	315±56	0

3.2.1.2 Antimicrobial Gluten Film Formation by Sol-gel Coating Method

The antimicrobial effect of gluten films, coated with procedure 1 was tested according to Chapter 2, section 2.7. Coated film samples had 104±60 CFUs while uncoated samples had 156±109 CFUs, showing 33% antimicrobiality.

For the gluten films coated by second procedure, number of *E. coli* cells were counted as 536±142 CFUs on uncoated control film samples by plate count method. On the other hand, coated film samples had no colony showing 100% antimicrobial effect of the coating after illumination. The result of the antimicrobial property was illustrated in Figure 3.24.

(A)

(B)

Figure 3.24 Antimicrobial test result of (A) the second procedure coated wheat gluten film (B) uncoated wheat gluten film.

Our light source and UV filter had been reported to provide no cell inactivation by UV illumination itself (Erkan, 2006).

Titanium coating on the surface was observed in EDX spectra (in Figure 3.25) from the SEM image as seen in Figure 3.26. As mentioned in Chapter 2, section 2.8, all samples to be investigated in SEM are coated with gold in order to achieve sufficient conductivity for analysis.

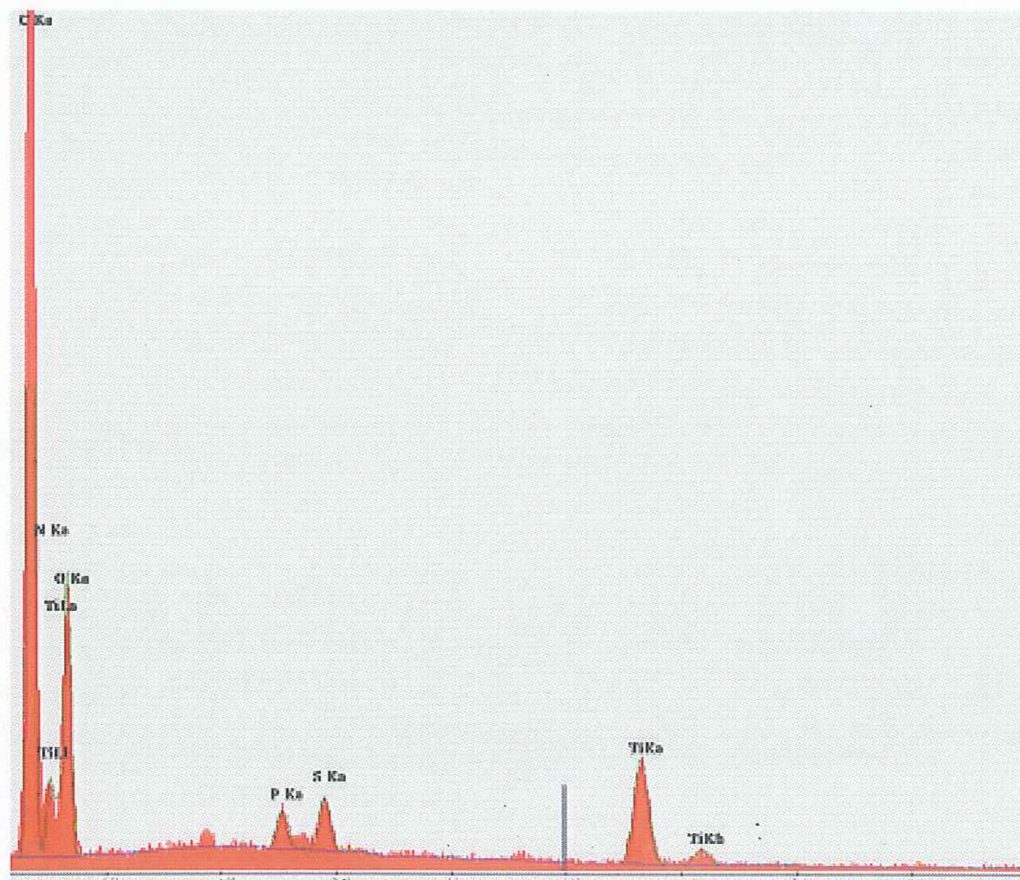
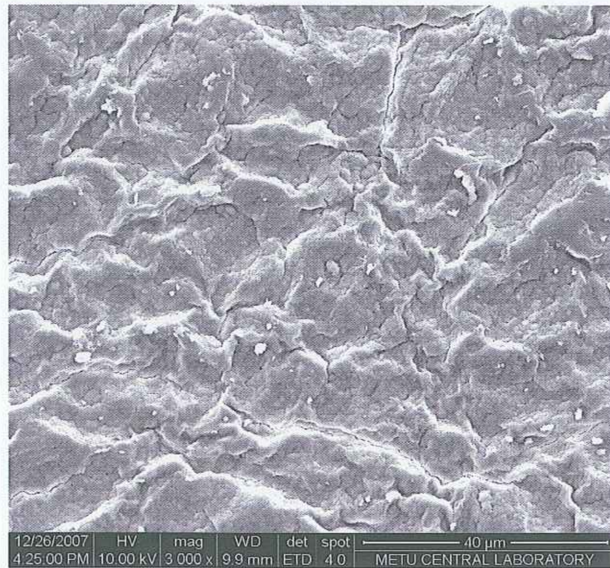
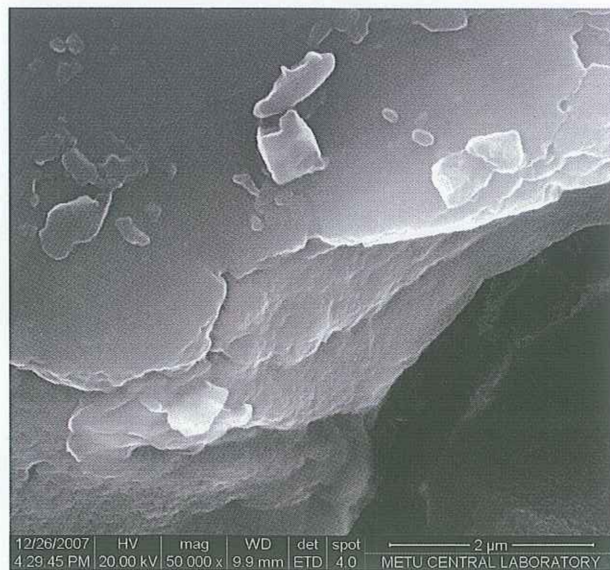


Figure 3.25 EDX spectra of surface area of wheat gluten film coated with second procedure.



(A)



(B)

Figure 3.26 SEM image of the surface area of the gluten film coated with titania using procedure 2 (A) at 3.000x (B) at 50.000x.

Titanium on the surface gave the antimicrobial property as TiO_2 could react with the *E. coli* cells on the surface of the film because when there is an absorption of a quantum of light by TiO_2 particles on the surface it leads to the formation of charge-carrier pairs of electrons in the conduction band and holes in the valence band (reaction (3.1)) and some of the charge carriers are trapped on the surface of TiO_2 particles and can undergo reactions with adsorbed species. From the Figure 3.26 (B), TiO_2 particles are seen at a higher magnification like 50.000x and the brighter sides of particles possibly show the electron runs, proving this reaction.

(3.1)

Thus, the holes react with adsorbed water molecules or hydroxyl groups bound to the TiO_2 surface (reaction (3.2)) to form highly reactive hydroxyl radicals. The hydroxyl radicals initiate the oxidation of organic molecules. It reacts with them and forms organic radicals which are further oxidized by molecular oxygen to carbon dioxide and water.

(3.2)

The catalytic cycle is completed by the transfer of the electrons trapped in the TiO_2 particle surface to dissolved oxygen molecules to form superoxide radicals (reaction (3.3)).

(3.3)

These can react with hydrogen protons to form molecular oxygen and hydrogen peroxide (reaction (3.4)).

$$2O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3.4)$$

Those molecular oxygen and hydrogen peroxide can react with the photo generated electrons to form hydroxyl radicals (reaction (3.5)).

$$H_2O_2 + e_{cb}^- \rightarrow HO^\cdot + OH^- \quad (3.5)$$

Both hydroxyl and oxygen radicals are capable of performing many selective/nonselective oxidation reactions resulting in inactivation of microorganisms (Belhacova, 1999).

Antimicrobial activity of TiO_2 has been observed in many studies. Huang et al. (2000) reported, that when TiO_2 was stirred with *E. coli* and illuminated for 1 hour, complete inactivation was observed. Kühn et al. (2003) coated slices of UVA-transparent Plexiglas with TiO_2. When *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecium* and *Candida albicans* were illuminated on the sample specimen for 1 hour, reduction efficiency of *E. coli* was more than the other tested microorganisms.

In another study by Erkan et al., 2006, there was a 85% decrease in survival ratio of *E. coli* due to photocatalytic effect after 2 hours of illumination on TiO_2 coated Pyrex glass plates. Pd doped TiO_2 coated glasses had higher photocatalytic efficiency than the ones coated only with TiO_2 showing 98% decrease in survival ratio of *E. coli*. Palladium doping increased the photocatalytic activity possibly by increasing the conductivity on surface of the PdO-TiO_2 coated glass.

Although photocatalytic antimicrobial activity has been observed on different inorganic surfaces such as glass and ceramics, there has not been any study on antimicrobial coating of biological films. In this study, antimicrobial film

production was achieved using method 2, however, the films were very brittle and their shapes were deformed after coating as shown in Figure 3.27.

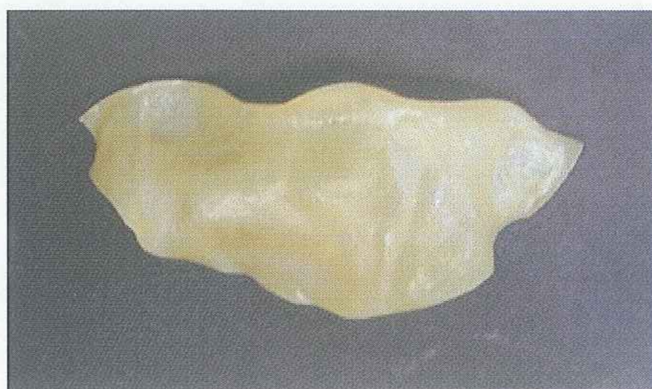


Figure 3.27 Wheat gluten film coated with second procedure.

In order to improve film's physical mechanical properties firstly, glycerol (plasticizer) concentration was increased to 4%, 8%, 10% and 20% (w/w). Glycerol is a plasticizer as it is a low volatile organic compound causing an increase in flexibility and extensibility. By decreasing intermolecular forces between polymer coils, it causes an increase in material flexibility and conversely a decrease in the barrier properties due to the augmentation of the free volume. To summarize, an initially hard and brittle material becomes soft and flexible when plasticized enough (Audic and Chaufer, 2005).

Therefore in the first step, increasing glycerol concentration resulted in very flexible gluten films. When coated, wheat gluten films with 20% glycerol content were not brittle but their shapes were destroyed as they were hardly

peeled from the petri dish before the coating procedure. The films containing 4-10% glycerol also turned into brittle and deformed films.

About 28% (w) titanium is observed in EDX spectra of cross sectional area of wheat gluten films coated with procedure 2, given in Figure 3.28. As seen in the SEM image of the same film in Figure 3.29 and 3.30, the coating is too thick and well adsorbed on film's surface which probably caused deformation in shape and brittleness. So that, after forming the coating solution in this procedure, it was diluted to make the coating solution thinner which resulted in thinner coats. The solution was diluted as 1/2, 1/5, 1/10, 1/20, 1/50, 1/100 in water, however, again highly brittle and deformed films were obtained as shown in Figure 3.31.

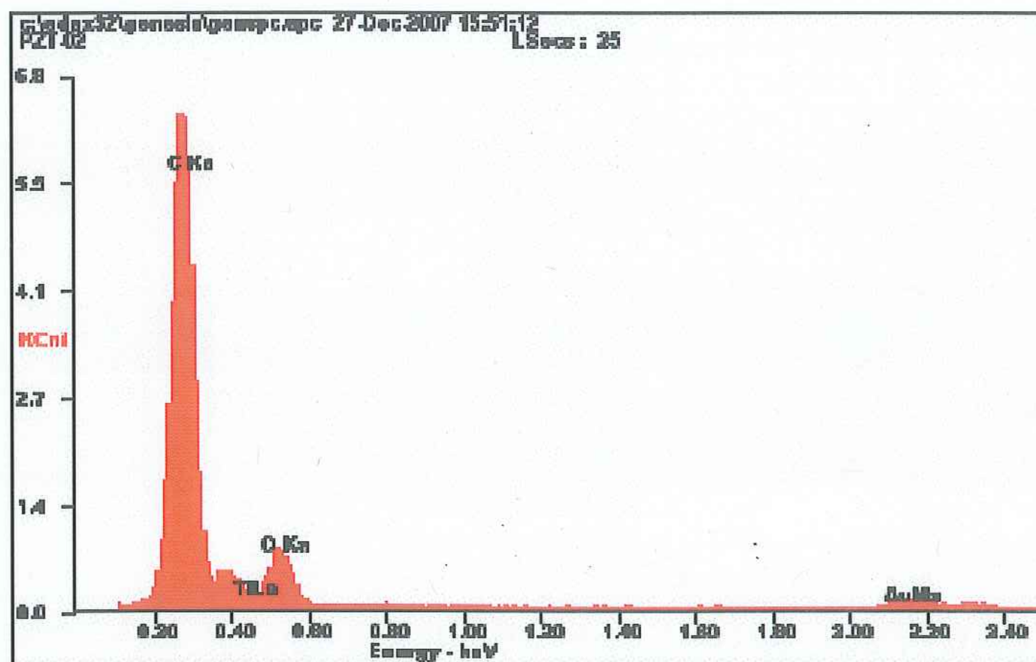


Figure 3.28 EDX spectra of the cross sectional area of wheat gluten films coated with procedure 2.

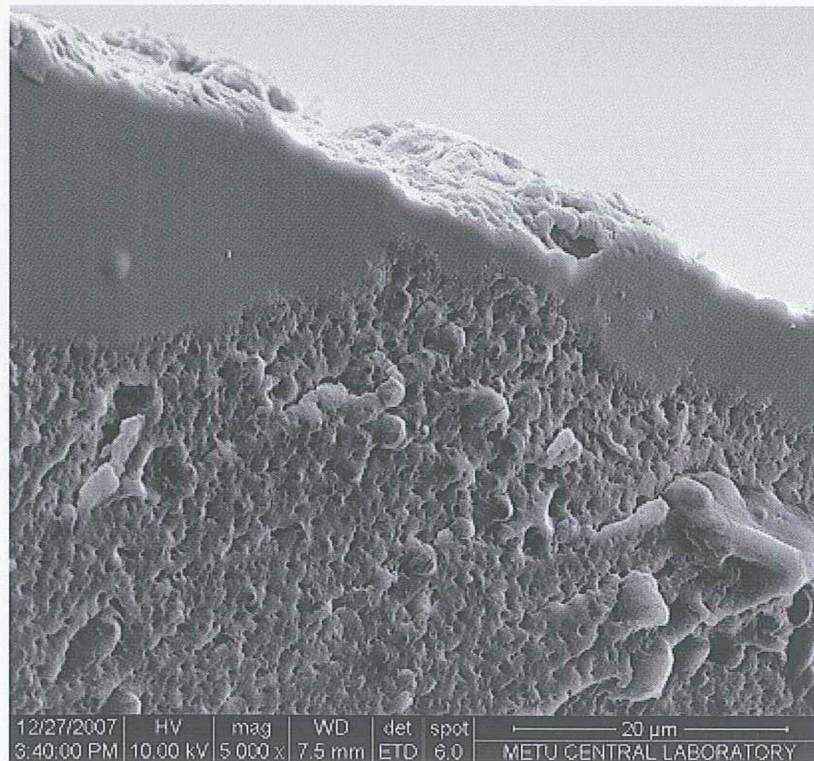


Figure 3.29 SEM image of the cross sectional area of the procedure 2 coated wheat gluten film.

More detailed, at 10.000x magnification, SEM image of the cross sectional area of the coating seen on the surface (in Figure 3.30) is comparable to the study by Erkan et al., 2006. In both of them the formation of continuous, defect-free, smooth TiO_2 structure was observed over the samples that were examined, where neither particle structure, nor grains were observed.

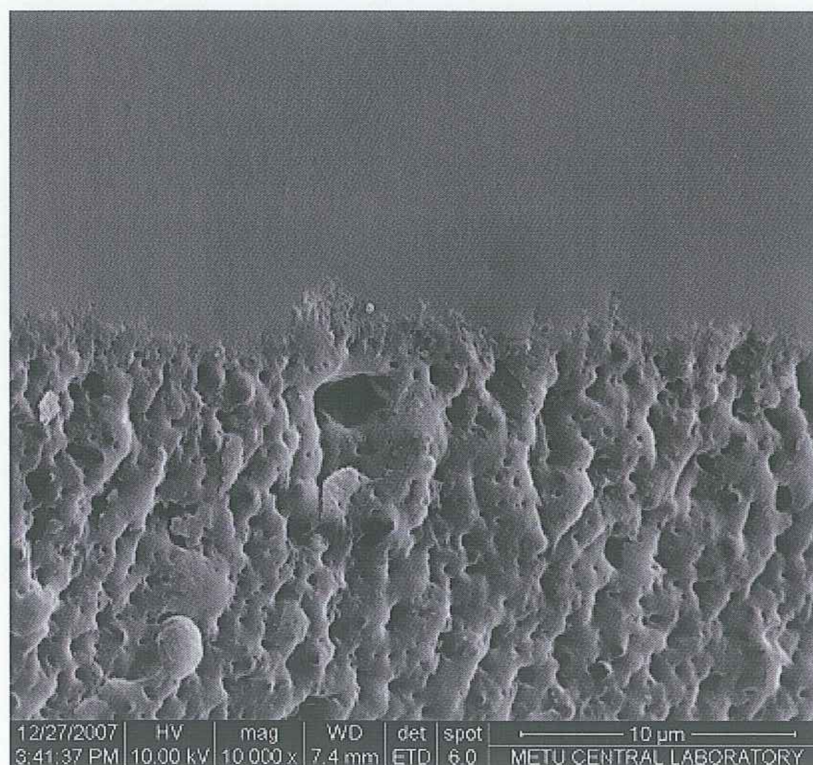


Figure 3.30 A more detailed SEM image of the cross-sectional area of the procedure 2 coated wheat gluten film at 10.000x magnification.

Figure 3.31 Films coated by the 1/2, 1/5, 1/ 10, 1/20, 1/50, 1/100 diluted TiO_2 solution from left to right.

In order to improve films' flexibility, another method, procedure 3 was used in which HCl was used instead of HNO_3 in procedure 2. The number of *E. coli* cells were counted as 575±16 CFUs on uncoated control film samples by viable count method. On the other hand, coated film samples had no colony showing 100% antimicrobial effect.

As in procedure 2, although antimicrobial films were produced, again films turned out to be brittle and their shapes were deformed as shown in Figure 3.32.

Figure 3.32 Wheat gluten film coated with third procedure.

In order to improve films' flexibility, another coating procedure, procedure 4 was used using isopropyl alcohol instead of water in procedure 2 which was explained in Chapter 2, section 2.6.3.4. However, the coated films were not antimicrobial. The number of *E. coli* cells were counted as 295±30 CFUs on uncoated control film samples by viable count method and coated film samples had 308±45 CFUs.

Although films were flexible and their shape were as the one before

coating as shown in Figure 3.33, they were not antimicrobial.

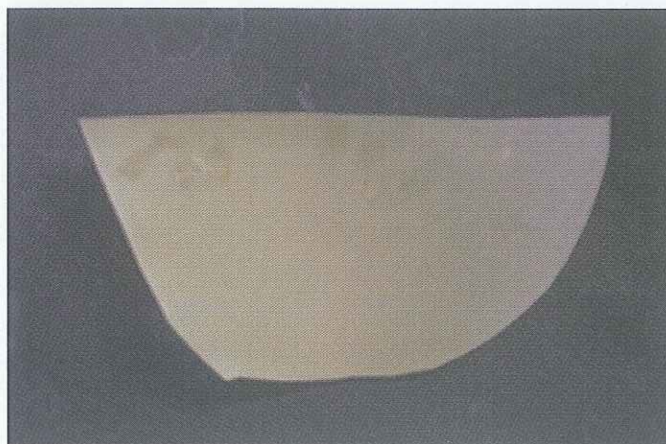
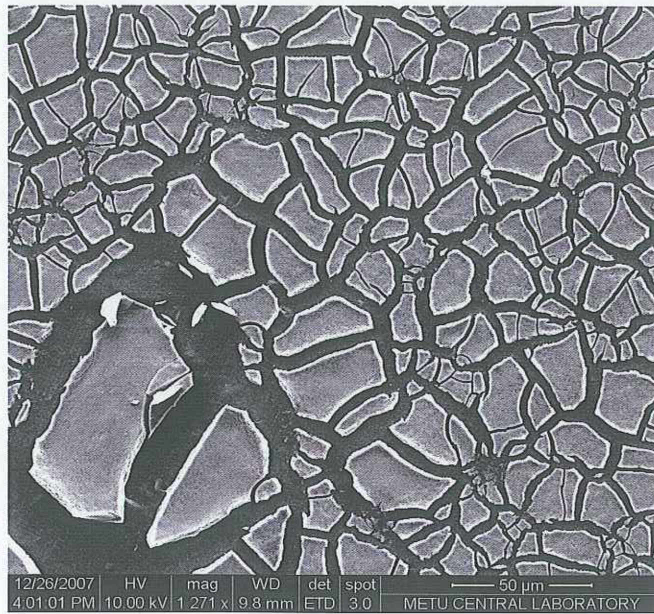


Figure 3.33 Wheat gluten film coated with fourth procedure.

As seen in Figure 3.34, the SEM image of the coated gluten film surface obtained by method 4, a non-continuous formation of coating unlike the coating in second procedure was obtained. This cracked structure may have lead to the loss of appearent electrical conductance over the surface so that antimicrobial property was not observed.



A

B

Figure 3.34 SEM image of the surface area of the procedure 4 coated wheat gluten film at (A) 1.271x magnification of general surface (B) 50.000x magnification of cracked flake surface.

EDX spectra in Figure 3.35 verifies the presence of titanium in the surface area of wheat gluten films coated by procedure 4.

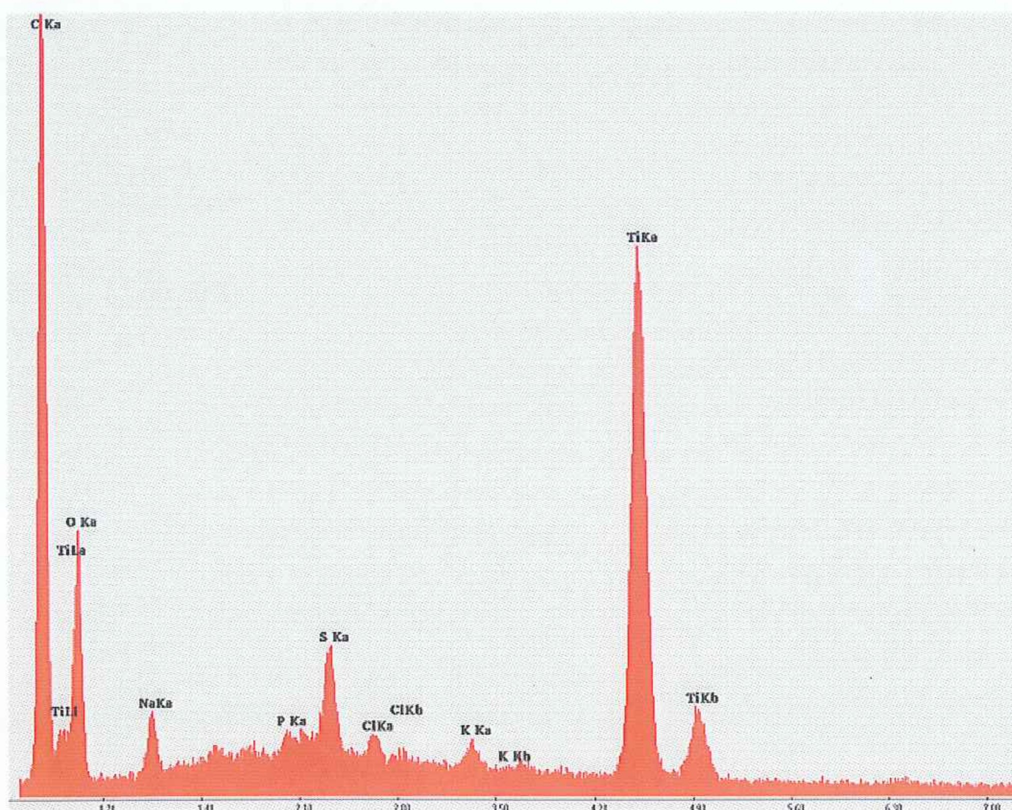
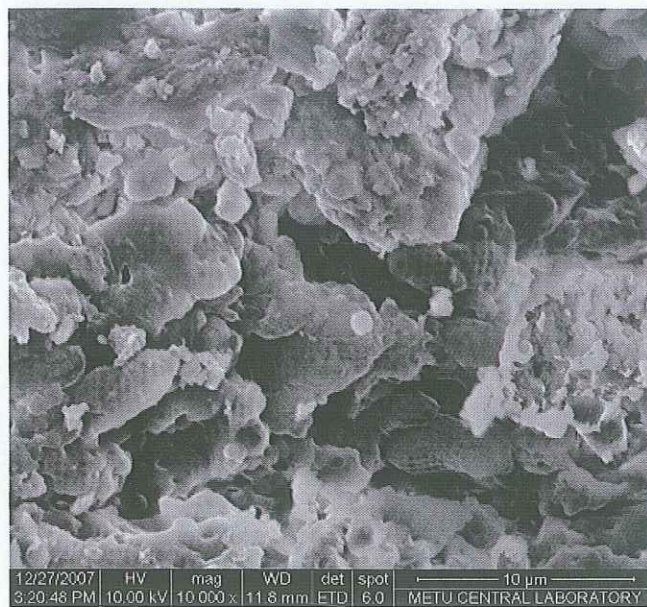


Figure 3.35 EDX spectra of the surface area of wheat gluten films coated with procedure 4, where titanium peaks are shown.

The SEM image of the cross sectional area as shown in Figure 3.36, was also unlike the one in second procedure, as there is not a continuous, uniform structure on the surface of the film.

A



B

Figure 3.36 SEM images of cross-sectional areas of the wheat gluten film coated with procedure 4 (A) a general view of the whole cross sectional area (B) more detailed view of the coating part on the surface.

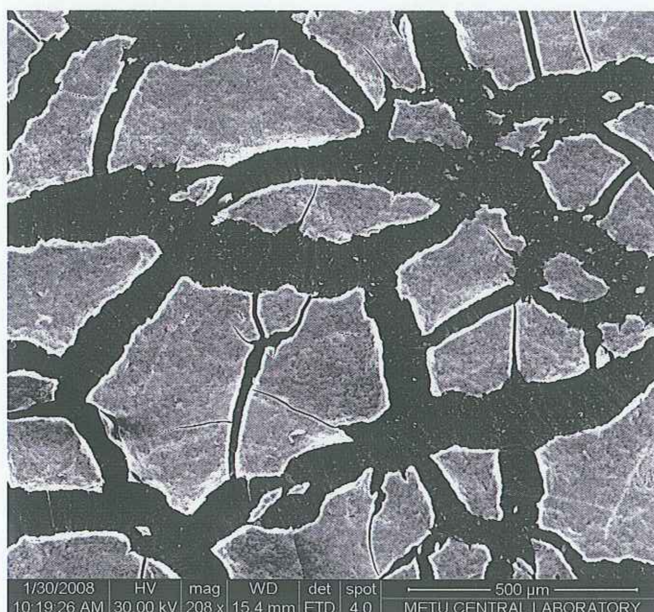
Another procedure, using the sol-gel prepared in the second method, the aqueous-nitric acid method, was also carried out as the fifth procedure to improve films' mechanical properties. After spreading the sol-gel on the surface of the semi-dried films, (gluten films which had their pH adjusted to 11 either by KOH or NaOH), they were completely dried. Films were kept at 60% RH for at least two days. After that, films were self-supporting and elastic as seen in Figure 3.37. However, films of pH adjusted by NaOH were only 42% antimicrobial. They had 188±52 CFUs while the control films had 327 CFUs. In addition films of pH adjusted by KOH were only 40% antimicrobial as they had 196±76 CFUs.

A B

Figure 3.37 After drying of the wheat gluten films coated by the fifth method, pH adjusted to 11 by (A) KOH (B) NaOH.

SEM images belonging to surface area of fifth procedure coated gluten films, in which NaOH was used to adjust pH, are shown in Figure 3.38. According to them, a non-continuous coating like the coating in fourth procedure, shown

previously in Figure 3.34 (A), was obtained. Again, this cracked structure may have lead to the loss of appearent electrical conductance over the surface so that antimicrobial property was only 42%. A higher magnification of the cracked flake at 50.000x, shown in Figure 3.38 (B), was also similar to the one in coating in fourth procedure as previously shown in Figure 3.34 (B).



A

B

Figure 3.38 SEM image of the surface area of the procedure 5 coated wheat gluten film, which had pH adjustment by NaOH, at (A) 208x magnification of general surface (B) 50.000x magnification of cracked flake surface.

Also the low level of titanium on the surface, which is verified by EDX spectra shown in Figure 3.39, can be the other reason for low antimicrobial property.

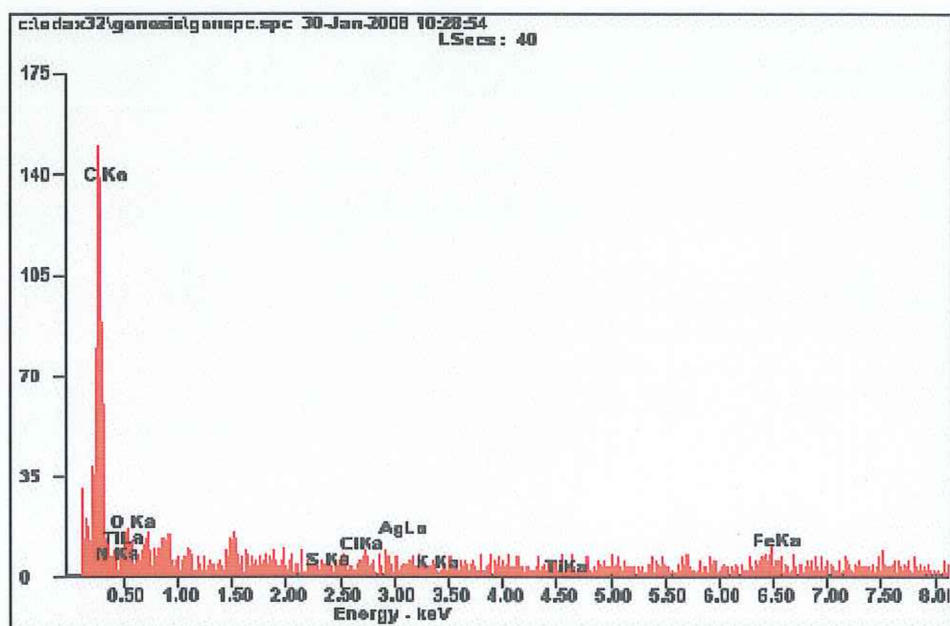
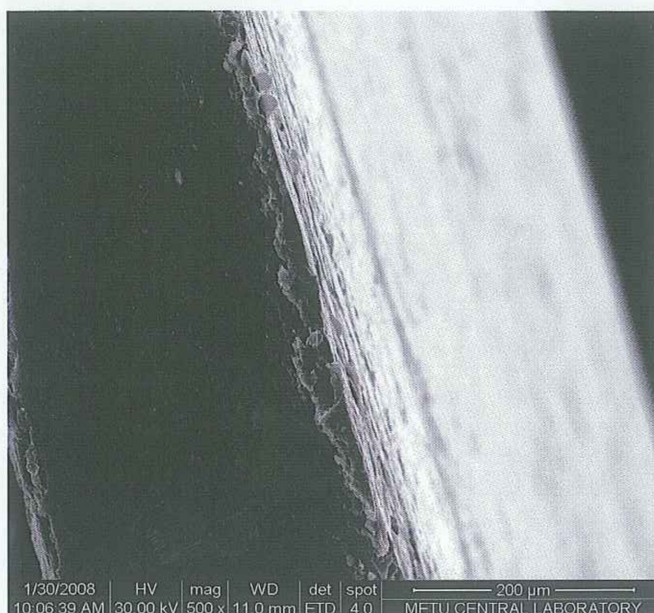
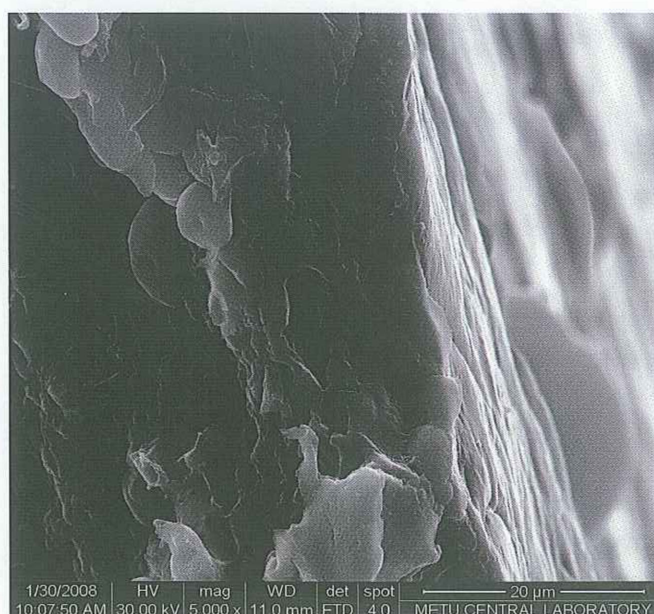


Figure 3.39 EDX spectra of the surface area of the procedure 5 coated wheat gluten film, which had its pH adjustment to 11 by NaOH.

SEM image of cross sectional area of the fifth procedure coated gluten film, which had NaOH to adjust its pH to 11, is shown in Figure 3.40 . Again a low level of titanium was observed accoring to EDX spectra given in Figure 3.41.



A



B

Figure 3.40 SEM image of cross-sectional area of the fifth procedure coated gluten film, which had NaOH to adjust its pH to 11 (A) at 500x magnification (B) at 5.000x magnification of the surface part.

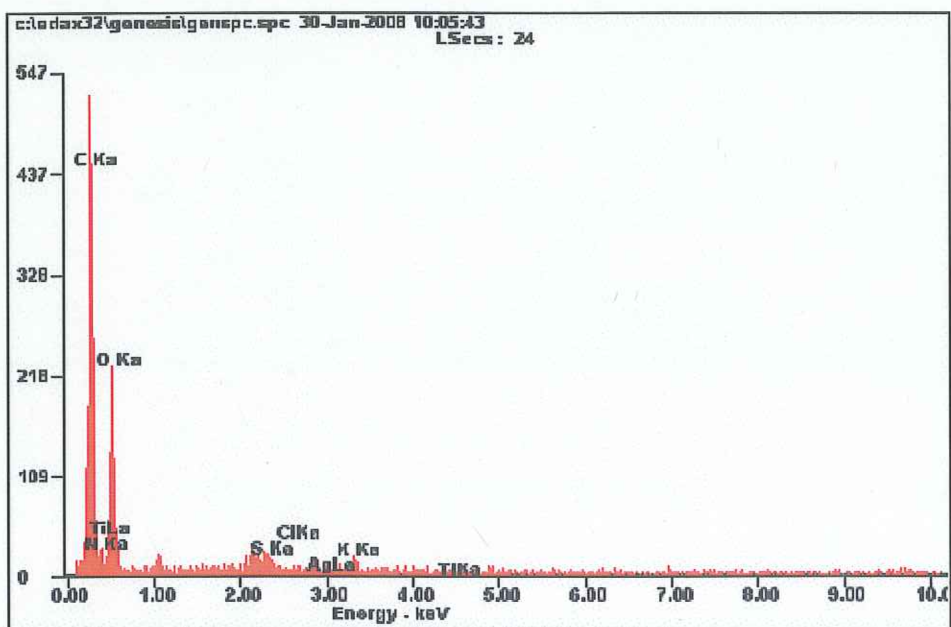


Figure 3.41 EDX spectra of cross-sectional area of the fifth procedure coated gluten film, which had NaOH to adjust its pH to 11.

SEM image of the surface area of the 40% antimicrobial, fifth procedure coated gluten film (pH adjusted by KOH), is also given in Figure 3.42. Again low titanium level, verified by the EDX spectra in Figure 3.43, gave the low percent of antimicrobial property.

Figure 3.42 SEM image of the surface area of the procedure 5 coated wheat gluten film, which had pH adjustment by KOH.

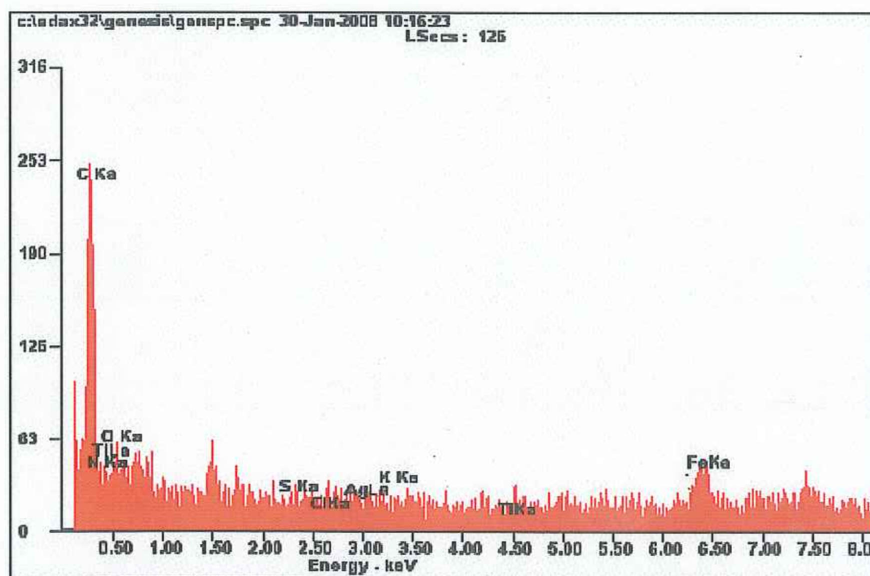
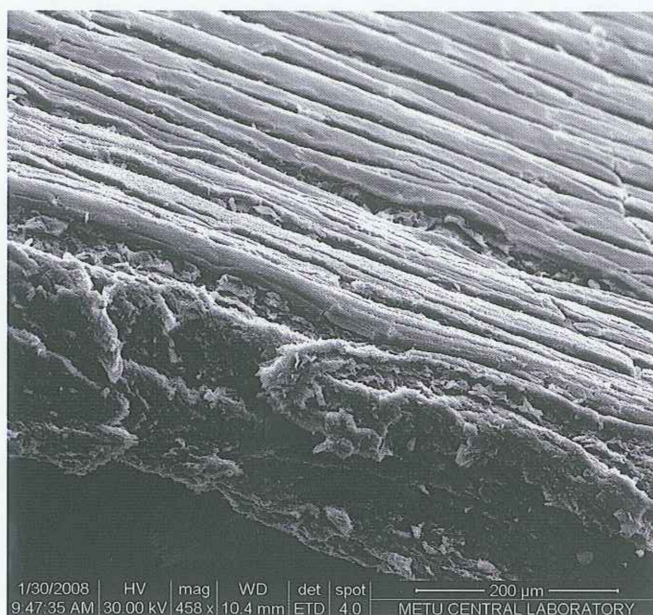
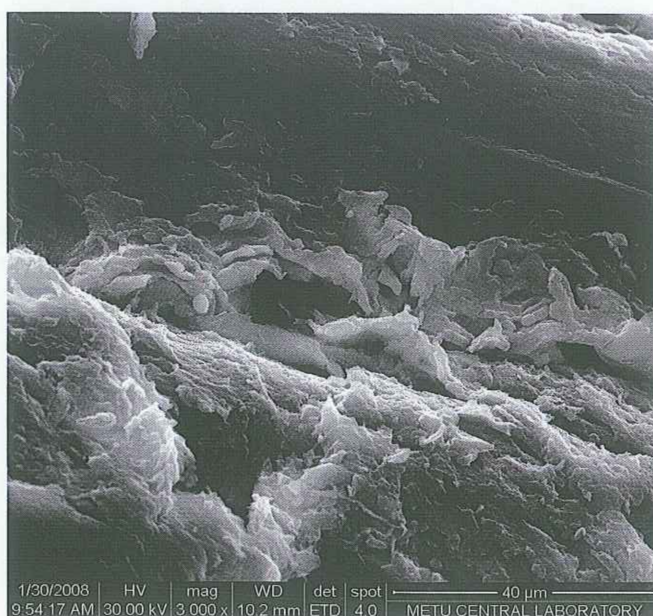


Figure 3.43 EDX spectra of surface area of the fifth procedure coated gluten film, which had KOH to adjust its pH to 11.

A layer of titanium coating is assumed to be on the surface of cross-sectional area which is given in Figure 3.44. Because according to the EDX spectra of the image which is surface of the cross-sectional area, in Figure 3.44 (A), titanium level is higher, but in the lower part of the image, which is under the surface layer, in Figure 3.44 (B), the titanium percent in weight is less. The EDX spectras are given in Figure 3.45. However, in overall, average titanium level is low which caused the less antimicrobial property.

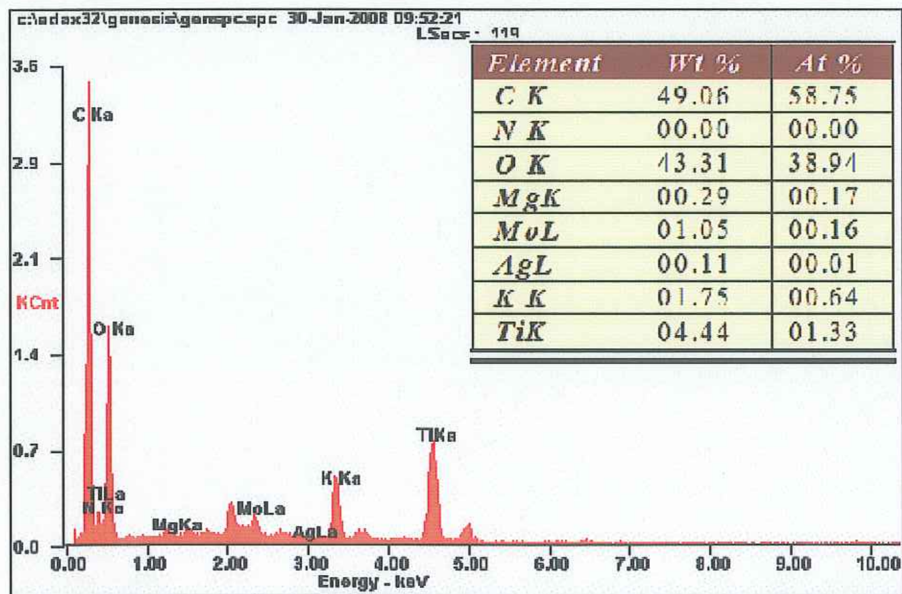


A



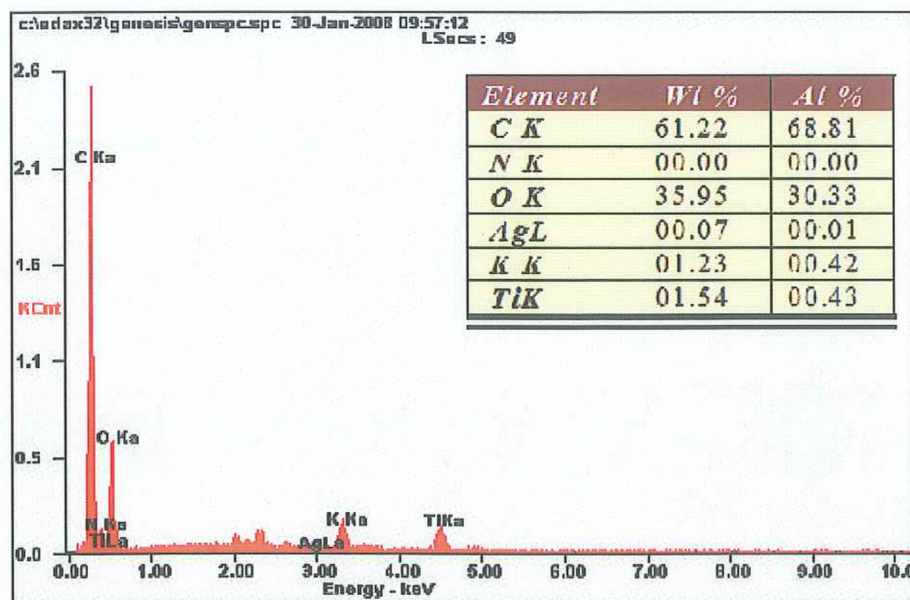
B

Figure 3.44 SEM image of cross-sectional area of the fifth procedure coated gluten film, which had KOH to adjust its pH to 11 (A) at 458x magnification (B) at 3.000x magnification of the part under the surface layer.



Element	Wt %	At %
C K	49.06	58.75
N K	00.00	00.00
O K	43.31	38.94
MgK	00.29	00.17
MoL	01.05	00.16
AgL	00.11	00.01
K K	01.75	00.64
TiK	04.44	01.33

A



Element	Wt %	At %
C K	61.22	68.81
N K	00.00	00.00
O K	35.95	30.33
AgL	00.07	00.01
K K	01.23	00.42
TiK	01.54	00.43

B

Figure 3.45 EDX spectras belonging to SEM images given in, (A) Figure 3.40 (A), 458x magnification (B) Figure 3.40 (B), 3.000x magnification of the part under the surface layer.

All results having the viable cell counts of different coating procedures after antimicrobial tests including photocatalytic inactivation is given in Table 3.5.

Table 3.5 Results of viable cell counts of different coating procedures after illumination.

Coating procedure	*Blank, CFUs*	*Coated, CFUs*	*% antimicrobial activity*
1st	156±109	104±60	33
2nd	536±142	0	100
3rd	575±16	0	100
4th	295±30	308±45	0
5th with NaOH	327	188±52	42
5th with KOH	327	196±76	40

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

In the first part of the study, lignin was demonstrated as a necessary additive to produce a self-supporting, continuous birchwood xylan film and the minimum lignin concentration was determined as about 1% lignin in xylan (w/w) for film formation. Xylan-lignin composite films were determined as biodegradable using an accelerated-enzymatic biodegradability test. The effect of lignin concentration on biodegradation, thickness and color of the films was determined as not significant in the range of 1.13-1.63% lignin/xylan.

In the second part of the study, wheat gluten-TiO_2 films were prepared to produce antimicrobial films. First of all, antimicrobial property could not be given to the films by direct addition of TiO_2 to film forming solutions. Antimicrobial films were obtained by wheat gluten film coating with sol-gel methods. Some of the films turned out to be deformed, brittle but 100% antimicrobial. Some of them were flexible, non-deformed but slightly antimicrobial. The best results were obtained by spreading sol-gel on semi-dried films. However, the antimicrobial/mechanical/physical properties of the films should be measured in detail.

For further studies, without removal of lignin, cotton stalk-lignin composite film production can be improved by ultrafiltrating the resulting salts after adjusting pH. Wheat gluten films' mechanical properties could be improved by trying different coating procedures. Methods given in this study can also be

altered. For instance, for the aqueous-nitric acid spreading method, a thicker coating on the semi-dried gluten films could be tried. Also coating of cotton stalk xylan films can be investigated. Effect of coating on mechanical properties of films should also be characterized.

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APPENDIX A

MEDIUM AND AGAR BASES

1. Luria-Bertani (LB) Broth:

Basis: 1L (in distilled water)

Tryptic soy broth (Sigma), 10g

Yeast Extract (Merck), 5g

NaCl, 10g

2. Luria-Bertani (LB) Agar:

Basis: 1L (in distilled water)

Tryptic soy broth (Sigma), 10g

Yeast Extract (Merck), 5g

NaCl, 10g